Functional, biochemical and structural characterization of Hepatitis C virus non-structural protein 5A

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**FUNCTIONAL, BIOCHEMICAL AND STRUCTURAL
CHARACTERIZATION OF HEPATITIS C VIRUS
NON-STRUCTURAL PROTEIN 5A**

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Content

ACKNOWLEDGEMENTS	I
CONTENT	II
LIST OF FIGURES	VI
SUMMARY	VIII
ABBREVIATIONS	XI
1. INTRODUCTION.....	1
1.1. BRIEF OVERVIEW OF HEPATITIS C VIRUS	1
1.1.1. Hepatitis C virus (HCV).....	1
1.1.2. Systems for studying Hepatitis C Virus.....	4
1.1.3. Therapy strategies for Hepatitis C.....	5
1.2. HCV NON-STRUCTURAL PROTEIN 5A (NS5A)	7
1.2.1. Some Structural features of NS5A protein	7
1.2.1.1. Post-translational modification of NS5A protein.....	7
1.2.1.1.1. Two phosphorylated forms of NS5A protein	7
1.2.1.1.2. Other possible observed post-translational modifications.....	10
1.2.1.2. N-terminal α -helical membrane anchor domain NS5A	11
1.2.1.3. Three domains of HCV NS5A.....	12
1.2.1.4. Other structural features of NS5A	16
1.2.2. Functions of NS5A protein.....	17
1.2.2.1. Roles in Hepatitis C virus replication.....	17
1.2.2.1.1. NS5A is a part of HCV replication complex.....	17
1.2.2.1.2. NS5A interacts with NS5B and other non-structural proteins.....	18
1.2.2.1.3. NS5A is a zinc metalloprotein	23
1.2.2.1.4. NS5A participates in virus-host interactions.....	23
1.2.2.2. Roles in resistance to IFN.....	24
1.2.2.3. Anti-apoptosis	26
1.2.2.3.1. NS5A is a potential viral Bcl-2 homologue	29
1.2.2.3.2. NS5A impairs TNF-Mediated hepatic apoptosis.....	32
1.2.2.3.3. NS5A interacts with p53.....	33
1.2.2.4. Transcriptional modulations.....	34
1.2.2.5. Perturbation of mitogenetic signaling pathway and growth control	35
1.2.2.6. Modulation of intracellular calcium and reactive oxygen.....	38
1.3. OBJECTIVE OF THIS PROJECT	39
2. MATERIALS AND METHODS.....	41
2.1. MATERIALS.....	41
2.1.1. Chemicals	41
2.1.2. Bacterial strains and mammalian cell lines	42
2.1.3. Media.....	42
2.1.3.1. Luria-Bertani (LB) medium	42
2.1.3.2. LB agar plates.....	42

2.1.3.3.	M9 medium (mineral medium).....	43
2.1.3.4.	NZCYM medium	43
2.1.3.5.	Media for mammalian cell culture.....	43
2.1.4.	Reagents for cell culture.....	43
2.1.4.1.	Transfection reagents.....	43
2.1.4.2.	Small interfering RNAs.....	44
2.1.4.3.	Anti-cancer drugs	44
2.1.4.4.	Other reagents	44
2.1.5.	Solutions and buffers.....	44
2.1.5.1.	Buffers for agarose gel	44
2.1.5.2.	Buffers for SDS-PAGE gel.....	45
2.1.5.3.	Buffers for Ni ²⁺ -NTA purification	45
2.1.5.4.	Buffers for FPLC purification	46
2.1.5.5.	Western-blot buffers	46
2.1.5.6.	Buffers for cell culture and analysis	47
2.1.5.7.	Other stock buffers	47
2.2.	METHODS.....	47
2.2.1.	Plasmid construction	47
2.2.1.1.	Constructions for NS5A proteins for pET-ubiquitin expression system in <i>E. coli</i>	47
2.2.1.2.	Constructed vectors for NS5B proteins expressed in <i>E. coli</i>	49
2.2.1.3.	Constructed vectors for NS5A protein expressed in mammalian cells	50
2.2.2.	Preparation of competent cells	51
2.2.3.	Plasmid transformation and purification	51
2.2.4.	Protein purification.....	52
2.2.4.1.	Expression and purification of the full length NS5A and NS5A-ΔN (without the N-terminal membrane anchor domain)	52
2.2.4.2.	Expression and purification of NS5A domain 1 (NS5A-D1), NS5A domain 2 (NS5A-D2), and NS5A domain 3 (NS5A-D3)	54
2.2.4.3.	Expression and purification of ¹⁵ N- and ¹⁵ N/ ¹³ C- labeled NS5A-D2.....	55
2.2.4.4.	Expression and purification of GST-NS5Bt	56
2.2.4.5.	Expression and purification of NS5Bt.....	57
2.2.5.	Western Blot analysis	58
2.2.6.	GST pull-down assay	58
2.2.7.	Surface plasmon resonance	59
2.2.8.	Analytical ultracentrifuge sedimentation equilibrium analysis	60
2.2.9.	Gel-filtration analysis	61
2.2.10.	Circular dichroism (CD) spectroscopy	61
2.2.11.	Dynamic light scattering	61
2.2.12.	NMR experiments	62
2.2.10.1.	NMR sample preparation.....	62
2.2.10.2.	NMR data collection and assignment	62
2.2.10.2.1.	1D ¹ H NMR experiment	62
2.2.10.2.2.	NMR experiments for structural determination	63
2.2.10.2.3.	2D ¹ H- ¹⁵ N HSQC.....	63
2.2.10.2.4.	HNCACB.....	63
2.2.10.2.5.	CBCACONH	64
2.2.10.2.6.	HNCA and HNCOCA.....	64
2.2.10.2.7.	HNCO.....	65
2.2.10.2.8.	HNHA.....	65
2.2.10.2.9.	HCCCONH and _CCCONH.....	66
2.2.10.2.10.	¹⁵ N-HSQC-NOESY	66
2.2.10.2.11.	NMR titration to study NS5A-D2 and NS5B peptide interaction	66
2.2.10.2.12.	NMR relaxation measurements.....	67
2.2.13.	Cell culture	68
2.2.14.	Transfection of pcDNA 3.1 (+)-NS5A into Human Hepatoma Cells HepG2.....	68

2.2.15.	RT-PCR and semi-quantitative RT-PCR.....	69
2.2.16.	siRNAs transfection	70
2.2.17.	Cell proliferation assay.....	71
2.2.18.	Protein concentration measurement	71
2.2.19.	Immunostaining.....	72
3.	RESULTS.....	73
3.1.	EXPRESSION, PURIFICATION AND CHARACTERIZATION OF FULL LENGTH NS5A AND NS5A-ΔN.....	73
3.1.1.	Expression and purification of full length NS5A and NS5A-ΔN	74
3.1.2.	Secondary structure analysis of NS5A-ΔN by CD spectrum	78
3.1.3.	Molecular interaction between NS5A-ΔN and its biological ligands.....	79
3.1.3.1.	Expression and purification of GST-NS5Bt and NS5Bt.....	79
3.1.3.2.	Interaction between NS5A-ΔN and GST-NS5Bt by GST pull-down assay.....	86
3.1.3.3.	Interaction between NS5A-ΔN and GST-PKR by GST pull-down assay.....	87
3.2.	EXPRESSION, PURIFICATION AND CHARACTERIZATION OF NS5A DOMAIN 1 (NS5A-D1) AND DOMAIN 3 (NS5A-D3)	89
3.2.1.	Expression and purification of NS5A-D1 (amino acids 28-213) and NS5A-D3 (amino acids 356-447).....	89
3.2.2.	1D ¹ H NMR evaluations and CD analysis for NS5A-D1 and NS5A-D3	92
3.3.	NS5A DOMAIN 2 IS NATIVELY UNFOLDED	96
3.3.1.	Expression and purification of NS5A Domain 2 (NS5A-D2)	97
3.3.2.	Identification of NS5A-D2 by MS spectrum.....	99
3.3.3.	Analytical ultracentrifuge sedimentation equilibrium analysis	99
3.3.4.	Gel filtration analysis of NS5A-D2	102
3.3.5.	Molecular interaction between NS5A-D2 and its biological ligands by <i>in vitro</i> GST pull-down assay and surface plasmon resonance	103
3.3.6.	Structural study of NS5A-D2	106
3.3.6.1.	Secondary structural analysis of NS5A-D2 by CD spectrum.....	106
3.3.6.2.	DLS analysis of NS5A-D2.....	107
3.3.6.3.	NMR ¹ D ¹ H and 2D ¹ H- ¹⁵ N HSQC spectrum of NS5A-D2.....	110
3.3.6.4.	NMR backbone ¹ H, ¹³ C and ¹⁵ N resonance assignments of NS5A-D2.....	112
3.3.6.5.	Secondary structure prediction of NS5A-D2 based on the NMR backbone assignment ..	117
3.3.6.6.	NMR sidechain assignment of NS5A-D2.....	118
3.3.6.7.	Analysis of NMR coupling constant and NOE data for NS5A-D2.....	119
3.3.6.8.	Backbone dynamics of NS5A-D2	122
3.3.6.9.	Effect of chemical denaturant on the conformation of NS5A-D2.....	125
3.3.6.10.	TFE induced α-helical transition of NS5A-D2	127
3.3.6.11.	Effect of low temperature on the conformation of NS5A-D2.....	129
3.3.7.	Binding of NS5A-D2 to HCV NS5B	133
3.3.7.1.	NS5A Domain 2 interacts with NS5B MK-17 peptide in a NMR-based titration assay..	133
3.3.7.2.	Secondary structure analysis of NS5A-D2 upon binding MK-17 peptide by CD.....	138
3.4.	ANTI-APOPTOSIS FUNCTION OF NS5A <i>IN VIVO</i>	139
3.4.1.	Anti-serum deprivation effect of NS5A in HepG2 cells and 293T cells.....	141
3.4.2.	NS5A is associated with endoplasmic reticulum (ER) and mitochondria	146
3.4.3.	Anti-apoptosis effect of NS5A protein on 293T cells treated with cisplatin	148
3.4.4.	siNS5A inhibits anti-apoptosis function of NS5A.....	151
3.4.4.1.	siNS5A inhibits NS5A expression in 293T cells	153
3.4.4.2.	siNS5A inhibits anti-apoptosis effects of NS5A in 293T cells	159
4.	DISCUSSION	161
4.1.	EXPRESSION AND PURIFICATION OF NS5A PROTEINS.....	161
4.2.	THE DOMAIN 2 OF NS5A PROTEIN	163

4.3.	METHODS FOR ANALYZING UNSTRUCTURED PROTEINS.....	172
4.4.	ANTI-APOPTOSIS OF NS5A	174
4.5.	EFFECTS OF SINS5AS.....	177
CONCLUSION		180
AUTHOR’S PUBLICATIONS		182
REFERENCE		183
APPENDIX.....		215

List of Figures

Fig. 1-1 Summary of HCV genome and the processing of gene products.....	3
Fig. 1-2 Structural and functional scheme of NS5A protein	8
Fig. 1-3 Structure of NS5A domain 1.....	15
Fig. 1-4 Structure of HCV NS5B RNA-dependent RNA polymerase.....	19
Fig. 1-5 NS5A binding sites on HCV NS5B polymerase.....	21
Fig. 1-6 Anti-apoptosis pathways of NS5A protein	28
Fig. 2-1 pET-ubiquitin expression vector for NS5A proteins	49
Fig. 3-1 Purification of full length NS5A	75
Fig. 3-2 Purification of NS5A-ΔN.....	77
Fig. 3-3 Western blot identification of full length NS5A and NS5A-ΔN.....	78
Fig. 3-4 Analysis NS5A-ΔN by CD	79
Fig. 3-5 Amino acids sequence alignment of the NS5A and NS5B binding sites from HCV-1b and -1a.....	81
Fig. 3-6 Purification of GST-NS5Bt	83
Fig. 3-7 Purification of NS5Bt	85
Fig. 3-8 <i>In vitro</i> binding studies of NS5A-ΔN and NS5B by GST pull-down assay.....	87
Fig. 3-9 <i>In vitro</i> binding studies of NS5A-ΔN and PKR by GST pull-down assay	88
Fig. 3-10 Purification of NS5A-D1 (amino acids 28-213).....	90
Fig. 3-11 Purification of NS5A-D3 (amino acids 356-447)	91
Fig. 3-12 1D ¹ H NMR spectrum of the NS5A-D1 (amino acids 28-213).....	93
Fig. 3-13 Amino acid sequence alignment of the NS5A domain 1 region between HCV-1a and -1b	94
Fig. 3-14 1D ¹ H NMR spectrum of the NS5A-D3 (amino acids 356-447).....	95
Fig. 3-15 Analysis of NS5A-D3 by CD	96
Fig. 3-16 Expression, purification and western blot analysis of NS5A-D2 (amino acids 240-335)	98
Fig. 3-17 MS analysis of purified NS5A-D2 from <i>E. coli</i>	99
Fig. 3-18 Analytical ultracentrifugation sedimentation equilibrium data	101
Fig. 3-19 Gel filtration analysis of NS5A-D2	102
Fig. 3-20 <i>In vitro</i> binding studies of NS5A-D2 and NS5B or PKR by GST pull-down assay ..	104
Fig. 3-21 Binding of NS5A-D2 to NS5Bt analyzed by surface plasmon resonance	105
Fig. 3-22 Analysis of NS5A-D2 by CD	107
Fig. 3-23 Dynamic light scattering analysis of NS5A-D2	109
Fig. 3-24 Initial analysis of NS5A-D2 by NMR 1D ¹ H and 2D ¹ H- ¹⁵ N HSQC spectra	111
Fig. 3-25 The 3D experiments of NS5A-D2 backbone assignment	114
Fig. 3-26 The backbone assignment of NS5A-D2.....	115
Fig. 3-27 2D ¹ H- ¹⁵ N HSQC spectrum of assigned NS5A-D2	116
Fig. 3-28 Chemical shift index (CSI) plots of CA, CB, HA, and CO of NS5A-D2 at 298 K....	118
Fig. 3-29 The 3D experiments of NS5A-D2 side chain assignment.....	119
Fig. 3-30 Summary of the NMR ³ J _{HNα} coupling constants and 3D NOE parameters defining the conformational characteristics of NS5A-D2 at 298 K	121
Fig. 3-31 3D ¹ H- ¹⁵ N NOESY-HSQC	122
Fig. 3-32 Relaxation measurements of NS5A-D2	124
Fig. 3-33 Effect of chemical denaturants on the structure of NS5A-D2.....	126
Fig. 3-34 TFE-dependent conformational transition of NS5A-D2 measured by CD and 2D ¹ H	

-¹⁵N HSQC at 25 °C	128
Fig. 3-35 Temperature dependence of the ¹H-¹⁵N HSQC spectrum of ¹⁵N labeled NS5A-D2.	129
Fig. 3-36 Chemical shift index (CSI) plots of CA, CB, HA, and CO of NS5A-D2 at 278 K	130
Fig. 3-37 Summary of the NMR parameters defining the conformational characters of NS5A-D2 at 278 K.....	132
Fig. 3-38 NS5A-D2 interacts with NS5B MK-17 peptide up to 1.6 mM	135
Fig. 3-39 NS5A-D2 interacts with NS5B MK-17 peptide up to 5 mM	136
Fig. 3-40 The relationship between chemical shift changes and the molar ratio of ligand to protein (L/P).....	137
Fig. 3-41 Analysis of NS5A-D2 upon NS5B MK-17 binding by CD at 25 °C	139
Fig. 3-42 Amino acids sequence alignment of BH domains in NS5A derived from HCV-1b and HCV-1a	141
Fig. 3-43 Western blot assay for the over-expression of NS5A in human hepatoma cell HepG2	142
Fig. 3-44 Anti-serum deprivation effect of NS5A in human hepatoma cells HepG2.....	143
Fig. 3-45 Two stable cell lines expressing flag-NS5A or flag-NS5B upon tetracycline (4 µg/ml)	144
Fig. 3-46 Anti-serum deprivation effect of NS5A in 293T-flag-NS5A stable cell line.....	145
Fig. 3-47 Subcellular localization of NS5A in 293T cells and HepG2 cells	147
Fig. 3-48 Anti-apoptosis effect of NS5A protein on 293T cells treated with cisplatin	150
Fig. 3-49 Western blot of caspase-3 protein.....	151
Fig. 3-50 HCV 1b NS5A cDNA sequence and the two siRNA targeting sites	153
Fig. 3-51 Inhibitions on NS5A protein expression in 293T-flag-NS5A cells treated with different concentrations of siNS5A D1-1 or siNS5A D2-1	156
Fig. 3-52 Inhibitions on NS5A protein expression in 293T-flag-NS5A cells treated with 100 nM siNS5A D1-1 or siNS5A D2-1 after different time of transfection	157
Fig. 3-53 Inhibition of NS5A expression at mRNA level	158
Fig. 3-54 siNS5A inhibits anti-apoptosis effects of NS5A in 293T cells	160
Fig. 4-1 Charge-hydrophobicity phase space plotted for NS5A-D2	165

Summary

Non-structural protein 5A protein (NS5A) of Hepatitis C virus (HCV) plays an important role in the regulation of viral replication, interferon resistance, and apoptosis. The detailed mechanism and molecular characteristics of NS5A remains unclear. In this study we first aimed to perform biophysical and biochemical characterization of the full length HCV NS5A. To this end, an efficient bacterial expression system was established to express NS5A and the system allowed us to purify the full length NS5A protein. The CD analysis of the full length NS5A suggested that this protein mainly consists of β -sheet at secondary structural level. *In vitro* GST pull-down assay proved that NS5A is functionally active and is capable of binding to its biological partners such as NS5B polymerase and PKR, a cellular interferon-inducible serine/threonine specific protein kinase.

NS5A comprises three domains. Recently the structure of the domain 1 has been determined, revealing a structural scaffold with a novel zinc-binding motif and a disulfide bond. At present, the structures of the domains 2 and 3 remain undefined. Domain 2 of HCV NS5A (NS5A-D2) is important for functions of NS5A and involved in molecular interactions with its own NS5B and PKR. In this study we focused our study on the domain 2. Like the full length NS5A protein, the

expression and purification of NS5A-D2 was efficient, allowed us to obtain a large amount of protein, and subsequently enabled us to perform biochemical and structural studies. The purified domain 2 was active and was able to bind NS5B and PKR, biological partners of NS5A. The results from gel filtration, CD analysis, DLS, 1D ^1H NMR and 2D ^1H - ^{15}N heteronuclear single quantum correlation (HSQC) spectroscopy indicate that the domain 2 of NS5A is flexible and disordered. The analysis of the backbone ^1H , ^{13}C , and ^{15}N resonances, $^3J_{\text{HN}\alpha}$ coupling constants and 3D Nuclear Overhauser Effect (NOE) data indicates that NS5A-D2 lacks secondary structural elements and reveals characteristics of unfolded proteins. NMR relaxation parameters confirmed the lack of a rigid structure in the domain 2. NMR titration results showed that NS5A-D2 is active and capable of binding to peptides derived from NS5B. The absence of an ordered conformation and the observation of a highly dynamic behavior of NS5A-D2 may provide an underlying molecular basis on its physiological function to allow NS5A-D2 to interact with a variety of biological partners.

NS5A protein was predicted to be an anti-apoptotic Bcl-2 homologue. It was reported to interact with FKBP38 and probably mimics the actions of Bcl-2 to facilitate anti-apoptosis and viral persistence. NS5A was found to interact with pro-apoptotic protein Bax or Bad and remove them from mitochondria to nucleus or cytoplasm, thereby disturbing the pro-apoptotic functions of these proteins. Additionally NS5A was able to sequester p53 inside the cytoplasm to prevent it

from exerting apoptotic effects. In this study, we investigated the anti-apoptotic functions of NS5A protein in hepatoma HepG2 cells and 293T cells. We found that the cells over-expressing NS5A protein showed a higher survival than the cells without NS5A expression under serum deprivation condition or treatment with chemical apoptotic inducer such as cisplatin. The expression of NS5A protein inhibited the activation of caspase-3, and important executor of apoptosis, and the cleavage of Poly (ADP-ribose) Polymerase (PARP), a substrate of caspase-3.

RNA interference has been emerging as an important tool for studying protein functions. In this study, small interfering RNAs (siRNAs) targeting domain 1 and 2 of NS5A were designed and used. The results showed that the siNS5As were able to suppress the expression of NS5A protein and consequently the anti-apoptotic effect of NS5A was abolished. Our results might shed light on the feasibility of applying siRNAs in combating HCV.

Abbreviations

ASFV.....	African swine fever virus
AV.....	adenovirus
BH.....	Bcl-2 homologues
CD.....	Circular dichroism
CKII.....	casein kinase II
CKI- α	casein kinase I- α
Cisplatin.....	<i>Cis</i> -Diammineplatinum (II) dichloride
CSI.....	Chemical shift index
DLS.....	dynamic light scattering
DMEM.....	Dulbecco's Modified Eagle Medium
DPC.....	Diphenyl carbazide
DTT.....	1, 4-Dithiothreitol
dsRNA.....	double-stranded RNA
EB.....	Ethidium bromide
EBV.....	Epstein-Barr virus
<i>E.coli</i>	<i>Escherichia coli</i>
EGF.....	epidermal growth factor
EHV-2.....	equine herpesvirus-2

eIF2- α	eukaryotic initiation factor 2
ERK.....	Extra-cellular signal-regulated kinases
FADD.....	Fas-associated death protein
FBS.....	fetal bovine serum
FRET.....	Fluorescence Resonance Energy Transfer
GAP.....	GTPase-activating protein
GAPDH.....	glyceraldehyde-3-phosphate dehydrogenase
Grb2.....	growth factor receptor-bound protein 2
GSK-3.....	glycogen synthase kinase 3
GST.....	glutathione S-transferase
HCC.....	hepatocellular carcinoma
HCV	Hepatitis C Virus
HVS.....	herpesvirus saimiri
hB-ind1.....	human butyrate-induced transcript 1
IFN.....	Interferon
IL-8.....	interleukin-8
IPTG.....	Isopropyl-thio- β -D-galactopyranoside
ISDR.....	interferon sensitivity determining region
JNK.....	c-Jun N-terminal kinase
LB.....	Luria-Bertani
LCS.....	low complexity sequence
MAPK.....	mitogen activated protein kinases

MS.....	Mass spectrometry
NaPB.....	sodium phenylbutyrate
NLS.....	nuclear localization signal
NMR.....	Nuclear magnetic resonance
NS5A.....	Non-structural protein 5A
PARP.....	poly ADP-ribose polymerase
PCD.....	programmed cell death
PKR.....	protein kinase RNA-activated
PMSF.....	Phenylmethylsulphonylfluoride
PPIase.....	peptidylprolyl cis/trans isomerases
PRE.....	p53 response-elements
PVDF.....	polyvinylidenedifluoride
RdRP.....	RNA-dependent RNA polymerase
ROS.....	reactive oxygen species
RT-PCR.....	reverse transcription-polymerase chain reaction
SAPK.....	stress-activated protein kinase
SDS.....	sodium dodecyl sulfate
SDS-PAGE.....	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
siRNAs.....	small interfering RNAs
SH3.....	Src-Homology 3
SM-FRET.....	single-molecule fluorescence resonance energy transfer
SRCAP.....	snf2-related CREB-binding protein activator protein

Abbreviations

SV40.....	simian virus 40
TAE.....	Tris_acetate_EDTA
TBC	TRE-2/BUB2/CDC16 genes
TBS.....	Tris-buffered saline
TFE.....	2, 2, 2-trifluoroethanol
TRADD.....	TNF receptor-associated death domain protein
TRAF2.....	TNF receptor associated factor-2
Ubp1.....	ubiquitin-specific carboxy-terminal protease
5'UTR.....	5'-untranslated region

Introduction

1.1. Brief overview of Hepatitis C virus

1.1.1. Hepatitis C virus (HCV)

Hepatitis C virus (HCV), identified in 1989 (Choo *et al*, 1989), has been a major cause of parenteral non-A, non-B hepatitis worldwide. Globally, more than 170 million people have been suffered with chronic infection of HCV (Lavanchy, 1999). Each year, an estimated 3 to 4 million people are newly infected according to WHO statistics (World Health Organization, 2004). With the development of the methods for diagnosing HCV infection, it becomes apparent that HCV infection shows high rate of progression (about 30%) to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Ohsawa *et al*, 1999; Okuda *et al*, 1998; Trepo *et al*, 1998). One major population of liver transplantation comes from the patients infected with HCV and suffering the complications of end-stage liver diseases (Wasley & Alter, 2000).

HCV is a distinct member of the Flaviviridae family (hepacivirus). HCV is a linear, positive-sense, single-stranded RNA virus, approximately 9600 nucleotides long (Miller & Purcell, 1990). Like the other members of the Flaviviridae family, there is a single open reading frame in HCV genomic RNA, which encodes a polyprotein

precursor of 3010–3033 amino acids (Saito & Miyamura, 1990). Host cellular signal peptidase and virally encoded proteases further process the polyprotein to at least ten mature viral proteins, in the order of the amino terminal structural proteins (core-E1-E2-p7) and the six carboxyl terminal non-structural proteins (NS2-NS3-NS4A-NS4B- NS5A-NS5B) (Miller & Purcell, 1990). The protein p7 is a membrane-associated protein that oligomerizes to form cation channels. The six non-structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B are not components of the virus particle. The functions of two non-structural proteins are well defined. NS3 acts together with NS4A and works as a proteinase to cleave between the non-structural proteins (Failla *et al*, 1994). NS5B is an RNA-dependent RNA polymerase (RdRP) responsible for viral RNA synthesis (Behrens *et al*, 1996; Lohmann *et al*, 1997). The functions of other non-structural proteins are not well defined. They appear to be important in viral replication, perturbing cell signal transduction or mediating immune evasion.

Fig. 1-1 Summary of HCV genome and the processing of gene products

The upper shows the structure of HCV genome. The open reading frame is about 9030 nucleotides long, which encodes both of the structural proteins and the non-structural proteins. The lower part shows the processing of HCV gene products. The cellular signal-peptide peptidase and signal peptidase; NS2-NS3 proteinase and NS3 proteinase encoded by HCV are responsible for the polyprotein processing. The arrows indicate the cleave sites of the processing. The processed gene products are indicated with names and basic characters.

The HCV genome shows remarkable sequence variations and more than 30 genotypes have been identified (Bukh *et al*, 1995; Bukh *et al*, 1995; Tokita *et al*, 1996). Among them six HCV genotypes (HCV-1a, -1b, -2a, -2b, -3a and -3b) are distributed worldwide. Subtype HCV-1a is prevalent among North and South

America, Europe, and Australia. Subtype HCV-1b is common in North America and Europe, and in parts of Asia (Bukh *et al*, 1995). While over 90% homology at the nucleotide level is seen in the 5'-untranslated region and the coding region for the core protein, more than 15% difference at amino acid level deduced from the entire transcripts was regularly seen between each genotype. Within a single genotype, the sequence variation is 4-5% (Kato *et al*, 1990; Kato *et al*, 1992).

1.1.2. Systems for studying Hepatitis C Virus

1.1.2.1. Cell culture system

Many trials have been made to use cultured mammalian cells to study HCV replication. First, the cultured hepatocyte cell lines susceptible to HCV infection have been screened by inoculating with HCV-positive sera. For instance, primary human hepatocytes, HepG2, Huh-7, and non-neoplastic immortalized PH5CH cells have been reported to be susceptible to HCV infection (Iacovacci *et al*, 1997; Kato *et al*, 1996; Seipp *et al*, 1997; Tagawa *et al*, 1995). Second, infectious HCV cDNA clones were isolated after intrahepatic injection of chimpanzees with RNA transcripts (Hong *et al*, 1999; Kolykhalov *et al*, 1997). Third, HCV subgenomic replicons containing coding regions for minimum replicase proteins and an antibiotic gene for selection were developed as cell culture systems for HCV propagation (Blight *et al*, 2000; Lohmann *et al*, 1999). The subgenomic replicon systems supplied information about roles of non-structural proteins in HCV

replication. Full length HCV replicon systems have also been established (Lindenbach *et al*, 2005). Most recent achievement is a virus replication system established using cloned full length HCV genome derived from JFH-1 genotype 2a (Wakita *et al*, 2005). The improvement on this JFH-1-based system has been obtained by using permissive cells derived from Huh-7 cells (Zhong *et al*, 2005), facilitating the study of the virus-host interactions.

1.1.2.2. Animal model system

The chimpanzee is an animal model susceptible to HCV infection. The chimpanzee infected with HCV suffered acute and chronic hepatitis (Bradley, 2000). However, this large animal has scarcity value and costs much for maintenance. Recently, a mouse model based on an immunodeficient mouse transplanted with normal human hepatocytes has been established (Mercer *et al*, 2001). This first small animal model for studying HCV replication *in vivo* will promote the development of antiviral therapies.

1.1.3. Therapy strategies for Hepatitis C

Although considerable efforts are made to reduce the incidence of new HCV infections, it is predicted that in the near future the HCV infection will still keep prevalent (Brown & Gaglio, 2003). Interferon (IFN)-based therapies are major therapies for hepatitis C currently in clinics. However, the doctors are headached

about the limited efficiency and the side effects of interferon (Feld & Hoofnagle, 2005). Alteration of host signaling pathways by the interaction between specific viral proteins and cellular target proteins is another molecular basis of HCV pathogenesis and persistence (Davis *et al*, 1999; Neddermann *et al*, 1997) and another impediment for efficient anti-viral therapies. Thus, new treatment approaches for more efficacious and less resistant therapy are introduced. Among them, 'HCV-targeted drugs' that target on the specific steps in the viral life cycle, such as the NS3-4A protease inhibitors as well as inhibitors of the NS5B protein, are examples of novel anti-viral agent (Chen, 2005; Lamarre *et al*, 2003). Recently, RNA interference has also been developed in the trial of anti-HCV therapy (Benitec, 2005; Kapadia *et al*, 2003; Kronke *et al*, 2004). Novel immunomodulatory agents that can enhance the host immune response have also been investigated (Coley, 2005; Horsmans, 2004). In addition, both humans and chimpanzees have shown natural immunity to the hepatitis C virus (Bassett *et al*, 2001; Mehta *et al*, 2002), and the vaccine efficacy has been observed in the chimpanzee challenge model. The development of the vaccine against the heterogeneous HCV pathogens becomes a potential policy for HCV prevention. To this end, the use of the recombinant HCV E1 and E2 envelope glycoproteins as vaccine antigens has been studied (Choo *et al*, 1994; Ralston *et al*, 1993); and also the use of attenuated or defective vectors expressing multiple HCV gene products to stimulate host immune responses has been examined (Pearse & Drane, 2005; Polakos *et al*, 2001).

1.2. HCV non-structural protein 5A (NS5A)

1.2.1. Some Structural features of NS5A protein

1.2.1.1. Post-translational modification of NS5A protein

1.2.1.1.1. Two phosphorylated forms of NS5A protein

Native NS5A protein is a large (56-58kDa), hydrophilic phosphoprotein. It has two phosphorylated forms. One is termed as p56, basally with the phosphorylation at its serine residues in the C-terminal half of NS5A, located between amino acids 228-278 and 379-447 (Fig. 1-2). Another is termed as p58, with hyperphosphorylation in its central region and dependent on NS4A modulation (Kaneko *et al*, 1994; Tanji *et al*, 1995).

Fig. 1-2 Structural and functional scheme of NS5A protein

Full length NS5A protein is 447 amino acids long. Two basal phosphorylation regions of NS5A protein are positioned in the C-terminal part of the protein (amino acids 228-278 and amino acids 379-447). Four identified hyperphosphorylated sites (Ser2194, Ser2197, Ser2201 and Ser2204 (amino acid 222, 225, 229, 232)) are shown in red circled ‘P’ (Kaneko *et al*, 1994; Katze *et al*, 2000; Tanji *et al*, 1995). The caspase cleavage sites (Asp154, Asp389) for releasing a 31 kDa fragment with transcriptional activity localizing to nucleus are shown (Sato *et al*, 2000). The location of nuclear localization signal (NLS) is indicated (amino acids 354-362) (Ide *et al*, 1996). Three domains are identified by trypsin digestion (Tellinghuisen *et al*, 2004). The domain 1 (amino acids 1-213) contains N-terminal amphiphatic α -helix (Brass *et al*, 2002; Penin *et al*, 2004) and zinc binding motif (Tellinghuisen *et al*, 2004; Tellinghuisen *et al*, 2005). The C-terminal region of NS5A protein is domain 2 (amino acids 250-342) and domain 3 (amino acids 356-447). Two low

complexity sequence blocks (LCS I and LCS II) are loops connecting different domains. Two Src-Homology 3 (SH3)-binding motifs are also indicated (Tan *et al*, 1999). The BH domains in NS5A are shown (Chung *et al*, 2003). The presented major functional domains of NS5A protein are interferon sensitivity determining region (ISDR) (Enomoto *et al*, 1995; Enomoto *et al*, 1996), PKR interaction region (Gale *et al*, 1997), NS5B binding regions (Shirota *et al*, 2002), FKBP38 binding region (Wang *et al*, 2006), TRAF2 interaction region (Park *et al*, 2002), p53 interaction regions (Lan *et al*, 2002; Majumder *et al*, 2001), Grb2 interaction (Tan *et al*, 1999), and the transcriptional activation domain (Fukuma *et al*, 1998).

The basal phosphorylation of the NS5A p56 form is mediated by the CMGC kinase family, such as casein kinase II (CKII), mitogen activated protein kinases (MAPK) and glycogen synthase kinase 3 (GSK-3) (Reed *et al*, 1997). The major sites of hyperphosphorylation are within a serine-rich region in the central region of NS5A protein. However, the hyperphosphorylation sites are not well conserved throughout different HCV genotypes. It is demonstrated that Ser2194 (amino acid 222 of NS5A) was the major hyperphosphorylation site in an HCV-1b NS5A (Katze *et al*, 2000). Additionally, Ser2197, 2201 and 2204 (amino acids 225, 229, 232) are showed as hyperphosphorylated sites for HCV-1b NS5A (Fig. 1-2) (Tanji *et al*, 1995). In the case of an HCV-1a NS5A, Ser2321 (amino acid 349 in NS5A) is reported to be the major hyperphosphorylation site (Reed *et al*, 1997). CKI- α (casein kinase I- α) is newly discovered to be responsible for both basal and hyperphosphorylation of NS5A. *In vitro* experiments showed that NS5A after pre-phosphorylation of Ser2201, Ser2204 was favored to the phosphorylation by CKI- α (Quintavalle *et al*, 2007). The other candidate kinases responsible for NS5A

hyperphosphorylation are still waiting to be elucidated. However, it has been observed that hyperphosphorylation of NS5A is dependent on the presence of other HCV non-structural proteins. Except for NS4A, the NS3-NS4A-NS4B-NS5A complex is also suggested to participate in the differential phosphorylation of NS5A protein (Neddermann *et al*, 1999). In addition, the p58 form of NS5A with a half life of 7 hours in replicon cells is less stable than p56 form, which showed a half life of 16 hours. This suggests that the two different phosphorylated forms of NS5A protein might play distinct roles in the virus replication cycle (Huang *et al*, 2007; Pietschmann *et al*, 2001).

1.2.1.1.2. Other possible observed post-translational modifications

It was reported that the native intact NS5A is mainly localized on endoplasmic reticular (ER) membrane via its N-terminal membrane anchor domain (Brass *et al*, 2002) (Fig. 1-2). However, the N-terminal truncated NS5A localised to the nucleus (Satoh, S., et al., 2000). This supports the prediction that there is a putative nuclear localization signal (NLS) within the second C-terminal region of NS5A and the proteolytic processing is responsible for activating this functional NLS at the position of amino acids 354-362 (Ide *et al*, 1996). Cell-based cleavage in both of the N-terminal and C-terminal regions of NS5A protein took place in the cells transfected with full-length NS5A. This proteolytic processing was enhanced during the apoptosis progress and mediated mainly by caspase-like proteases (Fig.

1-2). Both of the resulting selective N-terminal or C-terminal truncations were reported to have the ability of migrating into nucleus (Sato *et al*, 2000). One 31 kDa truncated fragment, which is localizing at nucleus, showed transcriptional activation after phosphorylation by PKA (Ide *et al*, 1997). Controversially, other investigations showed that the caspase-mediated C-terminal truncated HCV-1a NS5A fragments were mainly localized in cytosol and behaved non-relevantly to transcriptional activation (Kalamvoki *et al*, 2006). The effects of caspase-mediated cleavage of NS5A on the viral life cycle still remain to be elucidated.

1.2.1.2. N-terminal α -helical membrane anchor domain NS5A

NS5A is a membrane associated, essential component of the viral replication complex. The three-dimensional structure of synthetic peptide derived from the membrane anchor domain of NS5A (N-terminal 30 amino acids) was determined by NMR spectroscopy (Penin *et al*, 2004), which shows an α -helix extending from 5 to 25 residues. The hydrophobic Trp-rich side of this helix was implanted in detergent micelles, while the charged polar side was exposed to the environmental solvent. The in-plane amphipathic α -helical structure of NS5A membrane anchor domain enabled the N-terminal region of this molecule embedded in the membrane bilayer. These are basic elements for localizing NS5A protein to the ER membrane (Brass *et al*, 2002) and prove its essential role in HCV RNA replication. The overall amphipathic nature of the helical NS5A membrane anchor domain 1 is

conserved in different HCV genotypes. Recent study, by using the quartz nanosensor coated with cell-derived membranes, proposed that the N-terminal amphipathic anchor helix of NS5A protein can bind to the model lipid membranes. Moreover, one protein receptor in the membrane is probably required for NS5A binding (Cho *et al*, 2007). This offers a dynamic description for how the NS5A protein is localized to the ER membrane.

1.2.1.3. Three domains of HCV NS5A

According to secondary structure prediction (Tellinghuisen *et al*, 2004), the N-terminal half of NS5A, which is the most conserved region of NS5A, contained β -strand and α -helix elements with a small number of random coil. The center part of the protein is primarily α -helix in secondary structural level, whereas the C-terminal region of the protein was predicted to be mostly random coil structure, which has lower sequence conservation. It is suggested that NS5A is a three-domain protein, with two regions of low complexity sequences (LCS), designated as LCS I (amino acids 214–249) and LCS II (amino acids 343–355). As shown in Fig. 1-2, based on the location of LCS and the results of trypsin cleavage, the NS5A protein was thought to be divided into three domains: domain 1 (amino acids 1–213), domain 2 (amino acids 250–342), and domain 3 (amino acids 356–447).

Structural study by x-ray crystallography revealed that the domain 1 (amino acids 36-198) of HCV-1b NS5A protein mainly has β -sheet conformation whereas with small helical regions and a disulphide bond. The NS5A domain 1 was reported in an asymmetric dimer formation with two identical monomers packed via contacts close to the N-terminal regions of each monomer.

Shown in Fig. 1-3, two subunits were defined within the domain 1. The Subdomain 1A (amino acids 36–100) includes an N-terminal stretched random coil, which is close to a three-stranded anti-parallel β -sheet, and a small α -helix adjacent to C terminus of the third β -strand. A novel structural scaffold for a four-cysteine zinc-atom-coordination site is formed based on the described structural elements of Subdomain 1A. The four cysteine residues (Cys 39, Cys 57, Cys 59 and Cys 80) participating in zinc coordination were highlighted in Fig. 1-3. The position of zinc coordination site suggests that zinc probably plays an important role in maintaining the fold status of NS5A domain 1.

Subdomain 1B (amino acids 101-198) comprises a four-strand anti-parallel β -sheet and a small, two-strand anti-parallel β -sheet adjacent to the C terminus, surrounded by broad regions of random coil structures. Close to the C terminus of domain 1, one disulphide bond was observed, connecting the conserved residues Cys 142 and Cys 190. One covalent link is formed between the loop of β -strand B6 and the C-terminal stretch of strand B9.

The domain 1 shows uneven charge distribution. The N-terminal subdomain 1A region is predominantly basic, whereas the C-terminal subdomain 1B has an almost exclusively acid surface. This contrast between the charge distributions of the two subunits of domain 1 is probably one cause of the dimeric formation of the protein. The basic surface of the N-terminal domain 1, which is near the anchor helix is consistent with the fact that the N terminus region of NS5A protein embeds in the membrane bilayer by contacting with the negatively charged polar head of the membrane. One dimer groove was identified on the domain 1 by analysis of the charge surface of the protein. In the modeling analysis, the groove faces away from the membrane, just like a RNA-binding pocket, showing the potential ability of binding either single- or double-stranded RNA. The structural characters of NS5A domain 1 supports that NS5A protein has an essential roles in HCV replication through RNA binding (Tellinghuisen *et al*, 2004; Tellinghuisen *et al*, 2005).

Fig. 1-3 Structure of NS5A domain 1

The structure of NS5A domain 1 in ribbon diagram (PDB ID: 1ZH1) (Tellinghuisen *et al*, 2005). It comprises subdomain 1A (amino acids 36-100) and subdomain 1B. The β -sheet regions are shown in yellow, the helical regions are shown in red and the loop regions are shown in green. The coordinated zinc atom is blue, surrounded by four cysteine residues (Pink) (Cys 39, 57, 59, 80). The figure was generated by PyMOL (DeLano, 2002).

However, similar predictive results for secondary structure composition were obtained for the C-terminal-half of the HCV NS5A protein. Contrast to the compact well-folded domain 1, the C-terminal-half of the protein was primarily random coil structure in prediction, with small parts of secondary α -helical structure, near the

central region of the NS5A (Tellinghuisen *et al*, 2004). The C-terminal region of NS5A (from amino acid 200 to the C-terminus) has recently been proposed to be one intrinsically disordered proteins (Penin *et al*, 2004). The domain 2 of NS5A genotype 1a has been characterized by NMR study as natively unfolded, but functionally active by interacting with its biological partners (Liang *et al*, 2006; Liang *et al*, 2007). The flexible nature of NS5A domain 2 is consistent with the multiple functions of this domain via binding to different biochemical molecules.

1.2.1.4. Other structural features of NS5A

Three proline-rich motifs, which presents in many signaling proteins responsible to cellular Src-Homology 3 (SH3)-binding, can be found in NS5A protein (Tan *et al*, 1999). The N-terminal motif (amino acids 26-32) is predicted to be imbedded in the previously mentioned amphipathic helical anchor domain and unavailable for binding to other proteins. Near the C-terminus of NS5A is the polyproline cluster (amino acids 343-348, 351-356), predicted to be exposed on the surface. The predicted extended α -helix can bind to SH3 domains (Fig. 1-2). The N-terminal motif is only observed in HCV-1 isolates, whereas the C-terminal SH3-binding cluster is definitely conserved all through the HCV isolates.

1.2.2. Functions of NS5A protein

Though the role of NS5A in the HCV life cycle is not fully understand, its multiple functions of involving in virus replication, viral resistance to IFN, anti-apoptosis, regulation of cellular calcium level, transcriptional activation or repression, have been reported and attract the attentions of many researchers.

1.2.2.1. Roles in Hepatitis C virus replication

As originally reported, (Lohmann *et al*, 1999), the stable human hepatoma cells transfected with HCV genomic RNA replicons, autonomously replicated RNA and expressed large amounts of NS3-NS5B proteins. This suggests that NS5A is involved in genomic RNA replication.

1.2.2.1.1. NS5A is a part of HCV replication complex

Studies via HCV replicon system have confirmed that the non-structural proteins of hepatitis C virus form a complex catalyzing the viral replication and NS5A is one element of this complex (Mottola *et al*, 2002). Later, the identification of all the HCV non-structural proteins from an extracted RNA-protein complex of replicon cells proved that NS5A is a participant (Waris *et al*, 2004). After three mutations were introduced into the N-terminal α -helical membrane anchor domain of NS5A, the HCV replicons lost their resistance to G418 (Elazar *et al*, 2003). This implies

that NS5A is involved in HCV RNA replication, also suggests that its membrane association is indispensable during this replication.

1.2.2.1.2. NS5A interacts with NS5B and other non-structural proteins

The formation of the multiple complexes in viral replication suggests specific interaction between NS5A and other non-structural proteins.

NS5B is the C-terminus 66 kDa membrane-associated protein encoded by the HCV genome. NS5B is an RNA-dependent RNA polymerase (RdRP) responsible for viral RNA synthesis (Miller & Purcell, 1990). As a key component involved in HCV RNA genome replication, this enzyme uses the viral genome RNA as a template to catalyze the synthesis of a complementary (-)-stranded RNA, and the subsequent synthesis of genomic (+)-stranded RNA from this (-) RNA template. To date, the molecular structures of HCV-1b NS5B polymerase have been revealed in several crystal forms (Ago *et al*, 1999; Bressanelli *et al*, 1999; Lesburg *et al*, 1999). Like the structures of the other RNA-dependent RNA polymerase, HCV NS5B polymerase can be subdivided into the fingers, palm and thumb subdomains. However, the notable structural difference between NS5B polymerase genotype 1b and other classes of polymerases is the completely encircled active site of this polymerase (Fig. 1-4) (Lesburg *et al*, 1999). Based on 75% amino acids sequence identity, the overall structure of the HCV-2a NS5B polymerase of crystal form I or

II is similar to that of HCV-1b NS5B polymerase, except that the crystal form II of HCV-2a showed more variations compared to that of HCV-1b (Biswal *et al*, 2005).

Fig. 1-4 Structure of HCV NS5B RNA-dependent RNA polymerase

A ribbon diagram of the structure of HCV NS5B RNA-dependent RNA polymerase (PDB ID: 1C2P). It comprises three subdomains: fingers (red), thumb (pink) and palm (light blue). The two antiparallel helices connecting the subdomains of palm and fingers are shown in yellow. C-terminal region that is partly responsible for membrane anchor of the polymerase is shown in cyan. The active sites of NS5B polymerase are indicated by residue names. The figure was generated by PyMOL (DeLano, 2002).

Recently, a more detailed study was performed on the formation of HCV NS5A and NS5B complex. In vitro glutathione S-transferase (GST) pull-down assay proved that purified bacterial recombinant NS5A and NS5B directly interact with

each other. Two regions of NS5A (amino acids 105-162 and 277-334) were found to be responsible for NS5B binding (Shirota *et al*, 2002). Additionally, four discontinuous sequences of NS5B (amino acids 139-145, amino acids 149-155, amino acids 365-371, and amino acids 382-388) were found to be essential for binding to NS5A (Qin *et al*, 2001). The interacting surface for NS5A protein on NS5B polymerase may be on the shorter loop and on β 14, β 15, and β 16 of the early parts of the thumb subdomain (Fig. 1-5). The important NS5A-NS5B complex was also detected *in vivo* (Qin *et al*, 2001). Interestingly, NS5A at a low concentration (<1:10 ratio to NS5B) had a positive role on NS5B RNA-dependent RNA polymerase activity, but became inhibitory at higher concentrations (Shirota *et al*, 2002). Based on these observations, it is possible that NS5A has a modulating role of in HCV replication, through regulating NS5B activity. More recent analysis addressed NS5A-NS5B interaction on HCV RNA replication in an HCV replicon system. It was shown that the non-function of subgenomic replicon could be caused by deletions of the regions within NS5A to interact with NS5B (Shimakami *et al*, 2004). This strongly suggests that the interaction between NS5A and NS5B is critical for HCV RNA replication.

Fig. 1-5 NS5A binding sites on HCV NS5B polymerase

A) Ribbon diagram of HCV NS5B polymerase with four NS5A binding sites shown in yellow (amino acids 139-145, amino acids 149-155, amino acids 365-371, and amino acids 382-388) (PDB ID: 1C2P). Three subdomains of NS5B polymerase are fingers (red), thumb (pink) and palm (light blue). B) A 90° rotation showing the top-down view of the NS5A binding sites (yellow) on NS5B polymerase. The figure was generated by PyMOL (DeLano, 2002).

Data has shown that NS5A is anchored to the ER membrane and NS5B is also localized to ER via its C-terminal 21 amino acids (Schmidt-Mende *et al*, 2001). Colocalization and specific interaction between NS5A and IFN-induced double-stranded RNA-activated PKR in cells were also detected by Fluorescence Resonance Energy Transfer (FRET) (Stefano, 2002). This suggests possible cellular colocalization of the three proteins. It was observed that NS5B might

undergo post-translational phosphorylation (Hwang *et al*, 1997). Certain serine and threonine residues in NS5B can be phosphorylated in cells. As demonstrated in the *in vitro* RNA polymerase assays (He J., 2001), such modification could positively regulate the catalytic efficiency of NS5B. However, during recent evaluation of HCV non-structural proteins as substrates for PKR, it was observed that NS5B can be phosphorylated quite efficiently by PKR. It is interesting that NS5A can inhibit PKR-mediated phosphorylation of NS5B *in vitro*, whereas the phosphorylation of eIF2- α , native substrate for PKR, was not affected (Huang *et al*, 2004). Probably the observed effect of NS5A was due to specific binding to NS5B rather than inhibition of PKR. Nevertheless, the exact effect of the interaction between NS5A and NS5B on the activities of NS5B polymerase remains to be deeper addressed.

Evidences from studies of protein-protein interaction demonstrate that NS5A was capable of interacting with all the other non-structural proteins (Dimitrova *et al*, 2003). The previous observations show that the formation of hyperphosphorylated NS5A required the presence of other non-structural proteins. So it is clear that NS5A is one part of a multi-protein replication complex. Further researches revealed the importance of phosphorylated NS5A in the structure and function of this multi-component replication complex. It is proposed that basally phosphorylated NS5A binding to hVAP-A may help or stabilize the interaction between NS5A and NS5B. Hyperphosphorylated NS5A was unable to bind to hVAP-A and results in a disrupted replicase complex and inhibited viral RNA

replication (Evans *et al*, 2004; Gao *et al*, 2004).

1.2.2.1.3. NS5A is a zinc metalloprotein

Comparative sequence analysis of NS5A suggested the presence of a highly conserved zinc metal ion coordination motif within the N-terminal domain. In the replicon system mutation analysis indicated that the conserved central CPC (Cys Pro Cys) tripeptide sequence of the HCV NS5A zinc binding site is required for RNA replication. Atomic absorption analysis and x-ray crystallography demonstrated that each NS5A molecule contained one zinc atom, coordinated with Cys39, Cys57, Cys59, and Cys80 (Tellinghuisen *et al*, 2004; Tellinghuisen *et al*, 2005) (Fig. 1-2, Fig. 1-3). It has been demonstrated that zinc coordination in proteins participates in enzyme activities, nucleic acid interactions, metabolic regulations, protein-protein interactions, and structural fold maintenance (Berg & Shi, 1996). The observations of the mutations of central cysteine residues were lethal for RNA replication suggests that the NS5A zinc binding site is vital for HCV replication (Tellinghuisen *et al*, 2004).

1.2.2.1.4. NS5A participates in virus-host interactions

NS5A has been reported to interact with host proteins and participate in the regulation of HCV replication. Subgenomic replicons (Blight *et al*, 2000) and full

length HCV replicon system based on JFH-1 genotype 2a (Wakita *et al*, 2005; Zhong *et al*, 2005) have facilitated the study of biological roles of NS5A in HCV replication. For instance, the recent study about the functions of domain II and III of NS5A protein in HCV RNA replication employed the subgenomic replicons (Tellinghuisen *et al*, 2008). NS5A protein was reported to interact with human butyrate-induced transcript 1 (hB-ind1), which was indicated in regulation of HCV replication by using JFH-1-based system (Taguwa *et al*, 2008). Disruption of the interaction between NS5A and another host protein, a TBC (TRE-2/BUB2/CDC16 genes) domain protein containing Rab-GTPase-activating protein (Kolykhalov *et al*) domain, was reported to impair HCV RNA replication (Sklan *et al*, 2007). Ten host proteins including HSP90-associated protein 1, HSPA1A and the receptor recycling protein, VPS35, were identified as interacting proteins with NS5A (Randall *et al*, 2007).

1.2.2.2. Roles in resistance to IFN

The current therapy for HCV combines ribavirin and α interferon (IFN- α), whereas the response rate for the treatment is between 48% (HCV-1, -4, -5 and -6) and 88% (HCV-2 and -3). Two predominant HCV genotypes, HCV-1a and HCV-1b, both exhibit a high level of resistance to IFN therapy. Sequence heterogeneity in NS5A was identified in complete HCV genomic sequences from IFN responder and nonresponder patients, which is defined as an interferon sensitivity-determining

region (ISDR) (amino acids 237-276) (Fig. 1-2) (Enomoto *et al*, 1995; Enomoto *et al*, 1996). This attracts great attention on the possible role of NS5A in resistance to IFN therapy.

IFN-induced double-stranded RNA-activated kinase (PKR) is one of the prominent, well-characterized mechanisms by which IFN mediates antiviral activity. Once activated by IFN, PKR undergoes autophosphorylation and inhibits initiation of translation by phosphorylation of α subunit of eukaryotic initiation factor 2 (eIF2- α) (Hershey, 1991; Williams, 2001). Early data have shown that NS5A bound to and inactivated PKR (Gale *et al*, 1997), which required the ISDR and an additional 26 residues C-terminal to the ISDR in NS5A (Gale *et al*, 1999) (Fig. 1-2, Fig. 1-6). Within PKR the binding site was identified as the dimerisation domain (amino acids 244-366) including T258, a regulatory autophosphorylation site and the following protein kinase catalytic domain conservation regions. However, NS5A itself is not a substrate of PKR. Binding of NS5A to PKR resulted in the disruption of protein kinase dimerization and inhibition of both PKR autophosphorylation and phosphorylation of eIF2- α , its own substrate (Gale *et al*, 1999). The colocalization and specific interaction between NS5A and PKR in mammalian cells have also proved (Stefano, 2002). These suggest that the NS5A protein is contributed to viral survival and resistance to the inhibition of viral mRNA translation via activating PKR upon IFN treatment.

As a proinflammatory chemokine, IL-8 also can disrupt IFN-induced antiviral responses perhaps at a post-transcriptional stage (Khabar *et al*, 1997). The capability of NS5A to induce interleukin-8 (IL-8) expression both at mRNA and protein level gives another explanation for viral resistance to IFN. The increased expression level of IL-8 regulated by NS5A protein resulted in the inhibited IFN efficiency and viral survival (Girard *et al*, 2002; Polyak *et al*, 2001). Additionally, patients infected with hepatitis C virus but having higher levels of IL-8 in serum are particularly non-responsive to IFN therapy (Polyak *et al*, 2001). Other possible mechanisms for NS5A mediated IFN resistance probably include suppressing STAT1 phosphorylation (Lan *et al*, 2007), removing nuclear translocation activators and signal transducer, which are induced by IFN- α treatment (Gong *et al*, 2007). However, some contradictory reports suggest that NS5A protein is not responsible for viral IFN resistance (Aus dem Siepen *et al*, 2005; Brillet *et al*, 2007).

1.2.2.3. Anti-apoptosis

Apoptosis, named as programmed cell death (PCD), is a mechanism for cells suicide. Its characters include blebbing of the cell membrane, chromatin condensation, cleavage of DNA into nucleosomal size fragments, fragmentation of the cell with retention of cell membranes and rapid engulfment of the dying cell by

macrophages. Apoptosis plays an important role in normal development and as a defense against viral infection. Inhibition of apoptosis can lead to cancer (Thompson, 1995).

It is reported that PKR and phosphorylation of eIF-2 α are necessary components in the pathway of double-stranded RNA (dsRNA) mediated apoptosis (Neddermann *et al*, 1997; Srivastava *et al*, 1998). During HCV infection NS5A protein can disrupt PKR-dependent dsRNA signaling and apoptotic programs (Gale *et al*, 1999). It was observed that constitutive expression of NS5A from IFN-resistant HCV can block Ser51 phosphorylation of eIF2 α , which is an essential factor for dsRNA-induced apoptosis (Fig. 1-6). This suggests that during infection HCV can not only resist the antiviral effects imposed by IFN but also escape from the host clearance via apoptotic pathway - the activation of PKR by viral dsRNA. Naturally, the anti-apoptosis functions of NS5A are retrieving more and more attentions.

Fig. 1-6 Anti-apoptosis pathways of NS5A protein

Activated PKR and phosphorylation of eIF-2 α are required in the apoptosis induced by dsRNA. NS5A protein inactivates PKR and phosphorylation of eIF-2 α , resulting in resistance to the IFN-induced apoptosis (Gale *et al*, 1999). NS5A is capable of translocating pro-apoptotic Bax from mitochondria to the nucleus (Chung *et al*, 2003), or Bad to cytosol (Street *et al*, 2004). Binding of FKBP38 might facilitate the localization of NS5A to mitochondria and exert its anti-apoptosis functions (Wang *et al*, 2006). NS5A binds to the TNF α adaptor proteins, TRADD and TRAF2 and inhibits the activation of cellular caspases and execution of apoptosis (Park *et al*, 2002). Finally, NS5A can sequester p53, the tumour suppressor protein, in cytoplasm from translocation to nucleus (Lan *et al*, 2002; Majumder *et al*, 2001). In all, NS5A mediated signaling perturbation is indicated by red lines whereas ‘normal’ host signaling pathway is in black lines. The red ‘//’ indicates the block of normal host pathway by NS5A.

1.2.2.3.1. NS5A is a potential viral Bcl-2 homologue

Bcl-2 is an important regulator of apoptosis, which enhances cell survival by inhibiting apoptosis rather than promoting cell proliferation. Bcl-2 family contains multigenes localize to the mitochondria and nuclear membranes, which are highly conserved evolutionarily with viral homologues, generally share 20-30% homology. At least 16 Bcl-2 homologues have been discovered In humans (Reed, 1999), including Bcl-2, Bcl-X_L, Bcl-w and Mcl-1, which are anti-apoptotic, and Bad, Bak, Bax, Bid, Bim and Bcl-Xs, which are pro-apoptotic. The latter antagonize inhibition of apoptosis by binding to Bcl-2 and other anti-apoptotic members, through Bcl-2 homology (BH1, BH2, BH3, and BH4) domains. Hence the equilibrium between the pro-and anti-apoptotic Bcl-2 family proteins determines the progress of the cell death (Cory & Adams, 2002). Nearly all viral Bcl-2 homologues contain conserved BH1 and BH2 domains. However, in viral homologues the BH3 domain probably important for killing cells is poorly conserved. Little homology is shared among the viral homologues in the N terminal BH4 domain. Interestingly, viral Bcl-2 proteins only keep the anti-apoptotic function (Hardwick & Bellows, 2003).

In hepatocellular carcinoma (HCC) cells treated with sodium phenylbutyrate (NaPB), NS5A can antagonize the apoptosis program, which is independent with p53. To illustrate more mechanisms about the anti-apoptosis functions of NS5A

proteins, Chung *et al* points out that though the overall sequence identity between NS5A and other Bcl-2 homologues is low, NS5A is a potential viral Bcl-2 homologues with conserved BH3, BH1 and BH2 domains, in the same linear order as other anti-apoptotic viral Bcl-2 homologues (Fig. 1-2) (Chung *et al*, 2003). In NS5A the BH3 and BH2 are highly conserved, while the BH1 domain is less conserved. In the NS5A expressing Hep3B cells, NaPB treatment triggered relocalization of both Bax and NS5A from the mitochondria to the nucleus interior (Fig. 1-6). NS5A co-localizes and interacts with Bax in the nucleus. Consequently, Bax was retained inside the nucleus without finally moving to the nuclear membrane and initiate caspases activation. It was demonstrated that the BH domains especially BH2 domain are required components for the association between NS5A and Bax. Structural analysis of NS5A protein will give complete and detailed information for the interaction domains. BH domains were reported to be important for the evasion from apoptosis by several viruses such as Epstein Barr virus (EBV) and herpes virus saimiri (HVS) during their infection (Benedict, 2002; Tschopp *et al*, 1998). This implies that to achieve persistent infection, HCV might also adopt similar tactics to escape from apoptosis. Additionally, NS5A mediated Akt activation and induced the phosphorylation of Bad, another pro-apoptotic Bcl-2 homolog. The phosphorylated Bad was released from mitochondria to cytosol and lost its pro-apoptotic ability (Street *et al*, 2004).

38 kDa FK506-binding protein (FKBP38) is a member of immunosuppressant FK506-binding proteins (FKBPs) family, which belongs to the family of peptidylprolyl cis/trans isomerases (PPIase). PPIase is reported to participate in cellular signaling, transcription and protein tracking (Breiman & Camus, 2002). As a chaperone protein, FKBP38 can bind to anti-apoptotic molecule Bcl-2 or Bcl-X_L and target them into the mitochondria to exert their anti-apoptosis functions (Kang *et al*, 2005; Shirane & Nakayama, 2003). The removal of FKBP38 by specific siRNA resulted in the disruption of the anti-apoptotic function of Bcl-2 protein (Kang *et al*, 2005).

Recently studies showed that FKBP38 specifically interacted with the domain 1 of NS5A protein (Fig. 1-2) and the anti-apoptosis function of NS5A was inhibited by silencing FKBP38 expression (Wang *et al*, 2006). Another family member of FK506-binding proteins, FKBP8, which is similar to FKBP38 in sequence, but without several N-terminal residues for PPIase activity and FK506 binding (Lam *et al*, 1995), has also been reported to interact with NS5A protein. The complex formed by FKBP8, NS5A and Hsp90 was proved to be critical for viral replication. The TPR domain in FKBP8 was thought to be responsible for binding to NS5A protein (Okamoto *et al*, 2006). In addition to the fact that cyclophilin B can bind to NS5B polymerase and regulate viral replication (Watashi *et al*, 2005), the newly discovered molecular interactions between NS5A and FKBP38 or FKBP8 provide more information about the functions of the immunosuppressant FK506 binding

proteins in HCV infection. As previously described, three Bcl-2 homologues are positioned in NS5A protein. BH1 domain is inside the domain 1 of NS5A, which is thought to be the region interacting with FKBP38 or FKBP8. *In vitro* studies showed that the region between Bcl-2 BH3 and BH4 domains is responsible for the interaction with FKBP38 (Kang *et al*, 2005). This raises the speculation that NS5A may imitate Bcl-2 to exert anti-apoptosis functions.

1.2.2.3.2. NS5A impairs TNF-Mediated hepatic apoptosis

TNF or Fas both are important for initiating apoptosis to remove viruses in host upon viral infection (Gaur & Aggarwal, 2003; Itoh *et al*, 1991). Interruption of TNF or Fas activity can potentially disrupt cellular defense, thereby enhancing the likelihood of viral persistence. It has been demonstrated that HCV NS5A inhibits TNF-induced apoptosis in mammalian cells (Park *et al*, 2002). Functional investigation suggests the inhibitory role of HCV NS5A on TNF-mediated apoptosis is via an interaction between NS5A and the TNF α -responsive adaptor protein TRADD (TNF receptor-associated death domain protein). TRADD forms a complex with the activated TNF receptor, Fas-associated death protein - FADD and TRAF2 (TNF receptor associated factor-2) upon ligand binding. A variety of down-stream effectors such as caspases and NF κ B receive the apoptotic signals from this complex. By binding to TRADD and TRAF2, NS5A was able to block these signals and resist apoptosis (Fig. 1-2, Fig. 1-6) (Park *et al*, 2002).

1.2.2.3.3. NS5A interacts with p53

The tumor suppressor p53 protein is involved in the maintenance of genomic integrity (Lane, 1992). However, activated p53 can induce cell cycle arrest and apoptosis (Levine, 1997) by preventing entry into S phase and stopping cells replicating damaged DNA. Several viral proteins, such as SV40 T antigen and HBx from HBV, were reported to bind to p53 and block its function (Jiang *et al*, 1993). Inhibition of p53-mediated apoptosis by these viral oncoproteins may play an important role in carcinogenesis. For instance, HBx protein can form complex with p53 in cytoplasm to partially prevent the nuclear entry of p53 and disturb its ability to induce apoptosis. This may contribute to the early stage of hepatocellular carcinogenesis (Yun *et al*, 2000). It has been demonstrated that HCV NS5A can inactivate cellular genes regulated by p53 response-elements (PRE) and one downstream protein p21/waf1 (Majumder *et al*, 2001). The direct interaction between NS5A and p53 was positioned at the N-terminal region of p53, where other down regulators of p53 such as mdm2 bind. The interaction of NS5A with the p53 consensus DNA-binding site was thought to result in overall inhibition of p53 transcriptional activation. Similarly to the functions of HBx from HBV, via its C-terminal domain (amino acids 359-447), NS5A can bind to and partially sequester p53 in the cytoplasm, leading to the abrogation of p53-mediated function (Fig. 1-2, Fig. 1-6) (Lan *et al*, 2002). For any suppression on the activity of p53 will retard DNA repair and pool the hepatocytes with much unstable genetic

material. The interaction between NS5A and p53 may have some implications for hepatocarcinogenes.

1.2.2.4. Transcriptional modulations

Full-length NS5A was found to be incapable of transcriptional activation. The transcriptional activation region in NS5A protein was mapped to the central ISDR with another separate acidic area and one proline-rich region (amino acids 163-359). All these three parts are thought to be conserved motifs responsible for transcriptional activation (Fig. 1-2). Deletion mutation analysis further refined this transcriptional activation region from amino acid 228 to 284 which covers the ISDR and short flanking regions (Fukuma *et al*, 1998). An IFN-sensitive phenotype with six amino acids changes in the ISDR showed 90-fold higher transcriptional activity than a resistant phenotype without mutated ISDR. After the alanine residue at position A252 was mutated to phenylalanine, the transcriptional activity of NS5A was obviously increased. (Fukuma *et al*, 1998). Studies with yeast and Huh-7 cells have shown that NS5A truncated mutants (without N-terminus) fused with GAL4 DNA-binding domain could act as transcriptional activators (Chung *et al*, 1997; Kato *et al*, 1997). The compelling discovery about the possible nuclear localization of NS5A protein, which was even enhanced by apoptotic stimulation, supplies more evidence of transcriptional activity of this protein (Sato *et al*, 2000).

In contrast, studies by yeast two-hybrid screening has shown that NS5A was able to interact with Snf2-related CREB-binding protein activator protein (SRCAP), which is a newly identified ATPase that can facilitate CREB-binding protein to be a coactivator for several transcription factors (Johnston *et al*, 1999). NS5A regulated transcription via interaction with SRCAP (Ghosh *et al*, 2000). Additionally, perturbations in the p21/waf1 path way were also observed in these studies. Another viral protein, adenoviral E1A was thought to be an interferent on the interaction between SRCAP and CREB-binding protein and resulted in the repression of transcription (Kretsovali *et al*, 1998). HCV may exert the similar way through the interaction between NS5A with SRCAP to inhibit cellular transcription indirectly.

1.2.2.5. Perturbation of mitogenetic signaling pathway and growth control

Viruses have to regulate mitogenic signaling pathways of host cells to control cell growth and keep a persistent infection. MAPK, a serine/threonine kinases family, was thought to be critical mediators between the cellular response and different kinds of extracellular stimuli. Extracellular signal-regulated kinases (ERK) pathway, the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) pathway and p38MAPK pathway have been characterized as three main mitogenic signaling pathways (Chang & Karin, 2001). It has been suggested that NS5A regulates all of the three MAPK signaling pathways.

First, NS5A was capable of inhibiting the ERK MAPK pathway (Georgopoulou *et al*, 2003). Growth factor receptor-bound protein 2 (Grb-2) can mediate signal transduction (Fretz *et al*, 2000). Grb-2 has Src-Homology 3 (SH3) domains at both N-terminal and C-terminal regions. As described in Fig. 1-2, the interaction with Grb-2 was localized on the C-terminal region of NS5A (amino acids 277-447), which contains the highly conserved proline-rich SH3 binding motif. The binding of NS5A to Grb-2 inhibited the phosphorylation of extracellular signal-regulated kinases (ERK) 1 and 2 and blocked epidermal growth factor (EGF) signaling pathway (Tan *et al*, 1999). Consequently, NS5A was demonstrated to inactivate p38MAPK (He *et al*, 2001) and resulted in repressed phosphorylation of translation initiation factor, eIF4E (Wang *et al*, 1998). Finally, it has been shown that NS5A could activate the stress-stimulated JNK MAPK signaling pathway. However, NS5A indirectly activate JNK via TNF- α stimulation (Park *et al*, 2003). TRAF2 was thought to be a mediator during the activation of JNK by NS5A.

The activation of the ERK pathway is critical for normal hepatocytes to go through a check point in the late G1 stage (Talarmin *et al*, 1999). The down-regulation of MAPK signaling will result in perturbation of host cell cycle. Many viruses, such as reovirus HA3, perturb the cell cycle via G1 arrest to get viral replication in maximum (Saragovi *et al*, 1999). Induction of p21/waf1 is consistent with the arresting of cells at G0/G1 or G2/M stage. It has been described above that NS5A

interact with p53 and resulted in the repression of p21/waf (Majumder *et al*, 2001). NS5A probably promoted cell growth through repressing the levels of p21 and by pass the controls of check point. The final result would be the tumor formation. Direct interaction of NS5A with the cyclin dependent kinase Cdk1 was also reported. Additionally, NS5A was reported to inhibit cell growth by interacting specifically with Cdk1/2-cyclin complexes (Arima *et al*, 2001). The exact role of NS5A in cell cycle control is still ambiguous.

In general, mitotic check point controls generate cell cycle arrest and/or the removal of the cells with cell cycle defects. However, tumor related virus infections can lead to check point defects in cells and help the infected cells to evade from apoptosis, resulting in continuous progression in cell cycle and accumulation of chromosome instability (Jin *et al*, 1998; Lavia *et al*, 2003). For instance, HBx protein, which is expressed during hepatitis B virus infection and the hepatocellular carcinoma (HCC) development, has been reported to induce chromosome instability such as multi-polar spindles, centrosome amplification, and aneuploidy (Forgues *et al*, 2003). Other tumor virus proteins have been reported to promote mitotic abnormalities. They are E1A and E1B of adenovirus, E6 and E7 of papillomavirus, large and small T antigens of simian virus 40 (SV40) (Lavia *et al*, 2003). One recent compelling study shows that HCV NS5A protein over-expressed in hepatocytes was capable of promoting chromosome instability and aneuploidy, which is linked to aberrant mitotic regulations, such as delayed mitotic exit and

other mitotic abnormalities including multi-polar spindles. This permits the cells keep growing with cell cycle defects, which are characterized by irregular isolated chromatids at multiple spindle poles, and stepping forward into anaphase and telophase stages (Baek *et al*, 2006). This provides exciting evidence about direct role of NS5A protein involved in mitotic cell cycle perturbation and the initiation of chromosome instability.

1.2.2.6. Modulation of intracellular calcium and reactive oxygen

HCV NS5A was reported to change intracellular calcium levels, thus inducing oxidative products, reactive oxygen species (ROS) and up-regulating STAT-3 and NFκB (Gong *et al*, 2001). NFκB is critical for the apoptosis in host cells as a regulator on the expression of many proteins such as interferons, cytokines, and adhesion molecules (Kucharczak *et al*, 2003). NS5A is associated with ER and lead to the efflux of calcium from the ER to mitochondria, thereby activating NFκB and translocating NFκB to the nucleus (Waris *et al*, 2003). The cells over-expressing NS5A alone or the entire non-structural proteins, or HCV-infected liver cells also exhibited elevated levels of nuclear NFκB (Tai *et al*, 2000).

The proto-oncogene STAT-3 was also up-regulated by NS5A (Gong *et al*, 2001). It has been known that NS5A has the abilities of interacting with and regulating the activity of Fyn, one member of Src-family kinases (Macdonald *et al*, 2004).

STAT-3 was subsequently phosphorylated by the activated Fyn. The dysregulation of Stat3 was related with liver cancer (Yoshikawa *et al*, 2001). Free radicals, the byproducts of activating STAT-3, have been reported to participate in the cancer development (Sun, 1990). Steatosis and high level of reactive oxygen species were detected in the liver cells of HCV transgenic mice (Lemon, 2000). This suggests that free radicals might be a factor in the etiology of hepatocellular carcinoma. Controversially, shown in two different studies, ROS either inhibited (Choi *et al*, 2004), or stimulated (Qadri *et al*, 2004) viral replication. To understand the exact role of ROS activation in HCV replication, deeper study is clearly required.

1.3. Objective of this project

As described above, HCV non-structural protein 5A (NS5A), a membrane anchored phosphoprotein with two phosphorylated forms, possesses multiple functions in viral replication, IFN resistance (Blight *et al*, 2000; Gale *et al*, 1999; Gale *et al*, 1997; Lohmann *et al*, 2001), and pathogenesis (Harris *et al*, 2004). NS5A is also involved in anti-apoptosis (Chung *et al*, 2003), the disturbance of intracellular calcium and the elevation of reactive oxygen species in mitochondria (Gong *et al*, 2001). Furthermore, NS5A can interact with tumor necrosis factor receptor (TNFR)-associated factor 2, TRAF2 (Park *et al*, 2002), the growth receptor-bound protein 2, Grb2 (Macdonald *et al*, 2004; Tan *et al*, 1999) and p53 (Lan *et al*, 2002; Majumder *et al*, 2001), possibly having effects on their functions.

Structural information will help us to understand the multiple functions of HCV NS5A protein and facilitate drug design. Therefore, we aim to characterize NS5A by biochemical and biophysical methods and study three-dimensional structure of NS5A by NMR or x-ray crystallography. First, different constructs of NS5A protein including full-length or truncated mutations will be constructed in this study for achieving NS5A protein with high quantity and purity from *E. coli*. Circular dichroism (CD) and NMR will be then used to characterize the secondary structural elements of the purified protein. Dynamic laser scattering (DLS) will be also applied to analyze the physical properties of the molecule. The activity of NS5A to interact with its biological partners will be analyzed by *in vitro* GST-pull down assay. We will analyze the three dimensional structure of NS5A protein by NMR or x-ray crystallography.

Although multiple functions of HCV NS5A protein have been reported, the precise functions of this protein remain to be addressed. In this study, we also aim to investigate biochemical function of the NS5A in the mammalian culture model. Our strategies include observation of cell proliferations of the cells stably over-expressing NS5A protein in response to several apoptotic stimuli. The roles of NS5A protein in apoptosis will be analyzed through characterization of several biochemical apoptotic markers. NS5A specific RNA interference will be applied to identify the anti-apoptosis functions of NS5A and give some information about the feasibility of anti-viral therapy by using RNA interference.

Materials and Methods

2.1. Materials

2.1.1. Chemicals

Monoclonal antibody against NS5A was purchased from ID labs (London, Canada). Polyclonal antibody against NS5B, polyclonal anti-PARP antibody, monoclonal anti-GAPDH antibody, and polyclonal antibody against PKR were purchased from Santa Cruz Biotech (CA, USA). Monoclonal anti-flag antibody was from Sigma (St. Louis, MO, USA). PKR-agarose was from Upstate Biotechnology (Lake Placid, NY, USA). Ni²⁺-NTA resin, RNeasy Mini Kit, plasmid purification kit, PCR purification kit, gel extraction kit, *Escherichia coli* BL21 (DE3), Dulbecco's Modified Eagle Medium (DMEM) and carbenicillin were from Qiagen (Hilden, Germany) and Invitrogen (Carlsbad, CA, USA). Glutathione-Sepharose 4B was from Amersham Biosciences (Buckinghamshire, UK). The dNTP (dTTP, dATP, dGTP and dCTP) mixtures were purchased from Invitrogen or Finnzymes (Espoo Finland). The DNA polymerase pfu was purchased from Novagen (Madison, WI, USA). Immun-Star Chemiluminescent protein detection system and protein molecular weight marker were from Bio-Rad Laboratories (Hercules, CA, USA). Isopropyl-thio- β -D-galactopyranoside (IPTG) was from Promega (Madison, WI, USA). HiPrep 26/60 Sephacryl S-100, S- 200

gel filtration columns were from Amersham Biosciences (Buckinghamshire, UK). Phenylmethylsulfonyl fluoride (PMSF), Reverse transcription-polymerase chain reaction (RT-PCR) kit, restriction enzymes, T4 ligase, 1, 4-Dithiothreitol (DTT), Protease inhibitors complete-mini protease tablets were from Roche (Indianapolis, IN, USA). ^{15}N - NH_4Cl and ^{13}C -glucose was from Cambridge Isotope Laboratories (USA). NS5B peptides for NMR titration assay were obtained from GL Biochem. Ltd. (China). All the other chemicals were obtained from Sigma (St. Louis, MO, USA)

2.1.2. Bacterial strains and mammalian cell lines

DH5 α was used for the plasmid purification and *E.coli* (Escherichia coli) BL21 (DE3) was used for the protein expression. Cancer cells HepG2, 293T were used for the analysis of protein expression.

2.1.3. Media

2.1.3.1. Luria-Bertani (LB) medium

1 L LB medium:

10 g bacto-tryptone, 5 g bacto-yeast extract, 5 g NaCl, in 1000 ml ddH₂O, autoclaved.

2.1.3.2. LB agar plates

In the LB medium, 2% agar was added and autoclaved. Plates were poured after

the LB agar cooled to about 50-60 °C.

2.1.3.3. M9 medium (mineral medium)

1 L M9 medium:

52.7 ml 1 M Na₂HPO₄, 26.5 ml 1 M KH₂PO₄, 2 ml 5 M NaCl, 1.2 ml 1 M MgSO₄, 1.2 ml 100 mM CaCl₂, 1.2 ml 0.5%(w/v) thiamine HCl, 22.7 ml 20% glucose, 1 ml antibiotic (1000× stock), 22 ml 1 M NH₄Cl, added autoclaved water to 1 L.

2.1.3.4. NZCYM medium

10 g NZ amine, 5 g NaCl, 5 g yeast extract, 1 g casamino acids and 2 g MgSO₄ • H₂O in 1 L autoclaved water pH 7.0

2.1.3.5. Media for mammalian cell culture

The media for mammalian cell culture include DMEM and Opti-MEMI reduced serum medium, purchased from Invitrogen.

2.1.4. Reagents for cell culture

2.1.4.1. Transfection reagents

OligofectamineTM and Lipofectamine 2000 were purchased from Invitrogen

(Carlsbad, CA, USA), stored at 4 °C.

2.1.4.2. Small interfering RNAs

The small interfering RNAs (siRNAs) used were purchased from Dharmacon (Lafayette, CO, USA), stored at -20 °C.

2.1.4.3. Anti-cancer drugs

Cis-Diammineplatinum (II) dichloride (Cisplatin) was solubilized in DMSO to a stock concentration of 2 mM, 10 mM and 20 mM respectively.

2.1.4.4. Other reagents

MitoTracker® Deep Red 633, Alexa Fluor® 488 goat anti-mouse IgG, and R6, rhodamine B hexyl ester perchlorate were from Molecular Probes (Eugene, OR, USA).

2.1.5. Solutions and buffers

2.1.5.1. Buffers for agarose gel

1 L 50×TAE (Tris-acetate-EDTA)

242 g of Tris base, 57.1 ml of glacial acetic acid, 100 ml of 0.5 M EDTA, pH 8.0, add H₂O to 1 L.

6×DNA loading buffer

0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose

in H₂O.

Ethidium bromide (EB)

10 mg/ml stock in H₂O.

2.1.5.2. Buffers for SDS-PAGE gel

1 L 5×Tris-glycine electrophoresis buffer

5×25 mM Tris (15.1g/L), 5×250 mM glycine (94 g/L) pH 8.3, 5×0.1% SDS (50 ml 10% SDS stock/L)

2×SDS-PAGE gel-loading buffer

100 mM Tris-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2% Bromophenol blue, 20% (v/v) glycerol

1 L Destain solution

450 ml Methanol, 100 ml glacial acetic acid, 450 ml H₂O

Stain solution

0.25% Brilliant Blue R-250, 0.25 g in 100 ml of destaining solution

2.1.5.3. Buffers for Ni²⁺-NTA purification

Lysis buffer

100 mM Tris-Cl, pH 7.0, 200 mM NaCl, 20 mM Imidazole, 1 mM PMSF, 5 mM 2-mercaptoethanol

Washing buffer

100 mM Tris-Cl, pH 7.0, 1 M NaCl, 50 mM Imidazole, 1 mM PMSF, 5 mM 2-

mercaptoethanol

Elution buffer

100 mM Tris-Cl, pH 6.4, 200 mM NaCl, 500 mM Imidazole, 1 mM PMSF, 5 mM

2- mercaptoethanol

2.1.5.4. Buffers for FPLC purification

Gel filtration buffer

50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 0.01% NaN₃.

20 mM NaPO₄, pH 6.5, 50 mM NaCl, 1 mM DTT, 0.01 % NaN₃

Ion exchange buffer

Buffer A: 20 mM NaPO₄, pH 6.5, 1 mM DTT, 0.01% NaN₃

Buffer B: 20 mM NaPO₄, pH 6.5, 1 M NaCl, 1 mM DTT, 0.01% NaN₃

2.1.5.5. Western-blot buffers

1 L Membrane transfer buffer

3.03 g Tris, 14.4 g glycine, add water to 1 L

1 × Tris-buffered saline (TBS)

20 mM Tris, 500 mM NaCl, pH 7.5

Wash solution (TTBS)

20 mM Tris, 500 mM NaCl, 0.1% Tween-20, pH 7.5

Blocking solution

0.2% non-fat dry milk in TBS

Antibody buffer

0.2% non-fat dry milk in TTBS

2.1.5.6. Buffers for cell culture and analysis

PBS

137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄

Cell lysis buffer

50 mM Tris-Cl pH=7.4, 150 mM NaCl, 1% TritonX-100

2.1.5.7. Other stock buffers

1 L 1 M Tris buffer, pH 8.0

121.12 g Tris to 800 ml ddH₂O and using HCl (~50 ml) adjust pH to 8.0, and then adjust to 1000 ml.

1 L 5 M NaCl

292 g NaCl into 1000 ml, filter through 0.2 mm membrane.

2.2. Methods

2.2.1. Plasmid construction

2.2.1.1. Constructions for NS5A proteins for pET-ubiquitin expression system in *E. coli*

The DNA sequence for HCV 1a NS5A was amplified from HCV-1a cDNA (a generous gift of Dr Ding Xiang Liu) by polymerase chain reaction. Table 1 lists the

primers used in the constructions for NS5A proteins expressed in *E. coli*.

Table 1 Oligonucleotides used for the constructions for pET-ubiquitin expression system of NS5A proteins

Oligo name	Sequence
pET-Ub-NS5A-full length-His-for (<i>SacII</i>)	5'-GCGGGTACCCCGCGGTGGATCCGG TTCCTGGCTAAGGGACATC-3'
pET-Ub-NS5A-full length-His-rev (<i>HindIII</i>)	5'-GCGGGTACCAAGCTTCTATTAATGG TGGTGATGGTGGTGACCAGAGGATCC GCACACGACATCCTCCGCGTTGGC-3'
pET-Ub-NS5A-ΔN-His-for (<i>SacII</i>)	5'-GCGGGTACCCCGCGGTGGACTGCC TGGGATTCCCTTTGTGTCC-3'
pET-Ub-NS5A-ΔN-His-rev (<i>HindIII</i>)	5'-GCGGGTACCAAGCTTCTATTAATGG TGGTGATGGTGGTGACCAGAGGATCC GCACACGACATCCTCCGCGTTGGC-3'
pET-Ub-NS5A-D1-His-His-for (<i>SacII</i>)	5'-GCGGGTACCCCGCGGTGGAATGCC ACAACGCTGGGATTCCC-3'
pET-Ub-NS5A-D1-His-rev (<i>HindIII</i>)	5'-GCGGGTACCAAGCTTCTATTAATGG TGGTGATGGTGGTGACCAGAGGATCC CGCCTCTGCTGTTATATGGGAGGG-3'
pET-Ub-NS5A-D2-His-for (<i>SacII</i>)	5'-GCGGGTACCCCGCGGTGGAAAGGC AACTTGCACCGCCAACCA T-3'
pET-Ub-NS5A-D2-His-rev (<i>HindIII</i>)	5'-GCGGGTACCAAGCTTCTATTAATGG TGGTGATGGTGGTGTTTCGTAGTCAGG CTTTTCCACGT-3'
pET-Ub-NS5A-D3-for (<i>SacII</i>)	5'-GCGGGTACCCCGCGGTGGACGGAA AAAGCGTACGGTGGTCCTC-3'
pET-Ub-NS5A-D3-rev (<i>HindIII</i>)	5'-GCGGGTACCAAGCTTCTATTAATGG TGGTGATGGTGGTGACCAGAGGATCC GCACACGACATCCTCCGCGTTGGC-3'

All the forward primers include a *SacII* restriction enzyme site, and the reverse primers include a *HindIII* restriction enzyme site. The PCR product was cloned into

the *Sac*II and *Hind*III sites of pET-UbCHis vector (a generous gift of Prof. Craig Cameron) (Huang *et al*, 2004). The resulting plasmids are listed in Table 1, coding for the different proteins, with C-terminal (His)₆-tag to facilitate purification. All the coding sequences were verified by DNA sequencing.

Fig. 2-1 pET-ubiquitin expression vector for NS5A proteins

pET-UbCHis: This vector will direct expression of a protein with a carboxy-terminal hexahistidine tag. The PCR product was cloned into *Sac*II and *Hind*III sites (Huang *et al*, 2004).

2.2.1.2. Constructed vectors for NS5B proteins expressed in *E. coli*

The DNA sequence for GST-NS5Bt (the truncated mutant of HCV NS5B, which lacks the 21 amino acids at the C-terminal) was amplified from HCV-1a cDNA. The DNA sequence for HCV 1b NS5Bt was amplified from pXJ40flag-NS5B vector (a generous gift from Dr. Yee-Joo Tan) (Choi *et al*, 2004). The next table lists the primers used in the constructions for NS5B proteins expressed in *E. coli*.

Table 2 Oligonucleotides used for the constructions for NS5B proteins expressed in *E. coli*

Oligo name	Sequence
pGEX-4T-1-NS5Bt-for (<i>Nde</i> I)	5'-CACTCAGCATATGTCAATGTCTTATTCCTG GACAGG-3'
pGEX-4T-1-NS5Bt-rev (<i>Xho</i> I)	5'-GCGATCCTCGAGTTACTAGCAGCCCTGCCT CCTCTGGACAGA-3'
pET29-NS5Bt-for (<i>Nde</i> I)	5'-CACTCAGCATATGTCCTACACGTGGACAG GCGCT-3'
pET29-NS5Bt-rev (<i>Xho</i> I)	5'-GCGATCCTCGAGGCGGGGTCGGGCGCGAG ACAGGCT-3'

The PCR products were cloned into the pGEX-4T-1 vector or pET29 vector at *Nde*I and *Xho*I cloning sites, respectively. The resulting plasmids were pGEX-4T-1-NS5Bt, coding for the HCV 1a NS5Bt and an N-terminal GST-tag to facilitate purification; and pET29-NS5Bt, coding for HCV 1b NS5Bt and a C-terminal (His)₆-tag to facilitate purification.. The coding sequences were verified by DNA sequencing.

2.2.1.3. Constructed vectors for NS5A protein expressed in mammalian cells

The DNA sequence for full length HCV 1a NS5A was amplified by polymerase chain reaction. Table 3 lists the primers used in the constructions for full length NS5A protein expressed in mammalian cells.

Table 3 Oligonucleotides used for the constructions for full length NS5A protein expressed in mammalian cells

Oligo name	Sequence
pcDNA 3.1 (+)-NS5A-for (<i>Kpn</i> I)	5'-TCAGGGTACCATGTCCGGTTCCTGGCTACGT GACATCTGGGACTGG-3'
pcDNA 3.1 (+)-NS5A-rev (<i>Xho</i> I)	5'-GCGATCCTCGAGCTACATCCTCCGCGTTGGC CTCACTACTGAC-3'

The PCR product was cloned into the pcDNA 3.1 vector at *Kpn*I and *Xho*I cloning sites, respectively. The resulting plasmids were pcDNA 3.1 (+)-NS5A, coding for the HCV 1a NS5A protein. The coding sequence was verified by DNA sequencing.

2.2.2. Preparation of competent cells

One colony was picked into a 2 ml of LB medium without antibiotics and incubated at 37 °C overnight. The 2 ml of *E. coli* culture was transferred into 50 ml of fresh LB medium and continue to be incubated at 37 °C with shaking until OD600 reached to 0.4-0.6. The cells were spun down at 2600 g for 10 min at 4 °C. The supernatant was discarded and the cells were resuspended in 25 ml of ice cold sterile 100 mM of MgCl₂. The cells were spun down at 2600 g for 10 min at 4 °C. The cell pellets were resuspended in 5 ml of ice cold sterile 100 mM of CaCl₂. The transformation competency was obtained by keeping the *E. coli* cells in CaCl₂ on ice for 2 h. The competent *E. coli* cells were kept in 50% glycerol at – 80 °C.

2.2.3. Plasmid transformation and purification

The DH5α competent cells were used for the transformation and purification of

plasmids. The DH5 α competent cells were thawed on ice, 1 μ l of plasmids were added into 20 μ l of competent cells and incubated on ice for 30 min. The competent cells were heat-shocked for 45 s at 42 °C. The cells were immediately put on ice with the addition of 100 μ l of LB medium without antibiotics to the tube. The competent cells were incubated at 37 °C for 1 h to recovery and make the expression of the antibiotic-resistant gene in the plasmids. After recovery, the cells were spreaded evenly on the LB agar plate with the antibiotic. Then the plate was kept at 37 °C to keep the cells growing.

To amplify the plasmids, one colony was picked from the plate and inoculated into 2 ml of LB medium with the antibiotic. The cells were incubated overnight at 37 °C with shaking. The cells were spun down through the centrifuge at 2600 g for 5 min. The plasmids inside the cells were isolated by the QIAprep® Spin Miniprep kit (QIAGEN, Cat 27106). The absorbance of the purified plasmids was measured at 260 nm with the BECKMAN COULTER DU®530 Life Science UV/Vis Spectrophotometer. The concentration of the plasmid was equal to the absorbance unit at 260 nm of double-stranded DNA times 50 μ g/ml.

2.2.4. Protein purification

2.2.4.1. Expression and purification of the full length NS5A and NS5A- Δ N (without the N-terminal membrane anchor domain)

Co-transform pET-Ub-NS5A-full length-His/pET-Ub-NS5A- Δ N-His and pCG1

into BL21 (DE3) cells on plates with 30 µg/ml kanamycin and 25 µg/ml chloramphenicol. The cells were grown overnight in 25 ml NZCYM supplemented with 30 µg/ml kanamycin, 25 µg/ml chloramphenicol, and 0.1% dextrose at 37 °C. The overnight culture was used to inoculate 1 L NZCYM with 30 µg/ml kanamycin, 25 µg/ml chloramphenicol, 0.1% dextrose. The cells were grown at 37 °C to an OD₆₀₀ of 0.8–1.0 and then IPTG was added to a final concentration of 0.5 mM. The cells were grown for an additional 4-5 h at 20 °C. The cells were harvested by centrifugation at 8000 rpm for 5 min. The cell pellet was suspended in 30 ml lysis buffer (100 mM Tris-Cl, pH 8.0, 200 mM NaCl, 20 mM Imidazole, 0.1% NP40, 1 mM PMSF, 5 mM 2-mercaptoethanol), and sonicated at Amp 30%, pulse 6 s, for 20 min. The extract was clarified by ultracentrifugation for 30 min at 25,000 rpm at 4 °C. The supernatant was loaded onto 1 mL Ni²⁺-NTA-agarose column (Qiagen, Hiden, Germany) at a flow rate of 0.5 ml/min. The Ni²⁺-NTA-agarose column was washed further with 10 ml of the lysis buffer, followed by 20 ml washing buffer (100 mM Tris-Cl, pH 8.0, 1 M NaCl, 50 mM Imidazole, 0.1% NP40, 1 mM PMSF, 5 mM 2-mercaptoethanol). The purified NS5A-His was eluted by using elution buffer (100 mM Tris-Cl, pH 8.0, 200 mM NaCl, 500 mM Imidazole, 0.1% NP40, 1 mM PMSF, 5 mM 2-mercaptoethanol). Fractions (1 ml) were collected and analyzed for purity by SDS-PAGE. The protein was further purified by gel filtration (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM DTT, 0.1% NP40, 0.01% NaN₃).

2.2.4.2. Expression and purification of NS5A domain 1 (NS5A-D1), NS5A domain 2 (NS5A-D2), and NS5A domain 3 (NS5A-D3)

Plasmids pET-Ub-NS5A-D1-His/pET-Ub-NS5A-D2-His/pET-Ub-NS5A-D3-His and pCG1, which encodes for a yeast ubiquitin protease express vector, were co-transformed into *E. coli* BL21 (DE3) cells on plates with 30 µg/ml kanamycin and 25 µg/ml chloramphenicol. The cells were grown overnight in 25 ml NZCYM supplemented with 30 µg/ml kanamycin, 25 µg/ml chloramphenicol, and 0.1 % dextrose at 37 °C. The overnight culture was diluted 100-fold into 1 L NZCYM with 30 µg/ml kanamycin and 25 µg/ml chloramphenicol, 0.1 % dextrose. The cells were grown at 37 °C to the absorbance 0.8-1.0 at 600 nm, and protein was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for additional 4 hours at 20 °C. Approximately 5 g of the cell pellet was resuspended in 30 ml lysis buffer (100 mM Tris-Cl, pH 7.0, 200 mM NaCl, 20 mM Imidazole, 1 mM PMSF, 5 mM 2-mercaptoethanol), and lysed by sonication for 20min. The disrupted cell lysate was centrifuged at 20,000 x g for 30 min. The supernatant was loaded onto 1 ml Ni²⁺-NTA-agarose column (Qiagen, Hiden, Germany) pre-equilibrated with the lysis buffer. The Ni²⁺-NTA-agarose column was washed further with 10 ml of the lysis buffer, followed by 20 ml washing buffer (100 mM Tris-Cl, pH 7.0, 1 M NaCl, 50 mM Imidazole, 1 mM PMSF, 5 mM 2-mercaptoethanol). The purified NS5A-D2 was eluted by elution buffer (100 mM Tris-Cl, pH 6.4, 200 mM NaCl, 500 mM Imidazole, 1 mM PMSF, 5 mM 2-mercaptoethanol). The eluted protein sample was further purified by high-resolution Sephacryl S-200 gel filtration

column (Amersham Biosciences, Buckinghamshire, UK).

2.2.4.3. Expression and purification of ^{15}N - and $^{15}\text{N}/^{13}\text{C}$ - labeled NS5A-D2

Plasmids pET-Ub-NS5A-D2-His was co-transformed with pCG1, which encodes the yeast ubiquitin C-terminal protease, into *E. coli* BL21 (DE3) cells in the presence of 30 $\mu\text{g}/\text{ml}$ kanamycin and 25 $\mu\text{g}/\text{ml}$ chloramphenicol. A pre-culture (2 ml) was grown overnight in 25 ml M9 media containing $^{15}\text{N}\text{-NH}_4\text{Cl}$ or both $^{15}\text{N}\text{-NH}_4\text{Cl}/^{13}\text{C}\text{-glucose}$, supplemented with 30 $\mu\text{g}/\text{ml}$ kanamycin, and 25 $\mu\text{g}/\text{ml}$ chloramphenicol at 37 °C. The overnight culture was diluted by 100-folds into 1 L of M9 media containing $^{15}\text{N}\text{-NH}_4\text{Cl}$ or $^{15}\text{N}\text{-NH}_4\text{Cl}/^{13}\text{C}\text{-glucose}$ with 30 $\mu\text{g}/\text{ml}$ kanamycin and 25 $\mu\text{g}/\text{ml}$ chloramphenicol. Cells were grown at 37 °C to the absorbance of 0.8-1.0 at 600 nm and induced with 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for additional 4 hours at 20 °C. Approximately 5 g of cell pellet was resuspended in 30 ml lysis buffer (100 mM Tris-Cl, pH 7.0, 200 mM NaCl, 20 mM Imidazole, 1 mM Phenylmethylsulphonylfluoride (PMSF), 5 mM 2-mercaptoethanol), and lysed by sonication for 20 min in ice. The disrupted cell lysate was centrifuged at 20,000 x g for 30 min. The supernatant was loaded onto 1 ml Ni^{2+} -NTA agarose column (Qiagen, Hiden, Germany) pre-equilibrated with the lysis buffer, washed with 10 ml of the lysis buffer, followed by 20 ml washing buffer (100 mM Tris-Cl, pH 7.0, 1 M NaCl, 50 mM Imidazole, 1 mM PMSF, 5 mM 2-mercaptoethanol), and the protein was eluted by the elution buffer (100 mM Tris-Cl, pH 6.4, 200 mM NaCl,

500 mM Imidazole, 1 mM PMSF, 5 mM 2-mercaptoethanol). The eluted NS5A-D2 was further purified by high-resolution Sephacryl S-200 gel filtration column (Amersham Biosciences, Buckinghamshire, UK). The purified proteins samples were analyzed on 15% SDS-PAGE gel and protein concentration was determined by a protein assay kit from Bio-Rad Laboratories (Hercules, CA, USA). Proteins were stored at 4 °C before use.

2.2.4.4. Expression and purification of GST-NS5Bt

GST-fused HCV NS5Bt protein was expressed and purified as described before (Yamashita *et al*, 1998). The plasmid pGET-4T-1-NS5Bt was transformed into the *E. coli* strain BL21 (DE3), and the transformants were then cultured in 10 ml LB medium with 100 µg/ml Ampicillin at 30 °C overnight. The overnight culture was diluted 100-fold into 1 L LB with 100 µg/ml Ampicillin and cultured at 30 °C. Until the absorbance at 600 nm reached 0.6-0.7, the cultures were then induced by 0.4 mM IPTG and incubated at 30 °C overnight. The cells from 1 L were harvested by centrifugation and the pellet was resuspended in 30 ml lysis buffer (phosphate-buffered saline (PBS), pH 7.2, 1 mM dithiothreitol (DTT) and 1% Triton X-100). The suspension was sonicated on ice for 30 min and then centrifuged at 18,000 x g. The supernatant was passed through 1 ml glutathione-Sepharose 4B beads (Amersham Biosciences, Buckinghamshire, UK) equilibrated with the lysis buffer. The beads were washed with lysis buffer and then with 50 mM Tris-Cl, pH 8.0, and 1 mM DTT. The GST-NS5Bt was eluted with 2

ml of elution buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10 mM glutathione, 10 mM DTT, and 0.1% Triton X-100) and then eluted with 2 ml of elution buffer containing 500 mM NaCl. The eluted protein sample was further purified by high-resolution Sephacryl S-200 gel filtration column (Amersham Biosciences, Buckinghamshire, UK). The purified proteins samples were analyzed on 10% SDS-PAGE gel and stored at 4 °C before use.

2.2.4.5. Expression and purification of NS5Bt

The expression and purification of NS5B protein was described before (Ferrari *et al*, 1999). The plasmid pET29-NS5Bt was transformed into the *E. coli* strain BL21 (DE3), and the transformants were then cultured in 10 ml LB medium with 100 µg/ml kanamycin at 30 °C overnight. The overnight culture was diluted 100-fold into 1 L LB with 100 µg/ml kanamycin and cultured at 30 °C. Until the absorbance at 600 nm reached 0.8-1.0, the cultures were then induced by 0.4 mM IPTG and incubated at 30 °C overnight. The cells from 1 L were harvested by centrifugation and the pellet was resuspended in 30 ml lysis buffer (25 mM Tris-Cl, pH 7.4, 500 mM NaCl, 20 mM Imidazole, 5% glycerol, 5 mM 2-mercaptoethanol, 1 mM PMSF). The supernatant was loaded onto 1 ml Ni²⁺-NTA agarose column (Qiagen, Hiden, Germany) pre-equilibrated with the lysis buffer, washed with 10 ml of the lysis buffer, followed by 20 ml washing buffer (25 mM Tris-Cl, pH 7.4, 1 M NaCl, 50 mM Imidazole, 5% glycerol, 5 mM 2-mercaptoethanol, 1 mM PMSF), and the protein was eluted by the elution buffer (25 mM Tris-Cl, pH 7.4, 350 mM NaCl,

500 mM Imidazole, 2.5% glycerol, 5 mM 2-mercaptoethanol, 1 mM PMSF). The eluted NS5Bt was further purified by high-resolution Sephacryl S-200 gel filtration column (Amersham Biosciences, Buckinghamshire, UK). The purified proteins samples were analyzed on 10% SDS-PAGE gel and protein concentration was determined by a protein assay kit from Bio-Rad Laboratories (Hercules, CA, USA). Proteins were stored at 4 °C before use.

2.2.5. Western Blot analysis

The protein samples were separated on a 12% or 15% SDS-polyacrylamide gel (PAGE) and then transferred onto polyvinylidenedifluoride (PVDF) membranes. The blotted membranes were treated with blocking solution (TBS with 5% milk), and incubated with the primary antibody for 16 h at 4 °C. The membranes were then incubated with the secondary antibody for 2 h at 4 °C. The membranes were rinsed, treated with enhanced chemiluminescent reagent (Bio-Rad Laboratories), and exposed to X-ray films for visualization.

2.2.6. GST pull-down assay

The binding of NS5A-D2 (or NS5A-ΔN) with NS5B was examined by GST pull-down assay. Approximately 1 μg GST or GST-NS5Bt was immobilized to 10 μl of glutathione-Sepharose 4B at 4 °C for 2 h. The immobilized beads were incubated with approximately 5 μg of NS5A-D2 (or NS5A-ΔN) in 50 μl of modified GBT buffer (10% glycerol, 50 mM Hepes- NaOH (pH 8.0), 170 mM KCl,

7.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 1% Triton X-100) containing 1% bovine serum albumin. After extensively washing with GBT buffer, the bound proteins were analyzed by western blot using anti-HCV NS5A monoclonal and HCV NS5B polyclonal antibodies, respectively.

The interaction between NS5A-D2 (or NS5A-ΔN) and PKR was also examined by GST pull-down assay. Approximately 5 μg of NS5A-D2 (or NS5A-ΔN) was incubated with 24 μl PKR agarose (upstate), which binds approximately 1 μg GST-PKR protein. The mixture was incubated in 50 μl of modified GBT buffer (10% glycerol, 50 mM Hepes- NaOH (pH 8.0), 170 mM KCl, 7.5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 1% Triton X-100) containing 1% bovine serum albumin. After extensively washing, the bound proteins were analyzed by western blot using anti-HCV NS5A monoclonal antibody.

2.2.7. Surface plasmon resonance

Experiments were performed on BIACore 3000 (BIACore AB, Uppsala, Sweden) at 25 °C. Sensor chip CM5 was used for analysis. Immobilization of NS5Bt protein was conducted at a flow rate of 10 μl/min using amine coupling procedure (O'Shannessy *et al*, 1992). Ligand surface was activated by injecting a solution containing 0.2 M (EDC) N-ethyl-N'-[3-(dimethylamino)propyl]carbodiimide and 50 mM NHS (N-hydroxysuccinimide) for 10 min. 10 μg/ml NS5B was diluted in

HBS (10 mM HEPES, 150 mM NaCl, 5 mM MgCl₂, 0.005% p20, pH 7.4) and covalently immobilized to CM5 surface by amine coupling method. The surface was then deactivated by injecting 1 M ethanolamine at pH 8.5 for 7 min. 50 mM NaOH was injected in two-30 s pulses to wash off noncovalently-bound NS5Bt and to stabilize the baseline. Reference surface was both activated and deactivated like NS5Bt surface but without immobilization of NS5Bt. Final immobilization level was around 2500RU. Varying concentration (5 μ M - 80 μ M) of NS5A-D2 in Phosphate buffer (20 mM sodium phosphate, pH 7.0, 150 mM NaCl) was injected over both reference and ligand surface for 30 s at a flow rate of 40 μ l/min following two injections buffer only. The kinetic data were processed using BIAEvaluation software. Data obtained in the reference surface was subtracted from that obtained in NS5B surface. The response from an average blank injection was subtracted to remove any systematic artifacts observed between NS5B and reference surface (Myszka, 1999).

2.2.8. Analytical ultracentrifuge sedimentation equilibrium analysis

Equilibrium sedimentation experiments were performed on a Beckman Optima XL-A analytical ultracentrifuge with an An-50 Ti rotor. NS5A-D2 at concentration of 0.65, 0.32, and 0.16 mg/ml were analyzed at 4 °C in the gel filtration buffer (20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 1 mM DTT, 0.1% NaN₃), at 28000 rev/min (63221 x g) for 20 h, at 34500 rev/min (95981 x g) for 5 h, and 42000 rev/min (142248 x g) for 5 h. Absorbance measurements at 280 nm were taken.

Sedphat 5.14 was used for data processing and curve fitting (Vistica *et al*, 2004).

2.2.9. Gel-filtration analysis

Gel-filtration analysis was performed on a Sephacryl S-200 column pre-equilibrated with 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0 mM DTT and 0.01% NaN₃. The buffer flow rate is 0.5 ml/min. The molecular weight of the NS5A-D2 determined based upon the K_{av} values ($K_{av} = (V_e - V_o)/(V_t - V_o)$, $V_o = 37$ ml, $V_t = 125$ ml) using the standard proteins: bovine thyroglobulin, 670 kDa; bovine γ -globulin 158 kDa; chicken ovalbumin 44 kDa; horse myoglobin 17 kDa; vitamin B12, 1.35 kDa).

2.2.10. Circular dichroism (CD) spectroscopy

The CD spectrum of the proteins (0.1 mM) in 20 mM sodium phosphate, pH 6.5, 50 mM sodium chloride buffer was collected using Chirascan (Applied Photophysics Limited, Surrey, UK). The above mentioned buffer was used as a blank, and the spectrum with 4 scans was recorded from 180 to 280 nm at 25 °C. 2, 2, 2-trifluoroethanol (TFE) - induced structural change was examined using the 0.1 mM NS5A-D2 in the presence of various concentrations of TFE. Urea induced change was examined using the NS5A-D2 sample in the presence of 8 M urea.

2.2.11. Dynamic light scattering

The size distribution of the NS5A-D2 was determined by dynamic light scattering.

A light scattering spectrometer (90Plus/BI-MAS, Brookhaven Instruments Co. USA) was used. 1 ml of aqueous solution of NS5A-D2 (2 mg/ml) was loaded into the cuvette and put into the measuring compartment kept at 25 °C. The fluctuations in the scattering intensity at the scattering angle of 90 °C were recorded and transformed automatically.

2.2.12. NMR experiments

2.2.10.1. NMR sample preparation

The unlabeled, or uniformly ^{15}N - and $^{15}\text{N}/^{13}\text{C}$ - labeled NS5A-D2 samples for NMR experiments were used at a final concentration of 0.6 mM in 20 mM NaPO_4 , pH 6.5, 50 mM NaCl, 1 mM DTT, 0.01 % NaN_3 , and 90% $\text{H}_2\text{O}/10\% \text{D}_2\text{O}$.

2.2.10.2. NMR data collection and assignment

2.2.10.2.1. 1D ^1H NMR experiment

The pulse program is 'zgpgw5' plus water suppression of watergate W5 pulse sequences with gradients was used for the 1D ^1H experiment recording. The recording temperature is 298 K, TD (size of fid) is 12 K, the DS is 4 and a sweep width of is 16 ppm, and the o1p, decided with the gs mode, is 4.7 ppm. The receiver gain was achieved by the command 'rga'. The number of scan was 8. The D19 delay for water suppression was set to 100 μs for 700 MHz, 125 μs for 600 MHz. Other parameters are set according to the program from Bruker.

2.2.10.2.2. NMR experiments for structural determination

All backbone assignment experiments for the $^{15}\text{N}/^{13}\text{C}$ -labeled NS5A-D2 (102 residues) were done at 298 K on a Bruker AV700 spectrometer equipped with a cryoprobe accessory. Backbone ^1H , ^{15}N , ^{13}C resonances were assigned using data from 2D ^1H - ^{15}N HSQC, 3D HNCA, HN(CO)CA, HNCO, HNCACB, CBCA(CO)NH and HNHA spectra (Bottomley *et al*, 1999). The side chain ^1H and ^{13}C resonances were obtained from 3D HCC(CO)NH-TOCSY, HCCH-TOCSY and ^1H - ^{15}N NOESY-HSQC. To investigate conformation of NS5A-D2 at low temperature, backbone/side chain experiments and 3D ^1H - ^{15}N NOESY-HSQC were also collected at 278 K. All spectra were processed by Topspin version 1.3 (Bruker) and analyzed using Felix (Accelrys) and NMRView (Johnson, 1994).

2.2.10.2.3. 2D ^1H - ^{15}N HSQC

The pulse program 'hsqc3gppl19' with water suppression was used to record the 2D ^1H - ^{15}N HSQC. The acquisition mode was DQD in the ^1H dimension and States-TPPI in the ^{15}N dimension. The TD in the F1 is 256 and F2 is 4048. The CNST4, which is the J (NH), is set to 90. D1 is 1 s, and other parameters were set by the program. The number of scans was 4. The spectral width for ^1H is 16 ppm and ^{15}N is 40 ppm. The receiver gain was achieved by the 'rga' command.

2.2.10.2.4. HNCACB

The pulse program 'hncacbgpwg3d' and the water suppression with Watergate

were used for HNCACB experiment. Acquisition mode is DQD for ^1H , States-TPPI for both ^{13}C and ^{15}N . The spectral width is 16 ppm for ^1H , 34 ppm for ^{15}N and 80 ppm for ^{13}C . The o1p is 4.7 ppm, o2p is 39 ppm and o3p is 119 ppm. The TD for ^1H is 2048, for ^{13}C is 128, for ^{15}N is 96. The receiver gate was set by 'rga'. The shaped power (sp1) for water suppression was optimized in the gs mode. Other parameters were obtained by command 'getprosol'. The ^{15}N and ^{13}C hard pulses were adjusted according to the standard pulse program. The water flip pulse and the shape pulse for decoupling were from the programs provided by Dr. Sattler and Dr. Simon in EMBL.

2.2.10.2.5. CBCACONH

The pulse program 'cbcaconhgpwg3d' was used for recording CBCACONH spectra. The parameters were same as those of HNCACB. The scan number is 16. The shaped power (sp1) for water suppression was set in the prosol.

2.2.10.2.6. HNCA and HNCOCA

The water suppression using Watergate was used for both of HNCA and HNCOCA programs. The pulse program 'hncagpwg3d' was used for HNCA and 'hncocagpwg3d' was used for HNCOCA experiment. The acquisition mode for ^1H was DOD, for ^{13}C was States-TPPI, for ^{15}N was States-TPPI. The spectral width for ^1H was 16 ppm, for ^{13}C was 32 ppm, for ^{15}N was 34 ppm. The frequency offset for ^1H was 4.7 ppm, for ^{13}C was 54 ppm, and for ^{15}N was 119 ppm. The number of

scans was 32. The acquisition option was ‘-DLABEL_CN’. The receiver gain was set by command ‘rga’. Other parameters were obtained from ‘getprosol’ or set by the pulse program.

2.2.10.2.7. HNCO

The pulse program ‘hncogpwg3d’ with the water suppression using Watergate was used for recording HNCO spectra. The acquisition mode for ^1H was DOD, for ^{13}C was States-TPPI, for ^{15}N was States-TPPI. The spectral width for ^1H was 16 ppm, for ^{13}C was 24 ppm, for ^{15}N was 34 ppm. The frequency offset for ^1H was 4.7 ppm, for ^{13}C was 54 ppm, for ^{15}N was 119 ppm. The number of scans was 32. The TD in the ^1H was 2048, in the ^{13}C was 96, and in the ^{15}N was 96. The receiver gain was obtained by command ‘rga’. Other parameters were obtained by getprosol or set by the pulse program.

2.2.10.2.8. HNHA

The pulse program ‘hnhagp3d’ was used for HNHA. The acquisition mode for ^1H was DQD, for ^1H was States-TPPI, for ^{15}N was States-TPPI. The spectral width for ^1H was 14 ppm, for ^1H was 14 ppm, for ^{15}N was 35 ppm. The frequency offset for ^1H was 4.7 ppm, for ^{13}C was 54 ppm, for ^{15}N was 119 ppm. The number of scans was 32. The TD in the ^1H was 2048, for ^1H was 128, for ^{15}N was 96. The receiver gain was set by command ‘rga’. Other parameters were obtained by getprosol or set by the pulse program.

2.2.10.2.9. HCCCONH and _CCCONH

HCCCONH and _CCCONH experiments were performed using the ‘hccconhgpwg3d2’ pulse program and ‘hccconhgpwg3d3’ pulse program, respectively. The acquisition mode was the same as the previous one. The spectral width for ^1H was 14 ppm, for ^{13}C was 80 ppm, for ^{15}N was 35 ppm. The frequency offset for ^1H was 4.7 ppm, for ^{13}C was 39 ppm, and for ^{15}N was 119 ppm. The number of scans was 32. The TD was same as the previous ones. The receiver gain was set by command ‘rga’. Other parameters were obtained by getprosol or set by the pulse program.

2.2.10.2.10. ^{15}N -HSQC-NOESY

^{15}N -HSQC-NOESY experiment was performed using the Bruker standard pulse program, with the ‘noesyhsqcetf 3gp3d’. The parameters were same as the previous ones. The GPZ2 8.1% gradient was used. The mixing time was 100 ms. The ‘rga’ command was used for the receiver gain.

2.2.10.2.11. NMR titration to study NS5A-D2 and NS5B peptide interaction

Two peptides derived from NS5B (residues 139-155: MAKNEVFCVQPEKGGRK, referred as to MK-17; residues 365-388: SCSSNVSVAHGAGKRVYYLTRDP, hereafter referred as to SP-24) were synthesized (GL Biochem. Ltd., Shanghai, China). Uniformly ^{15}N -labeled NS5A-D2 with concentration of 0.2 mM was prepared in a buffer containing 20 mM NaPO_4 , pH 6.5, 50 mM NaCl, 0.1% NaN_3 ,

1 mM DTT. ^1H - ^{15}N HSQC spectra were collected at 298 K with varying molar ratio between ^{15}N -labeled protein and the peptides on a Bruker 600 MHz with a cryoprobe. For the NMR dissociation constant calculation experiment, lyophilized MK17 peptide aliquots were mixed with ^{15}N -labeled NS5A-D2 with different molar ratio and a series of 2D ^1H - ^{15}N HSQC were recorded. The one to one binding of protein (P) and peptide (L) to form a protein-peptide complex (PL) can be expressed as $[\text{P}]+[\text{L}]\leftrightarrow[\text{PL}]$. The equilibrium dissociation constant (K_D) is expressed as $[\text{P}]*[\text{L}]/[\text{PL}]$. Based upon the protein sample concentration ($[\text{protein}]_0$) and ligand total concentration ($[\text{L}]_0$), which is explained as $[\text{Protein}]_0=[\text{P}]+[\text{PL}]$, and $[\text{L}]_0=[\text{L}]+[\text{PL}]$, for one to one binding mode, the relationship between chemical shift perturbations of the protein $\Delta\delta=((\Delta\delta^1\text{H})^2+(0.2*(\Delta\delta^{15}\text{N})^2)^{0.5}$ and K_D can be obtained from following equations:

$$\Delta\delta(\text{ppm})=0.5*\Delta\delta_{\text{max}}(1+X+K_D/[\text{Protein}]_0-[(1+X+K_D/[\text{Protein}]_0)^2-4X]^{0.5}), \quad \text{where}$$

$[\text{Protein}]_0$ is the protein sample concentration; X is the molar ratio of ligand on protein; $\Delta\delta_{\text{max}}$ is $\Delta\delta$ value at the protein saturation level (Bayer *et al*, 2003; Smet *et al*, 2005). K_D can be obtained by fitting $\Delta\delta$ and X into the equation.

2.2.10.2.12. NMR relaxation measurements

Backbone ^{15}N relaxation measurements were carried out at 700 MHz as described previously (Farrow *et al*, 1994; Vojnic *et al*, 2006). Twelve and ten T delays were used for T_1 (21, 54, 108, 162, 270, 432, 648, 864, 1188, 1512, 1944, 2268 ms) and T_2 (14.4, 28.8, 43.2, 57.6, 72, 100.8, 129.6, 158.4, 187.2, 230.4 ms), respectively.

The inter-scan delay was 1.5 s for the ^{15}N T_1 and T_2 experiments. The rates were fit with the program Felix (Accelrys) to a two-parameter single-exponential decay. Heteronuclear NOE experiments were performed using a 2 s inter-scan delay followed by either 3 s of proton saturation using a series of 120° ^1H pulses or an additional 3 s delay at 298 K and 278 K respectively.

2.2.13. Cell culture

HepG2 and 293T cells were cultured in DMEM with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin. 293T-flag-NS5A and 293T-flag-NS5B cells were cultured in DMEM with 10% fetal bovine serum, 100 units/ml of penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin, 0.4 mg/ml G418. To induce the expression of NS5A or NS5B protein, 4 $\mu\text{g}/\text{ml}$ tetracycline was added. The cells were cultured at 37 $^\circ\text{C}$ in a humid environment with 5% CO_2 .

2.2.14. Transfection of pcDNA 3.1 (+)-NS5A into Human Hepatoma Cells HepG2

One day before transfection, 6×10^6 human hepatoma cells HepG2 was plated in 2 ml of Dulbecco's Modified Eagle Medium (DMEM) plus 10% fetal bovines Serum (FBS), but without antibiotics per well. At the time of transfection, the cells were 90-95% confluent. For each well, pcDNA 3.1 (+)-NS5A (4.0 μg) was diluted in 250 μl DMEM without serum and mixed gently. The transfection reagent-LipofectamineTM2000 (Invitrogen) was mixed gently before use and

diluted 10 μ l in 250 μ l DMEM without serum later. The LipofectamineTM2000 solution was mixed gently and incubated for 5 min at room temperature. Then the diluted LipofectamineTM2000 and DNA were combined for 20 min at room temperature. 500 μ l DNA-LipofectamineTM2000 complexes was added to each well and mixed gently. The cells were incubated at 37 °C in a humid environment with 5% CO₂ for 24-48 h.

2.2.15. RT-PCR and semi-quantitative RT-PCR

Total RNAs were isolated from harvested cells by using the RNeasy® Mini Kit (QIAGEN, Cat 74104). The concentrations of extracted total RNAs were measured at 260 nm on DU® 530 UV/Vis Spectrophotometer (BECKMAN COULTERTM). 5 μ g of total RNAs were mixed with 10 μ M of the corresponding reverse primer and 0.5 μ l of RNase inhibitor (40 U/ μ l), incubated for 10 min at 65 °C. The mixture was chilled on ice immediately after the incubation. To synthesize cDNAs, 0.5 μ l of dNTPs mixture (10 mM each, Invitrogen) and 1 μ l of Reverse Transcriptase (10000 units/ml, New England BioLabs) were added into the above RNA mixture and incubated at 42 °C for 2 h. The PCR reaction was set up by using the synthesized cDNAs as template. The temperature for the initial DNA denature is 94 °C for 5 min. The primers were annealed with the DNA template for 30 sec to 1 min at the temperature 5 °C lower than T_m ($T_m = 4 (G + C) + 2 (A + T)$). The DNA was extended at 72 °C with 1 min per 1 kb of the DNA fragment. The PCR was repeated for 20-30 cycles with a final extension at 72 °C for another 10 min to fill in the ends of reaction products. The PCR

products were run and viewed on 1% agarose with 1 µg/ml of ethidium bromide. The ethidium bromide stained gels were documented with UV illumination on Chemi Genius2 (SynGene).

2.2.16. siRNAs transfection

Oligofectamine (Invitrogen) was used to transfect the siRNAs into the 293T-flag-NS5A cells. The day before transfection, the mammalian cells were seeded into a 6-well plate (2×10^5 cells/well) or 96-well plate (5×10^3 cells/well) and cultured for about 24 h until 50-70% confluence in DMEM plus 10% fetal bovine serum (FBS), without antibiotics at 37 °C in a humid environment with 5% CO₂. Before transfection, the 20 µM stock siRNAs were diluted to different concentrations in the Opti-MEM I reduced serum medium, and the Oligofectamine was diluted according to the manual suggestions also. After incubation of 5-10 min at room temperature, the mixtures were mixed together gently and incubated for another 20-25 min at room temperature to allow the formation of siRNA:Oligofectamine complex. Extra Opti-MEM I medium were added into the above siRNA:Oligofectamine complex mixtures up to the 500 µl (6-well plate) or 50 µl (96-well plate) for each well, respectively. The old cell culture media were removed from the wells, then the whole siRNA:Oligofectamine complex mixtures were added. After incubation of the cells in the siRNA:Oligofectamine complex mixture for 2 h at 37 °C in a humid environment with 5% CO₂, 1.5 ml fresh DMEM with 10% FBS (6-well plate) or 150 µl fresh DMEM with 10% FBS were

added into each well.

2.2.17. Cell proliferation assay

Cells were seeded in a 96-well plate as 5×10^3 cells/well. After treatment the cells with serum free medium or cisplatin or siRNA, cell proliferations were determined using the Promega CellTiter 96® AQueous One solution reagent kit (Madison, WI, USA). First DMEM cell culture medium, MTS and PMS were mixed together with the ratio of 100 μ l: 20 μ l: 1 μ l per well. After removing the old media from the wells, 120 μ l of the above mixtures was added into each well. The cells were incubated for 2 h at 37 °C in a humidified, 5% CO₂ atmosphere. The absorbances of the products were read at 490 nm using the Benchmark plus microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was determined by the ratio of the absorbance in wells containing treated cells compared to non-treated cells. Statistical analysis was performed using Student's *t* test and *P* values less than 0.05 were considered to be statistically significant.

2.2.18. Protein concentration measurement

Cells were harvested by cell lysis buffer, and then centrifuged at 4 °C. The cell debris was discarded. The total protein concentrations were measured by the Bio-Rad Protein Assay (Bio-Rad, Cat 500-0006). After the assay dye was added to the protein, the absorbance was measured at 595 nm, and the protein concentrations were calculated according to the Beer-Lambert's law: $A = \epsilon bc$ (*A* is the absorbance, ϵ is the molar

extinction coefficient ($\text{L/mol}\cdot\text{cm}$). Bovine serum albumin was used as a standard protein.

2.2.19. Immunostaining

Cells on coverslips were incubated in DMEM plus 10% FBS containing 500 nM (final concentration) R6 (rhodamine B hexyl ester perchlorate; Molecular Probes) or 100 nM of MitoTracker at 37 °C, 5% CO₂ atmosphere for 30 min. Then took the coverslips out and washed with PBS for 3 times and fixed 3.7% paraformaldehyde in $1 \times$ PBS at 37 °C for 15 minute. The cells was washed twice immediately for 5 min with PBS. Then covered the cells with 8% BSA in PBS and incubated for 1 h at room temperature. The cells were washed twice for 5 min with PBS. The excess PBS was gently removed and the cells were covered with primary antibody, anti-NS5A monoclonal antibody (1:1000 diluted in 1% BSA in PBS), then incubated for 2 h at room temperature. The cells were washed again with PBS three times for 5 min. The excess PBS was gently removed and the cells were incubated with the secondary antibody, Alexa Fluor[®] 488 goat anti-mouse IgG (1:1000 diluted in 1% BSA in PBS) at room temperature for 2 h in the dark. The cells were washed three times for 5 min with PBS in the dark. After mounted, the fixed cells on slides were viewing by using Zeiss Meta 510 confocal. The excitation wavelengths used are 488 nm for FITC, 633 nm for Mito-Tracker and 405 nm for R6 ER Tracker.

Results

3.1. Expression, purification and characterization of full length NS5A and NS5A-ΔN

Non-structural protein 5A (NS5A) of Hepatitis C virus plays significant roles in the viral replication, IFN resistance, and pathogenesis (Blight *et al*, 2000; Gale *et al*, 1997). NS5A is also involved in the apoptotic regulation (Chung *et al*, 2003; Lan *et al*, 2002; Wang *et al*, 2006), the elevation of reactive oxygen species in the mitochondria and the regulation of intracellular calcium (Gong *et al*, 2001). NS5A protein is a large, hydrophilic phosphoprotein (56-58 kDa). Some structural information about NS5A has been obtained. The N-terminal 30 amino acids of NS5A is the membrane anchoring domain (Brass *et al*, 2002). The domain 1 of HCV 1b NS5A (amino acids 36-198) has been resolved to have major β -sheet conformation and small helical regions (Tellinghuisen *et al*, 2004; Tellinghuisen *et al*, 2005). The C-terminal region of NS5A (from amino acid 200 to the C-terminus) has recently been proposed to be similar to the natively unfolded proteins (Penin *et al*, 2004), with predicted primarily random coil structure and small regions of helical secondary structure, located near the center of the NS5A sequence (Tellinghuisen *et al*, 2004).

3.1.1. Expression and purification of full length NS5A and NS5A-ΔN

To study the functions and characters of NS5A protein, several systems, including yeast, mammalian cells and insect cells (Ide *et al*, 1997; Katze *et al*, 2000; Kim *et al*, 1999; Reed *et al*, 1997), have been used to isolate this protein. A pET-ubiquitin expression system of successfully isolating HCV-1b NS5A protein from the soluble fraction of *Escherichia coli*, has been established by the laboratory of Dr. Craig E. Cameron (Huang *et al*, 2004). In this system, upon the addition of IPTG, the cells produced ubiquitin (6-8 kDa)-NS5A-His fusion protein and processed the fusion protein co- and/or post-translationally by an ubiquitin-specific carboxy-terminal protease (Ubp1) that was constitutively expressed from a second plasmid – pCG1 in the BL21 (DE3) cells (Tobias & Varshavsky, 1991), thereby releasing the HCV-1b NS5A-His fusion protein *in vivo*.

In our study, we applied this system to express and purify full length HCV-1a NS5A. To obtain soluble protein, the cells were induced at 20 °C for 4 h. NP-40 was added to a final concentration of 0.1% in the lysis buffer before sonication. Also 0.1% of NP-40 was required in all the purification buffers to maintain solubility of the full length NS5A protein throughout the subsequent purification procedure. After elution with the buffer containing 500 mM imidazole, full length NS5A protein was normally concentrated in the first and second fraction (Fig. 3-1A). Rich in prolines, the full length NS5A protein showed larger than its actual

size (49.2 kDa) in SDS-PAGE. Western blot assay showed the successful cleavage of ubiquitin *in vivo* (Fig. 3-1C). Gel filtration by Sephacryl S-100 was used to further purify the protein (Fig. 3-1B).

Fig. 3-1 Purification of full length NS5A

A) Expression and purification of full length NS5A were analyzed on a 12% SDS-PAGE gel. Lane 1, molecular weight marker; lane 2, supernatant loaded on Ni²⁺-NTA column; lane 3, flowthrough from the Ni²⁺-NTA column; Lane 4, lysis buffer wash; lane 5, wash buffer wash; lane 6, the first fraction eluted from the Ni²⁺-NTA column; Lane 7, the second fraction eluted from the Ni²⁺-NTA column. Lane 8 and 9, fractions from S-100 gel filtration eluted by 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP40, 1 mM DTT. B) Gel filtration analysis and purification of full length NS5A. The full length NS5A purified by Ni²⁺-NTA column was loaded onto S-100 gel filtration column. The arrow indicates the elution position of NS5A. C) Western blot analysis for checking the cleavage of ubiquitin. The proteins were probed by a commercially available ubiquitin mouse monoclonal antibody (1:1000 diluted). Lane 1, ubiquitin protein; Lane 2, purified full length NS5A.

We also cloned and purified the truncated NS5A- Δ N, which was deleted of the N-terminal 30 amino acids, the membrane anchoring domain of NS5A protein. Although the same procedure was applied to purify both of the full length NS5A and the truncated NS5A- Δ N, the higher solubility of NS5A- Δ N led to its higher productivity compared to the yield of full length NS5A (Fig. 3-2). During the purification procedure, 0.1% of NP-40 was still necessary in all the purification buffers to maintain solubility and stability of the truncated NS5A protein. The yield of the purified NS5A- Δ N was approximately 0.75 mg/L to 1 mg/L. Due to the higher solubility and yield of NS5A- Δ N obtained from this pET-ubiquitin system, the purified NS5A- Δ N was chose for the later structural and functional studies.

Fig. 3-2 Purification of NS5A-ΔN

A) Expression and purification of NS5A-ΔN were analyzed on a 12% SDS-PAGE gel. Lane 1, molecular weight marker; lane 2, supernatant loaded on Ni²⁺-NTA column; lane 3, flowthrough from the Ni²⁺-NTA column; Lane 4, lysis buffer wash; lane 5, wash buffer wash; lane 6, the first fraction eluted from the Ni²⁺-NTA column; Lane 7, the second fraction eluted from the Ni²⁺-NTA column. Lane 8, 9 and 10, fractions from S-100 gel filtration eluted by 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% NP40, 1 mM DTT. B) Gel filtration analysis and purification of NS5A-ΔN. The NS5A-ΔN purified by Ni²⁺-NTA column was loaded onto S-100 gel filtration column. The arrow indicates the elution position of NS5A-ΔN.

The commercial HCV NS5A mouse monoclonal antibody (epitope: amino acids 2190-2300, ID Labs) was used to probe the purified full length NS5A and truncated NS5A- Δ N proteins. As shown in Fig. 3-3, the purified proteins can be specifically identified by the monoclonal antibody.

Fig. 3-3 Western blot identification of full length NS5A and NS5A- Δ N

The purified proteins were probed by a commercially available HCV NS5A mouse monoclonal antibody (1:1000 diluted). Lane 1, the truncated NS5A (NS5A- Δ N); Lane 2, full length NS5A

3.1.2. Secondary structure analysis of NS5A- Δ N by CD spectrum

CD spectrum was recorded to analyze the secondary structure of NS5A- Δ N. The representative CD spectrum of α -helices is characterized by minimum near 208 and 222 nm and the β -sheet structures yield a minimum at 215 nm, and random coil structures are characterized by a negative peak near 200 nm (Adler *et al*, 1973). The CD spectrum of NS5A- Δ N shows a minimum at around 214 nm (Fig. 3-4), suggesting that this protein is mainly β -sheet. This CD result is consistent with the later structural study of NS5A protein to some degree: the resolved crystal structure of HCV 1b NS5A domain 1 revealed that it mainly comprises β -sheet

(Tellinghuisen *et al*, 2005).

Fig. 3-4 Analysis NS5A-ΔN by CD

The CD spectrum was recorded on Chirascan circular dichroism spectrometer (Applied Photophysics Limited, Surrey, UK) at 25 °C, using NS5A-ΔN (0.1 mM) in 20 mM sodium phosphate, pH 7.0, 150 mM NaCl, 0.1% NP40, 1 mM DTT.

3.1.3. Molecular interaction between NS5A-ΔN and its biological ligands

3.1.3.1. Expression and purification of GST-NS5Bt and NS5Bt

Hepatitis C Virus NS5B has a GDD motif (Gly-Asp-Asp motif), which is common to all the RNA polymerases of RNA viruses, and has been reported to possess

RNA-dependent RNA polymerase (RdRP) activity (Behrens *et al*, 1996; Lohmann *et al*, 1997). It has been elucidated that HCV NS5A directly interacts with HCV NS5B as a component of HCV replicase complex. Two regions of NS5A (amino acids 105-162 and 277-334) were found to be responsible for NS5B binding. Additionally, four discontinuous sequences of NS5B (amino acids 139-145, amino acids 149-155, amino acids 365-371, and amino acids 382-388) were found to be essential for binding to NS5A (Shirota *et al*, 2002). Although there is about 15% amino acids difference between HCV-1a and -1b, the sequence of the binding sites of NS5A protein on NS5B polymerase is highly identical and conserved (Fig. 3-5A). The sequence of the binding sites of NS5B on NS5A is conserved (Fig. 3-5B). To study the interaction between NS5A and NS5B, GST-NS5Bt (without the c-terminal 21 amino acids, which is predicted be to an anchoring domain) (Yamashita *et al*, 1998) was purified for the GST pull-down assay and NS5Bt-His₆ was purified for later NMR study.

Fig. 3-5 Amino acids sequence alignment of the NS5A and NS5B binding sites from HCV-1b and -1a

A) Amino acids sequence alignment of the NS5A binding sites on NS5B polymerase between HCV-1b and -1a. The identical amino acids are shown in yellow and the less conserved amino acids are shown in green. The four NS5A protein binding sites on NS5B polymerase are shown in blue boxes. B) Amino acids sequence alignment of the NS5B binding sites on NS5A between HCV-1b and -1a. The identical amino acids are shown in yellow and the non-similar amino acids are shown in white. The two NS5B protein binding sites on NS5A are shown in blue boxes.

GST-fused HCV NS5Bt protein was expressed and purified as described before (Yamashita *et al*, 1998). Briefly, the GST-NS5Bt protein was expressed in *E. coli* BL21 (DE3) cells at 20 °C for 4 h. The harvest *E. coli* cells were sonicated and the cell lysate was passed through glutathione-Sepharose 4B beads. The GST-NS5Bt was first eluted with the elution buffer plus 150 mM NaCl and then eluted with the elution buffer containing 500 mM NaCl (Fig. 3-6a). The eluted protein sample was

further purified by high-resolution Sephacryl S-200 gel filtration column (Fig. 3-6b) and analyzed by 10% SDS-PAGE gel (Fig. 3-6c). After concentration, GST-NS5Bt seems to undergo some degradation. The identity of the GST-NS5Bt was confirmed by Western blot analysis (Fig. 3-6d).

Fig. 3-6 Purification of GST-NS5Bt

A) Expression and purification of GST-NS5Bt were analyzed on a 10% SDS-PAGE gel. Lane 1, molecular weight marker; lane 2, the first fraction eluted from the glutathione-Sepharose 4B beads; Lane 3, 4, the fractions eluted from the glutathione-Sepharose 4B beads after addition of the elution buffer with 500 mM NaCl. B) Gel filtration analysis and purification of GST-NS5Bt. The NS5Bt purified by glutathione-Sepharose 4B beads was loaded onto S-200 gel filtration column. The arrow indicates the elution position of GST-NS5Bt. C) GST-NS5Bt purified after S-200 gel filtration. Lane 1, molecular weight marker; Lane 2, GST-NS5Bt after purification by S-200 gel filtration with 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 10 mM DTT, 0.1% NaN₃. D) Western blot identification of GST-NS5Bt. The purified proteins were probed by a commercially available anti-HCV NS5B polyclonal antibody (1:1000 diluted).

The NS5Bt-His₆ protein was expressed *E. coli* BL21 (DE3) cells with the T7 polymerase system. As prescribed before (Ferrari *et al*, 1999), the NS5Bt-His₆ protein was eluted from Ni²⁺-NTA column with the elution buffer containing 350 mM NaCl and 2.5% glycerol (Fig. 3-7a). Then the eluted sample was passed through high-resolution Sephacryl S-100 gel filtration column to be further purified (Fig. 3-7b). The final concentrated protein was analyzed by 10% SDS-PAGE gel (Fig. 3-7c). Polyclonal anti-NS5B antibody was used to probe the NS5Bt-His₆ protein. Shown in Fig. 3-7d, the purified proteins can be specifically identified by the anti-NS5B polyclonal antibody.

Fig. 3-7 Purification of NS5Bt

A) Expression and purification of NS5Bt were analyzed on a 10% SDS-PAGE gel. Lane 1, molecular weight marker; lane 2, uninduced cells; lane 3, cells after 4 h induction; lane 4, supernatant loaded on Ni²⁺-NTA column; lane 5, cell pellet after sonication and centrifuge; lane 6, flowthrough from the Ni²⁺-NTA column; Lane 7, wash buffer wash; lane 8, 9, 10, fractions eluted from the Ni²⁺-NTA column. B) Gel filtration analysis and purification of NS5Bt. The arrow indicates the elution position of NS5Bt. C) NS5Bt purified after S-100 gel filtration. Lane 1, molecular weight marker; Lane 2, concentrated NS5Bt (50 mg/ml) after purification by S-100 gel filtration with 20 mM sodium phosphate, pH 6.5, 350 mM NaCl, 2.5% glycerol, 1 mM DTT, 0.1% NaN₃. D) Western blot identification of NS5Bt. The purified proteins were probed by a commercially available anti-HCV NS5B polyclonal antibody (1:1000 diluted).

3.1.3.2. Interaction between NS5A-ΔN and GST-NS5Bt by GST pull-down assay

To determine whether the purified NS5A-ΔN protein has the ability to bind NS5B, we performed an *in vitro* binding study between NS5A-ΔN and GST-NS5Bt by using the GST pull-down assay (Fig. 3-8). As shown in the results of western blot detection, NS5A-ΔN could be pulled down with GST-NS5Bt (Fig. 3-8A, lane 2) but not with GST (Fig. 3-8A, lane 3), indicating the specific interaction between NS5A-ΔN and NS5B *in vitro*. This proves that the purified NS5A-ΔN is functional and has its biological activity. Our results are consistent with the previous report (Shirota *et al*, 2002).

Fig. 3-8 *In vitro* binding studies of NS5A-ΔN and NS5B by GST pull-down assay

A) 5 μ g NS5A-ΔN was mixed with 1 μ g of GST-NS5Bt and pulled down with 10 μ l GST resin after preblocking by 1% bovine serum albumin. After washing with GBT buffer, each bound protein was analyzed by western blot with anti-HCV NS5A monoclonal antibody. Lane 1, NS5A-ΔN input; Lane 2, after loading NS5A-ΔN, protein bound to the GST resin immobilized with GST-NS5Bt; Lane 3, after loading NS5A-ΔN, protein bound to the GST resin immobilized with GST. B) Western blot analysis of the binding of GST-NS5Bt (1 μ g) to GST resin by anti-HCV NS5B polyclonal antibody. Lane 1, GST-NS5B input; Lane 2, protein bound to the GST resin immobilized with GST-NS5B; Lane 3, protein bound to the GST resin immobilized with GST.

3.1.3.3. Interaction between NS5A-ΔN and GST-PKR by GST pull-down assay

IFN-induced double-stranded RNA-activated PKR is one mechanism by which

IFN mediates antiviral activity (Hershey, 1991). Early data has shown that NS5A bound to PKR and prevented the activation of PKR (Gale *et al*, 1997), which required the ISDR and an additional C-terminal 26 residues to the ISDR in NS5A (Gale *et al*, 1998). Binding of NS5A to PKR resulted in inhibition of both PKR autophosphorylation and phosphorylation of its substrate, eIF2- α . To prove the purified NS5A- Δ N is functional and can bind to other biological partners than NS5B, the binding between the purified NS5A- Δ N and PKR was also analyzed by the *in vitro* GST pull-down assay. The result showed that NS5A- Δ N interacts with GST-PKR (Fig. 3-9, lane 2). This further proves that the purified NS5A- Δ N is active and functional by interacting with PKR, another characterized biological partner of HCV NS5A.

Fig. 3-9 *In vitro* binding studies of NS5A- Δ N and PKR by GST pull-down assay

5 μ g NS5A- Δ N was mixed with 24 μ l PKR-agarose beads (1 μ g GST-PKR bound) after preblocking by 1% bovine serum albumin. After extensive washing, each bound protein was analyzed by western blot with anti-HCV NS5A monoclonal antibody. Lane 1, NS5A- Δ N input; Lane 2, after loading NS5A- Δ N, protein bound to the PKR agarose beads; Lane 3, after loading NS5A- Δ N, protein bound to the GST resin immobilized with GST.

3.2. Expression, purification and characterization of NS5A Domain 1 (NS5A-D1) and Domain 3 (NS5A-D3)

In the purification of full length NS5A and NS5A- Δ N, we could not get the soluble protein without NP40 or using NP40 less than its critical micellar concentration, CMC (0.3 mM). Our attempt to crystallize of NS5A- Δ N failed probably due to the high concentration of NP40, which is essential for the stability of NS5A- Δ N. Other detergents such as n-Dodecyl α -D-maltoside and Octyl- β -D-glucopyranoside, were also chosen for purify NS5A- Δ N, but resulted in much less yields. Because three domains have been predicted inside the HCV NS5A protein, domain 1 (residues 1-213), domain 2 (residues 250-342), and domain 3 (residues 356-447) (Fig. 1-2) (Tellinghuisen *et al*, 2004), we changed our strategy by truncating the full length NS5A protein into the three domains to study their structural and functional properties, respectively.

3.2.1. Expression and purification of NS5A-D1 (amino acids 28-213) and NS5A-D3 (amino acids 356-447)

HCV 1b NS5A domain 1 was revealed to comprise a nascent zinc-binding motif and its zinc-binding property is essential for HCV replication (Tellinghuisen *et al*, 2004; Tellinghuisen *et al*, 2005). NS5A domain 3 is mainly responsible for interacting with growth factor receptor bound protein 2 (Grb2) and perturbing Grb2 mediated signaling pathway to inhibit mitogenic signaling (Tan *et al*, 1999). According to the three domain model (Tellinghuisen *et al*, 2004), we cloned the

cDNA of domain 1 and domain 3 of HCV-1a NS5A into the UbCHis vector, respectively. Both of the domain 1 (NS5A-D1, amino acids 28-213) and domain 3 (NS5A-D3, amino acids 356-447) could be purified by using the same purification procedure as that for purifying full length NS5A and NS5A- Δ N, but without adding NP40 or any other detergents in the purification buffers (Fig. 3-10, Fig. 3-11).

Fig. 3-10 Purification of NS5A-D1 (amino acids 28-213)

A) Expression and purification of NS5A-D1 were analyzed by 12% SDS-PAGE gel, Lane 1, molecular weight markers; lane 2, supernatant loaded on Ni-NTA column; lane 3, flow through from the Ni^{2+} -NTA column; Lane 4, lysis buffer wash; lane 5, wash buffer wash; lane 6, 7, 8, fractions eluted from the Ni^{2+} -NTA column; Lane 9, purified NS5A-D1 from S-100 gel filtration eluted by 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 1mM DTT, 0.1% NaN_3 . B) Gel filtration analysis and purification of NS5A-D1. The NS5A-D1 purified by Ni^{2+} -NTA column was loaded onto S-100 gel filtration column. The arrow indicates the elution position of NS5A-D1.

Fig. 3-11 Purification of NS5A-D3 (amino acids 356-447)

A) Expression and purification of NS5A-D3 were analyzed by 12% SDS-PAGE gel. Lane 1, molecular weight markers; lane 2, supernatant loaded on Ni²⁺-NTA column; lane 3, flowthrough from the Ni²⁺-NTA column; Lane 4, wash buffer wash; lane 5, 6, fractions eluted from the Ni²⁺-NTA column; Lane 7, purified NS5A-D3 from S-100 gel filtration eluted by 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 1mM DTT, 0.1% NaN₃. B) Gel filtration analysis and purification of NS5A-D3. The NS5A-D3 purified by Ni²⁺-NTA column was loaded onto S-100 gel filtration column. The arrow indicates the elution position of NS5A-D3.

3.2.2. 1D ^1H NMR evaluations and CD analysis for NS5A-D1 and NS5A-D3

To get some primary information about the NS5A domain 1 and domain 3, initial NMR evaluations for these two domains were conducted. 1D ^1H NMR spectra are the integrated intensity of resonances with α protons giving rise to signal. Even for small proteins, 1D ^1H NMR spectra are too complicated to be analyzed comprehensively. However, 1D ^1H NMR spectrum can be useful for evaluating the suitability or stability of a protein sample. With the help of the 1D ^1H spectrum, a qualitative distinction between folded and unfolded proteins is possible. Well folded proteins have relatively sharp peaks and good chemical shift dispersion. Additionally, the well dispersed NMR resonance signals in the regions of methyl protons (-0.5 - 1.5 ppm), α -protons (3.5 - 6 ppm), and amide protons (6 - 10 ppm) are good indicators of folded globular proteins (Page *et al*, 2005). As shown in Fig. 3-12, the 1D ^1H spectra of HCV 1a NS5A-D1 (amino acids 28-213) showed broadened (many protons per peak) and heterogeneous signals caused by conformational heterogeneity and increased internal mobility, which hints high aggregation. The high aggregation resulted in great precipitation during the concentration of this protein. We could not get enough NS5A-D1 protein for further study of its secondary or three dimensional structures. Our trial of structural study about HCV-1a NS5A-D1 failed due to the highly aggregated character of this protein. However, a recent structural study of the domain 1 (amino acids 36-198) of HCV-1b NS5A revealed that it mainly comprises β -sheet but with a small region of

α -helix (Fig. 1-3) (Tellinghuisen *et al*, 2005). Unlike the previous study, in our hands, even in the same buffers as described (Tellinghuisen *et al*, 2004; Tellinghuisen *et al*, 2005), the purified truncated domain 1 of HCV-1a NS5A (amino acids 36-198) was highly aggregated and not suitable for further structural study. A sequence alignment reveals 82% homology between the two genotypes. The observed aggregate might be attributable to the difference in protein sequence of the domain 1 (Fig. 3-13).

Fig. 3-12 1D ^1H NMR spectrum of the NS5A-D1 (amino acids 28-213)

Fig. 3-13 Amino acid sequence alignment of the NS5A domain 1 region between HCV-1a and -1b

The identical amino acids are shown in yellow, the less conserved amino acids are shown in green and the non-similar amino acids are shown in white.

The NS5A-D3 (amino acids 356-447) was indicated as unfolded protein by 1D ¹H NMR spectra, which is characterized by lost of many signals compared with well folded proteins and its small amount of signals with little chemical shift dispersion that means the ¹H frequencies of all residues are very similar and the signals overlap (Fig. 3-14). CD spectrum was also recorded to analyze the secondary structure of NS5A-D3 (Fig. 3-15). The spectrum exhibits a minimum at about 202 nm, indicating that NS5A-D3 is in random coil conformation.

Fig. 3-14 1D ^1H NMR spectrum of the NS5A-D3 (amino acids 356-447)

A) Aliphatic region in the 1D ^1H spectrum of the NS5A-D3; B) Aromatic region of the 1D ^1H NMR spectrum of the NS5A-D3.

Fig. 3-15 Analysis of NS5A-D3 by CD

The CD spectrum was recorded using the S-200-purified NS5A-D3 (0.1 mM) in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl.

3.3. NS5A Domain 2 is natively unfolded

The domain 2 of HCV NS5A protein is involved in several important biological functions of NS5A. The domain 2 participates in the molecular interaction with NS5B, the RNA-dependent RNA polymerase (RdRP) of HCV, which is essential in viral HCV RNA synthesis and replication (Shirota *et al*, 2002). The domain 2 also contains the interferon (IFN) sensitivity-determining region, ISDR, and the PKR

binding domain (Gale *et al*, 1997). The domain 2 is also a part of NS5A transcriptional activation domain (Kato *et al*, 1997; Tanimoto *et al*, 1997) and contributes to IRES suppression (Kalliampakou *et al*, 2005). In addition, the domain 2 is important for the interaction between NS5A and TRAF2 (Park *et al*, 2002) or Grb2 (Macdonald *et al*, 2004; Tan *et al*, 1999). A potential Bcl-2 homology region 2 (BH2) was predicted in the domain 2 (Chung *et al*, 2003). Furthermore, the domain 2 of the HCV NS5A protein activates PI3K by binding to the SH3 domain of the PI3K p85 regulatory subunit and result in the augmented phosphorylation of Akt and the inactivation of the Akt substrates FKHR and GSK-3 β (Street *et al*, 2004; Street *et al*, 2005). In view of this, HCV NS5A appears to be a multi-functional protein. The elucidation of the biochemical and structural characteristics of NS5A domain 2 will be helpful for the understanding of the biological functions of HCV NS5A protein and its molecular basis in depth.

3.3.1. Expression and purification of NS5A Domain 2 (NS5A-D2)

We designed and constructed an over-expression system for the domain 2 using ubiquitin and ubiquitin-specific, carboxy-terminal protease, which is constitutively expressed from the second plasmid, pCG1, to cleave ubiquitin off from the fusion protein and released NS5A-D2 protein in cells (Huang *et al*, 2004). The system was efficient in over-producing the NS5A-D2 and allowed us to obtain soluble NS5A-D2 (Fig. 3-16A). The purified protein was stable after storage for more than one month at 4 °C. We confirmed the identity of the NS5A-D2 by western blot

analysis (Fig. 3-16B).

Fig. 3-16 Expression, purification and western blot analysis of NS5A-D2 (amino acids 240-335)

A) Expression and purification of NS5A-D2 were analyzed on a 15% SDS-PAGE gel. Lane 1, molecular weight marker; Lane 2, uninduced cells; Lane 3, cells after 4 h induction; Lane 4, supernatant loaded on Ni²⁺-NTA column; Lane 5, eluted fraction from Ni²⁺-NTA column after addition of elution buffer (100 mM Tris-Cl, pH 7.0, 200 mM NaCl, 500 mM Imidazole, 1 mM PMSF, 5 mM β-ME); Lane 6, protein further purified by S-200 gel filtration eluted with 20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 1 mM DTT, 0.1% NaN₃. B) Western blot analysis of the NS5A-D2 by a commercially available HCV NS5A mouse monoclonal antibody (1:1000 diluted). Lane 1, uninduced cells; Lane 2, cells after induction; Lane 3, purified NS5A-D2.

3.3.2. Identification of NS5A-D2 by MS spectrum

The purified NS5A-D2 was sent to Mass Spectrometry (MS) analysis for further identification. The result of Mass Spectrometry and the Blast search showed that the purified protein was from Hepatitis C virus NS5A protein, with the molecular weight as expected (**Error! Reference source not found.**). Taken together with the positive result of western blot probed by monoclonal NS5A antibody, the data suggest that the purified protein is the domain 2 of NS5A protein.

dimeric form of NS5A-D2. Equilibrium sedimentation experiments were performed on a Beckman Optima XL-A analytical ultracentrifuge with an An-50 Ti rotor. NS5A-D2 at concentration of 0.65, 0.32, and 0.16 mg/ml were analyzed at 4 °C in the gel filtration buffer (20 mM sodium phosphate, pH 6.5, 50 mM NaCl, 1 mM DTT, 0.1% NaN₃), at 28000 rev/min (63221 x g) for 20 h, at 34500 rev/min (95981 x g) for 5 h, and 42000 rev/min (142248 x g) for 5 h. Absorbance measurements at 280 nm were taken. Sedphat 5.14 was used for data processing and curve fitting (Vistica *et al*, 2004). As shown by the arrows in the following Fig. 3-18A, aggregation (OD>1.5) was detected at all the concentration. Shown in Fig. 3-18B, the calculated molecular weight after fitting was 11,709 Daltons, which is close to the theoretical molecular weight of NS5A-D2 (11,856 Daltons), suggesting that NS5A-D2 is in its monomer form at the current conditions. The fitting root mean square deviation (RMSD) is 0.017047, which is relatively high, indicating that the fit is not perfect. This is probably due to the aggregation of the samples during the experiment.

Fig. 3-18 Analytical ultracentrifugation sedimentation equilibrium data

A) The NS5A-D2 protein was analyzed at three different concentrations at 28000 rev/min (63221 x g), 34500 rev/min (95981 x g), and 42000 rev/min (142248 x g). B) Representative result for the NS5A-D2 is shown. The upper panel shows the equilibrium profile for each velocity. The calculated molecular weight in Daltons is indicated. RMSD between the fitted and experimental values is also indicated. The lower panel shows the residue difference between the experimental and fitted values.

3.3.4. Gel filtration analysis of NS5A-D2

The theoretical molecular weight of NS5A-D2 (amino acids 240-335) is 11.856 kDa. On the S-200 gel filtration column, the elution volume of this protein is about 74 ml (Fig. 3-19). Using the equation described, the determined molecular weight of the NS5A-D2 is 20.3 kDa, which is significantly larger than the theoretical one. This suggests that the NS5A-D2 may contain a high degree of unstructured regions.

Fig. 3-19 Gel filtration analysis of NS5A-D2

The Ni^{2+} -NTA-purified NS5A-D2 was loaded onto an S-200 gel filtration column. The arrow indicates the elution position of NS5A-D2. The K_{av} ($K_{av} = (V_e - V_o) / (V_t - V_o)$, $V_e = 74$ ml, $V_o = 37$ ml, $V_t = 125$ ml) of NS5A-D2 was fitted to the standard curve (right above the gel filtration figure). The molecular weight of NS5A-D2 was calculated by fitting its K_{av} to the equation $K_{av} = -0.40907 * \log(\text{molecular weight}) + 2.182250$.

3.3.5. Molecular interaction between NS5A-D2 and its biological ligands by *in vitro* GST pull-down assay and surface plasmon resonance

It has been elucidated that HCV NS5A interacts with HCV NS5B directly as a component of HCV replicase complex (Shirota *et al*, 2002). To determine whether the purified NS5A-D2 still maintains the ability to bind NS5B, we performed an *in vitro* binding study between NS5A-D2 and GST-NS5Bt using the GST pull-down assays (Fig. 3-20C). Both NS5A-ΔN and NS5A-D2 were pulled down with GST-NS5Bt (Fig. 3-20C, lane 2, 5) but not with GST (Fig. 3-20C, lane 3, 6), indicating the specific interaction of NS5A-D2 with NS5B *in vitro*. To further prove that NS5A-D2 is in its functional form, the binding between the purified NS5A-D2 and commercially available PKR was also tested by the GST pull-down assay. The result showed that NS5A-D2 interacts with PKR (Fig. 3-20D, lane 5). This proves that the purified NS5A-D2 is active and functional in interacting with the NS5B and PKR, which are the characterized biological partners of HCV NS5A.

Fig. 3-20 *In vitro* binding studies of NS5A-D2 and NS5B or PKR by GST pull-down assay

A) Purified NS5A-ΔN (Lane 2) and NS5A-D2 (Lane 3), 12% SDS-PAGE. B) Purified GST-NS5Bt (Lane 2), 10% SDS-PAGE. C) GST pull-down assay to examine the interaction between NS5A-D2 and NS5B. Lane 1, NS5A-ΔN input; Lane 2, loading NS5A-ΔN, eluted from the resin immobilized with GST-NS5Bt; Lane 3, loading NS5A-ΔN, eluted from the resin immobilized with GST; Lane 4, NS5A-D2 input; Lane 5, loading NS5A-D2, eluted from the resin immobilized with GST-NS5Bt; Lane 6, loading NS5A-D2, eluted from the resin immobilized with GST. D) GST pull-down assay to check the interaction between NS5A-D2 and PKR. Lane 1, NS5A-ΔN input; Lane 2, after loading NS5A-ΔN, elution from the PKR agarose beads; Lane 3, loading NS5A-ΔN, eluted from the resin immobilized with GST; Lane 4, NS5A-D2 input; Lane 5, loading NS5A-D2, eluted from the PKR-agarose beads; Lane 6, loading NS5A-D2, eluted from the resin immobilized with GST. E) Western blot analysis of the binding of GST-NS5Bt immobilized on the resin. Lane 1 and 4, GST-NS5Bt input; Lane 2 and 5, protein bound to the resin immobilized with GST-NS5Bt; Lane 3 and 6, protein bound to the resin immobilized with GST. F) Scheme for GST pull-down assay of the interaction between NS5A-ΔN and NS5B, NS5A-D2 and NS5B.

Surface plasmon resonance was used to quantify the binding affinity of NS5A-D2 to NS5Bt. Shown in the following Fig. 3-21, the calculated binding affinity is 65.6 μ M. This suggests that NS5A-D2 is capable of binding to its biological partner. This result is consistent with our GST-pull down assay results.

Fig. 3-21 Binding of NS5A-D2 to NS5Bt analyzed by surface plasmon resonance

A) Fitting curve of the binding of NS5A-D2 to NS5Bt immobilized on CM5 chip. B) Sensorgrams of the binding of NS5A-D2 to NS5Bt immobilized on CM5 chip. Individual SPR profiles were obtained at increasing concentrations of NS5A-D2 in the range of 5 μ M - 80 μ M from the bottom to the top.

Furthermore, surface plasmon resonance was also used to determine the binding affinity of NS5A- Δ N to NS5Bt. The stability of the purified NS5A- Δ N was dependent on the presence of NP40 in the buffer. However, the use of NP40, even 0.1% in the running buffer, resulted in a difficulty stabilizing chip matrix and got high non-specific binding. Without NP40 in the running buffer, NS5A- Δ N was heavily aggregated. Unfortunately, under the experimental conditions, we failed to determine the binding affinity of NS5A- Δ N to NS5Bt.

3.3.6. Structural study of NS5A-D2

3.3.6.1. Secondary structural analysis of NS5A-D2 by CD spectrum

CD spectrum was recorded to analyze the secondary structure of NS5A-D2 (Fig. 3-22). The spectrum exhibits a minimum at about 202 nm with a slight negative ellipticity at about 224 nm, indicating that NS5A-D2 is mostly in random coils.

Fig. 3-22 Analysis of NS5A-D2 by CD

The CD spectrum was recorded using the S-200-purified NS5A-D2 (0.1 mM) in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl.

3.3.6.2. DLS analysis of NS5A-D2

Dynamic light scattering (DLS) has been used to characterize the ordered or disordered proteins, and the folding intermediates of proteins (Cleland & Wang, 1990; Havel *et al*, 1986). The scattering intensity of a small molecule is proportional to the square of the molecular weight. The molecular size of 2 mg/ml NS5A-D2 in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl was examined by DLS. An effective diameter measured is the average diameter which is weighted by

the intensity of light scattered by each particle (Berne & Percora, 1974). The different peaks shown in the graph are corresponding to different diameters of the molecule. As shown in Fig. 3-23A, the weighted average diameter calculated from the different peaks of NS5A-D2 is 10.5 nm, which is considered as its effective diameter. However, the effective diameter of the well folded Bcl-X_L at the same concentration and buffer condition was 6.3 nm (Fig. 3-23B). Theoretically the molecular weight of NS5A-D2 (amino acids 240-335) is 11.8 kDa, but the molecular weight of Bcl-X_L is 20 kDa. The larger diameter observed on NS5A-D2 by DLS is probably due to the extended and disordered conformation (Nicoli & Benedek, 1976). Polydispersity is the measurement of the size distribution width, which has no units. The increase of polydispersity results in the increase in size distribution width (Berne & Percora, 1974). The size distribution width of NS5A-D2 is wide with a polydispersity value of 0.533 (Fig. 3-23A), suggesting that the disordered NS5A-D2 is polydisperse and tends to aggregation.

Fig. 3-23 Dynamic light scattering analysis of NS5A-D2

A) The DLS experiment was performed on NS5A-D2 (2 mg/ml) in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl. B) The DLS experiment was performed on Bcl-X_L (2 mg/ml) in 20 mM sodium phosphate, pH 6.5, 50 mM NaCl.

3.3.6.3. NMR $^1\text{D } ^1\text{H}$ and $2\text{D } ^1\text{H}-^{15}\text{N}$ HSQC spectrum of NS5A-D2

NMR is a valuable tool to identify intrinsically unstructured proteins. To further define the structural characteristics of the NS5A-D2, we performed $1\text{D } ^1\text{H}$ and $2\text{D } ^1\text{H}-^{15}\text{N}$ HSQC NMR spectroscopy (Fig. 3-24A and Fig. 3-24B). The analysis of the dispersion of NMR resonance signals in the regions of methyl protons (-0.5 - 1.5 ppm), α -protons (3.5 - 6 ppm), and amide protons (6 - 10 ppm) is a good method for evaluating folded globular proteins (Page *et al*, 2005). Our NMR data demonstrated that the $1\text{D } ^1\text{H}$ spectrum of NS5A-D2 lacks NMR signals in the region of the methyl protons and the $2\text{D } ^1\text{H}-^{15}\text{N}$ HSQC spectrum show a narrow dispersion in the backbone amide proton resonance signals which only covers the range of 7.2-8.5 ppm. Additionally, the side-chain signals of asparagine accumulate at about 7.4 ppm/6.85 ppm, which also indicates this protein is highly unstructured. Taken together, our initial $1\text{D } ^1\text{H}$ and $2\text{D } ^1\text{H}-^{15}\text{N}$ HSQC NMR data suggest that the NS5A-D2 is natively disordered or dynamic and is not well structured.

Fig. 3-24 Initial analysis of NS5A-D2 by NMR 1D ^1H and 2D ^1H - ^{15}N HSQC spectra

The NMR samples contained 0.6 mM of either unlabeled or uniformly ^{15}N -labeled NS5A-D2. A) 1D ^1H spectrum of the NS5A-D2. B) 2D ^1H - ^{15}N HSQC spectrum for NS5A-D2.

3.3.6.4. NMR backbone ^1H , ^{13}C and ^{15}N resonance assignments of NS5A-D2

Many proteins are intrinsically unstructured but in their biologically functional states (Wright & Dyson, 1999). The unfolded proteins are unsuitable for X-ray crystallography study because of their disordered conformations. Highly overlapped resonances, particularly those of protons, provide the experimental difficulties in the NMR studies of unfolded proteins. However, high-resolution NMR experiments utilizing a triple-resonance strategy allow researchers to get detailed insights into the structure and dynamics of the unstructured proteins. The three- and four-dimensional NMR experiments introduce the assignments of the proton, nitrogen and carbon chemical shifts of proteins or protein complexes to determine their structures in solution (Lingel *et al*, 2004). Several NMR studies of the natively unfolded proteins or the proteins in their unfolded states have been reported, including cytoplasmic dynein intermediate chain (Benison *et al*, 2006), HIV-1 Tat (Shojania & O'Neil, 2006), lysozyme (Evans *et al*, 1991; Schwalbe *et al*, 1997), FK506-binding protein (Logan *et al*, 1994), and SH3 domain (Zhang & Forman-Kay, 1997).

Assignments of ^1H , ^{13}C , and ^{15}N resonances have been made by using three-dimensional heteronuclear NMR spectroscopy (**Error! Reference source not found.**, Fig. 3-26). We have assigned $^{13}\text{C}_\alpha$ resonances for 94 residues and $^{13}\text{C}_\beta$ resonances for 92 residues (two glycines with no $^{13}\text{C}_\beta$) out of 102 residues in

NS5A-D2, respectively. The unassigned residues are K1, P84 and the C-terminal His₆-tag. As shown in Fig. 3-27, 86 backbone residues out of 94 non-proline residues in NS5A-D2 were resolved, except for K1, A2 and the C-terminal His₆-tag. The chemical shifts of the backbone amide proton resonances are clustered in a narrow 1.3 ppm window from 7.2 ppm to 8.5 ppm, and the dispersion of the ¹⁵N chemical shifts is narrowed in 19.2 ppm, as typical signs of an unfolded protein (Peti *et al*, 2001). The backbone resonances are clustered in three regions in the spectra. A bulk of the ¹⁵N chemical shifts for the backbone amides are clustered between 116.4 ppm and 127.4 ppm. The ¹⁵N chemical shifts for Gly residues are resonated between 108.1 ppm and 109.3 ppm. The ¹⁵N chemical shifts for Thr/Ser residues are mostly clustered between 114.1 ppm and 116.5 ppm, consistent with the characteristic ¹⁵N chemical shifts of amino acid residues in unfolded conformations (Wishart *et al*, 1995). Excluding K1 and the C-terminal His₆-tag residues, the assignments of the side chains are about 80% complete. The cross peaks for the side-chain amides of the carboxyamide groups in four Asn (between 6.7 ppm and 7.5 ppm for ¹H, between 111.9 ppm and 113.1 ppm for ¹⁵N, respectively) and the NH of the rings of three Trp (between 10 ppm and 10.2 ppm for ¹H, between 129.3 ppm and 130.3 ppm for ¹⁵N, respectively) are also visible in the 2D ¹H-¹⁵N HSQC spectrum. The chemical shifts of the -NH₂ side chain protons of the four Asn resonate at the chemical shifts indicative of short unstructured peptides (Merutka *et al*, 1995). This feature is not usually displayed in the spectra of native folded proteins because longer range specific contacts always induce the

chemical shift perturbations (Schwalbe *et al*, 1997).

The assignment of NS5A-D2 has been deposited in the BioMagResBank with the accession number of 15225.

Fig. 3-25 The 3D experiments of NS5A-D2 backbone assignment

The experiment data were processed by Felix. A) HNCACB provides information about $C_{\alpha i}$, $C_{\alpha i-1}$, $C_{\beta i}$ and $C_{\beta i-1}$. B) CBCACONH provides information about $C_{\alpha i-1}$ and $C_{\beta i-1}$. C) HNCA provides information about $C_{\alpha i}$, $C_{\alpha i-1}$. D) HNHA provides information about H_A . E). HNCO provides information about C' (CO)

Fig. 3-26 The backbone assignment of NS5A-D2

This figure shows the assigned fragment of NS5A-D2 from Ser 35 to Asp 43, other fragments are also assigned with the same procedure. The upper strips are the connection of $C_{\alpha i}$ and $C_{\alpha i-1}$ from HNCA shown in **Error! Reference source not found.**, the line gives the connection. The lower strips are the connection of $C_{\beta i}$ and $C_{\beta i-1}$ from HNCACB. The green peaks are the $C_{\beta i}$ and $C_{\beta i-1}$, the blue peaks are the $C_{\alpha i}$ and $C_{\alpha i-1}$, which are same as the upper strips.

Fig. 3-27 2D ^1H - ^{15}N HSQC spectrum of assigned NS5A-D2

The uniformly ^{15}N -labeled NS5A-D2 was prepared at a final concentration of 0.6 mM in 20 mM NaPO_4 , pH 6.5, 50 mM NaCl , 1 mM DTT, 0.01% NaN_3 , and 90% $\text{H}_2\text{O}/10\%$ D_2O , and the spectrum was recorded at 298 K on a 700 MHz equipped with a cryoprobe. Backbone amide proton resonances are narrowly dispersed over the range of 7.2 ppm to 8.5 ppm. The rectangle region is expanded to allow labeling of the crowded central region of the spectrum. Backbone resonance assignments of 86 out of the 94 non-proline residues of NS5A-D2 are shown. Side chain Asn - NH_2 resonances and side chain Trp - NH resonances are also shown. The dashed ellipse outlines one of the last five histidines in His_6 -tag based on backbone and side chain assignments.

3.3.6.5. Secondary structure prediction of NS5A-D2 based on the NMR backbone assignment

As a sensitive indicator of conformation, NMR backbone chemical shift assignment allows secondary structure analysis through comparison with the random coil values (termed as secondary chemical shift, $\Delta\delta$) corrected for local sequence effects (Wishart *et al*, 1991). For $^{13}\text{C}_\alpha$ and ^{13}CO , positive secondary chemical shifts compared with those of random coils indicate a preference for helical conformations whereas negative values indicate a propensity toward extended conformations in β -sheet region. The trend is reversed for $^1\text{H}_\alpha$ and $^{13}\text{C}_\beta$, with negative values indicating helical conformations and positive values indicating β -sheet (Wishart & Case, 2001; Wishart & Nip, 1998; Wishart & Sykes, 1994). As shown in Fig. 3-28, multinuclear (CA, CB, HA and CO) chemical shift indexing, consensus CSI (Wishart & Sykes, 1994) values are zeros, suggesting that NS5A-D2 largely exists in a random coil conformation. Although the CSI values of HA suggest an indication of possible α -helix characters in some regions of NS5A-D2, similar to the previous prediction (Tellinghuisen *et al*, 2004), CSI values of CA, CB and CO do not support presence for consistent helical structures.

Fig. 3-28 Chemical shift index (CSI) plots of CA, CB, HA, and CO of NS5A-D2 at 298 K

Chemical shift values were taken from CSI v2.0 (Wishart & Sykes, 1994). The consensus CSI values are zeros, indicating a random coil conformation.

3.3.6.6. NMR sidechain assignment of NS5A-D2

The side chain ^1H and ^{13}C resonances were obtained from 3D HCC(CO)NH-TOCSY, CC(CO)NH-TOCSY based on the knowledge of the backbone ^1HN , ^{15}N , $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$, $^{13}\text{C}'$ and ^1HA . Excluding K1 and the C-terminal His₆-tag residues, the assignments of the side chains are about 80% complete.

Error! Reference source not found. shows the spectra of HCC(CO)NH and

CC(CO)NH.

A

B

Fig. 3-29 The 3D experiments of NS5A-D2 side chain assignment

Uniformly $^{13}\text{C}/^{15}\text{N}$ labeled sample was used for data collection. Data was processed with Felix. A)

HCC(CO)NH. B) CC(CO)NH.

3.3.6.7. Analysis of NMR coupling constant and NOE data for NS5A-D2

To evaluate if there are any folded structure present, we measured $^3J_{\text{HN}\alpha}$ coupling

constants from a 3D HNHA experiment (Vuister GW, 1993) and also analyzed 3D ^1H - ^{15}N NOESY-HSQC (Marion *et al*, 1989). As shown in Fig. 3-30A, the majority $^3J_{\text{HN}\alpha}$ coupling constants ranged from 5 to 8 with an average value of 6.607 ± 1.157 , giving no evidence of the presence of any α -helix, β -sheet or β -turns. A summary of the NOEs involving the HN, C_α and C_β protons is shown in Fig. 3-30B. Sequential $\text{NH}(i)$ - $\text{NH}(i+1)$ NOEs and $\text{C}_\alpha\text{H}(i)$ - $\text{NH}(i+1)$ NOEs are observed for several regions. $\text{C}_\beta\text{H}(i)$ - $\text{NH}(i+1)$ NOEs are also seen for some residues. Only two weak $\text{C}_\alpha\text{H}(i)$ - $\text{NH}(i+2)$ NOEs are observed (between E52 and E54; R72 and L74). The spectra from 3D ^1H - ^{15}N NOESY-HSQC are shown in **Error! Reference source not found.**, with the characters of lack of information from NOEs between protons that are far apart on the primary sequence. Overall, the NOE data of NS5A-D2 provided no evidence for any significant non-sequential NOEs, which is an indication for the presence of a folded conformation.

Fig. 3-30 Summary of the NMR $^3J_{HN\alpha}$ coupling constants and 3D NOE parameters defining the conformational characteristics of NS5A-D2 at 298 K

A) $^3J_{HN\alpha}$ coupling constants measured for NS5A-D2 at 298 K. B) variations for NOE intensities for the NS5A-D2 observed in 3D NOESY-HSQC spectrum recorded with a mixing time of 100 ms.

Fig. 3-31 3D ^1H - ^{15}N NOESY-HSQC

Uniformly $^{13}\text{C}/^{15}\text{N}$ labeled sample was used for data collection. A) The overlaid spectrum of ^1H - ^{15}N NOESY-HSQC is shown. B) Data was processed by Felix.

3.3.6.8. Backbone dynamics of NS5A-D2

The backbone dynamics of the uniformly ^{15}N -labeled NS5A-D2 were studied by 2D ^{15}N -relaxation measurements at 298 K. The steady-state heteronuclear ^1H - ^{15}N NOE for the backbone amide of NS5A-D2 exhibited a relatively featureless and flattened variation with the amino acid sequences (Fig. 3-32A), as seen in unfolded proteins (Hu *et al*, 1990). The mean value of heteronuclear ^1H - ^{15}N NOEs was 0.24 at 700 MHz, while those of folded proteins with similar lengths of polypeptide

chain were higher than 0.5 (Thormann *et al*, 2004; Ulrich *et al*, 2005). The lower NOE values for NS5A-D2 reflect more unrestricted dynamics on the nanosecond to picosecond time scales than those for the folded proteins. The region from I41-F70 shows slightly higher NOE values than the rest, suggesting the presence of relatively rigid structure. A significant deviation from the average values was observed for R72.

The resulting ^{15}N T_1 and T_2 data are summarized in Fig. 3-32B and Fig. 3-32C. The T_1 values appear to be uniform, with a mean value of 0.64 s. The mean T_2 value is 0.18 s. In folded proteins with the similar size to NS5A-D2, the average T_2 values are on the order of 0.12 s (Thormann *et al*, 2004). In general, high T_2 values indicate unrestricted fast dynamics, whereas low values suggest restricted fast dynamics and possible slow conformational exchange (Bhavesht *et al*, 2003). The high T_2 values measured for NS5A-D2 indicate large amplitude motions on the nanosecond to picosecond time scale, which is characteristic of a random coil conformation.

Fig. 3-32 Relaxation measurements of NS5A-D2

A) Steady-state heteronuclear ^1H - ^{15}N NOE for the backbone amide of NS5A-D2 at 298 K; B) Longitudinal relaxation, T_1 ; C) Transverse relaxation, T_2 . The schematic plots illustrate dynamics of the backbone.

3.3.6.9. Effect of chemical denaturant on the conformation of NS5A-D2

To analyze the stability of proteins, the two-state model of protein unfolding in chemical denaturants is commonly used. In this model, there is a native state and an unfolded state. The energetics of this transition depends on the difference in the structure between the two states (Dobson, 1992). To detect whether there is any structural difference between the native NS5A-D2 and the chemical denaturant-treated NS5A-D2, we performed the measurements of 2D ^1H - ^{15}N HSQC for this two states. Fig. 3-33 shows the effects of 8 M urea on the ^1H - ^{15}N HSQC correlation spectrum of NS5A-D2. The global patterns of the two spectra are similar, with the features of the unfolded proteins including the narrow bulk window, the three typical separated regions of the residue random coil positions (Gly, Thr/Ser, and other backbone amides), and the $-\text{NH}_2$ side chain protons of Asn resonating at the chemical shifts found for short unstructured peptides (Merutka *et al*, 1995; Schwalbe *et al*, 1997; Wishart *et al*, 1995). The similarity between the CD spectrum of native NS5A-D2 and NS5A-D2 treated by 8 M urea also suggests that the domain 2 is mainly unfolded at its native state (Fig. 3-33B). The results suggest that the native state NS5A-D2 by and large resembles the chemical-induced unfolded state. However, upon comparing the 2D ^1H - ^{15}N HSQC correlation spectra of two states, we noticed that there are a few residues undergoing chemical shift perturbations, suggesting the possible presence of transient secondary structures between the two states.

Fig. 3-33 Effect of chemical denaturants on the structure of NS5A-D2

A) Two dimensional ^1H - ^{15}N HSQC spectrum of NS5A-D2 were recorded in the absence and presence of 8 M urea. Concentrations of the uniformly ^{15}N - labeled samples for the NMR study were 0.2 mM. The spectra were collected at pH 6.5 and 298 K. The ^1H - ^{15}N HSQC spectrum of NS5A-D2 (red) was overlaid with 8 M urea-treated spectrum (black). B) CD spectra of 100 μM NS5A-D2 (blue line) and NS5A-D2 in the presence of 8 M urea (pink line) at pH 6.5.

3.3.6.10. TFE induced α -helical transition of NS5A-D2

As shown in Fig. 3-34a, the CD spectrum of NS5A-D2 without TFE exhibits a minimum near 202 nm, with a slight negative ellipticity at about 222 nm, suggesting that it is mainly unfolded. However, at lower TFE concentration (10% and 20%) the protein appeared highly aggregated (data not shown). TFE-induced cooperative transition to α -helix formation of NS5A-D2, which is characterized by minimum near 208 and 222 nm, was observed at 40% TFE and even more obviously at 60% TFE. We also recorded ^1H - ^{15}N HSQC spectrum of the ^{15}N labeled NS5A-D2 upon the addition of 60% TFE (Fig. 3-34b), showing broadening of the amide proton line-widths and significant chemical shift perturbations. The chemical shift perturbations in ^1H and ^{15}N in the presence of 60% TFE appears to favor a transition towards α -helix (Wang & Jardetzky, 2002).

Fig. 3-34 TFE-dependent conformational transition of NS5A-D2 measured by CD and 2D ^1H ^{15}N HSQC at 25 $^{\circ}\text{C}$

A) CD spectra of 100 μM NS5A-D2 (blue line), NS5A-D2 in the presence of 40% TFE (pink line) and in the presence of 60% TFE (yellow line) at pH 6.5. B) Chemical shift perturbations of the ^{15}N -labeled NS5A-D2 were monitored on a 2D ^1H - ^{15}N HSQC upon the addition of 60% TFE. The spectrum of NS5A-D2 in aqueous solution is shown in red while the spectrum of NS5A-D2 in 60% TFE is shown in blue.

3.3.6.11. Effect of low temperature on the conformation of NS5A-D2

To investigate any potential conformation change of NS5A-D2 at low temperature, backbone and side chains assignment data were collected at 278 K (5 °C). $^3J_{\text{HN}\alpha}$ coupling constants, 3D NOEs and dynamic heteronuclear ^1H - ^{15}N NOE were also measured. Compared to the 2D ^1H - ^{15}N HSQC spectrum at 298 K, the spectrum at 278 K still shows a lack of chemical shift dispersion but with overall upfield shift for ^1H (Fig. 3-35). The CSI values of HA for 278 K show largely a propensity to α -helix characters, but the consensus CSI values are zeros, indicating that NS5A-D2 is still predominantly unstructured at low temperature (Fig. 3-36).

Fig. 3-35 Temperature dependence of the ^1H - ^{15}N HSQC spectrum of ^{15}N labeled NS5A-D2

Spectra of 0.6 mM NS5A-D2, 20 mM NaPO_4 , pH 6.5, 50 mM NaCl, in 90% H_2O /10% D_2O were acquired as the temperature was lowered from 25 °C (blue) to 5 °C (red). As the temperature is lowered, the overall peaks of the backbone amide resonances show upfield shift for ^1H .

Fig. 3-36 Chemical shift index (CSI) plots of CA, CB, HA, and CO of NS5A-D2 at 278 K

Chemical shift values were taken from CSI v2.0 (Wishart & Sykes, 1994). The consensus CSI values are still zeros, indicating a random coil conformation.

As shown in Fig. 3-37A, at 278 K, the average value of $^3J_{\text{HN}\alpha}$ coupling constants was 6.1654 ± 1.028 , a little lower than that of 298 K, shifting from typical random coil value (6.607 ± 1.157) toward helical value. This suggests that at low

temperature NS5A-D2 has more propensity for helical conformation. The summary of the NOEs involving the HN, C $_{\alpha}$ and C $_{\beta}$ protons at the temperature is also shown in Fig. 3-37B. At lower temperature more sequential NH(*i*)-NH(*i*+1) NOEs, C $_{\alpha}$ H(*i*)-NH(*i*+1) NOEs and C $_{\beta}$ H(*i*)-NH(*i*+1) NOEs were observed compared to those of 298 K, especially for the region from T3 to V40. In addition, more NH(*i*)-NH(*i*+2) and C $_{\alpha}$ H(*i*)-NH(*i*+2) NOEs were seen at lower temperature.

As shown in Fig. 3-37C, the mean value of heteronuclear ^1H - ^{15}N NOEs is 0.3866 and is a little higher than that of 298 K, suggesting NS5A-D2 is more stable at low temperature, especially the region from T3 to V40. However, the spectrum still shows relatively flattened and featureless. The mean value is lower than that of the folded protein with similar size (Thormann *et al*, 2004; Ulrich *et al*, 2005), indicating NS5A-D2 is in unfolded conformation. The result is consistent with that of $^3J_{\text{HN}\alpha}$ coupling constants and 3D NOEs.

Fig. 3-37 Summary of the NMR parameters defining the conformational characters of NS5A-D2 at 278 K

A) $^3J_{\text{HN}\alpha}$ coupling constants measured for NS5A-D2 at 278 K. B) Variations for NOE intensities for the NS5A-D2 observed in 3D NOESY-HSQC spectrum recorded with a mixing time of 100 ms at 278 K. C) Comparison of steady-state heteronuclear ^1H - ^{15}N NOE for the backbone amide of NS5A-D2 at 278 K (in red) to that of 298 K (in black).

Taken together, there is a trend for NS5A-D2 to be helical and the protein appears

to be less flexible at low temperature. However, the protein is still not in well folded conformation.

3.3.7. Binding of NS5A-D2 to HCV NS5B

NS5B has a GDD motif common to all RNA polymerases of RNA viruses and has been shown to possess RNA-dependent RNA polymerase (RdRP) activity (Behrens *et al*, 1996; Lohmann *et al*, 1997). It has been elucidated that HCV NS5A directly interacts with HCV NS5B as a component of HCV replicase complex and NS5A-D2 contains the binding sites for NS5B protein (Shirota *et al*, 2002). To determine the binding ability of NS5A-D2 to HCV NS5B, we analyzed the molecular interaction by NMR spectroscopy.

3.3.7.1. NS5A Domain 2 interacts with NS5B MK-17 peptide in a NMR-based titration assay

Based upon the previously reported sequences of NS5B essential for binding to NS5A (Shirota *et al*, 2002), the MK-17 peptide, which contains residues 139-155 of NS5B, and SP-24 peptide which contains residues 365-388 of NS5B, were designed for NMR studies. Our NMR results showed that obvious perturbations in the chemical shifts were observed in ^1H - ^{15}N HSQC spectrum of ^{15}N -labeled NS5A-D2 in the presence of 5 mM of the MK-17 peptide (Fig. 3-39A), whereas

the titration with the peptide SP-24 caused a heavy precipitate upon its addition (data not shown). While many residues of NS5A-D2 experience chemical shift perturbations upon the addition of the MK-17 peptide, however, the residues most affected with an increasing concentration of the peptide MK-17 less than 2 mM appear to be localized to two regions: L48-V57 and L86-E96, which are within the previously reported NS5A domain involved in NS5B binding (amino acids 277-334, K38-Y95 in NS5A-D2) (Shirota *et al*, 2002) (Fig. 3-38). As shown in Fig. 3-39B, Y95 showed most obvious shift at this stage. Interestingly, as the ligand concentration gradually increased over 2 mM, we have observed that residues from other region began to show apparent chemical shift perturbation; for instance, amino acid R68 exhibited noticeable chemical shift perturbation in the presence of an increasing amount of the peptide ranging from 2 mM to 4 mM (Fig. 3-39B). Compared to the spectrum of free NS5A-D2, the wider line widths and more dispersed signals shown in the spectrum of NS5A-D2 with the MK-17 peptide suggest that NS5A-D2 might undergo some conformational changes upon binding with the MK-17 peptide. Consistent with the previous report (Shirota *et al*, 2002), the purified NS5A-D2, despite its disordered state, still maintains the binding capacity to its biological ligand.

Fig. 3-38 NS5A-D2 interacts with NS5B MK-17 peptide up to 1.6 mM

A) Chemical shift perturbations of the ^{15}N -labeled NS5A-D2 were monitored on a 2D ^1H - ^{15}N HSQC upon the addition of the unlabeled NS5B MK-17 peptide. The spectrum of free NS5A-D2 is shown in red while the spectrum of NS5A-D2 binding to NS5B MK-17 peptide is shown in black. Concentrations of ^{15}N -labeled NS5A-D2 and NS5B MK-17 peptide was 0.1 mM and 1.6 mM, respectively. B) Differences in chemical shifts $\Delta\delta = ((\Delta\delta \text{ } ^1\text{H})^2 + (0.2 * \Delta\delta \text{ } ^{15}\text{N}^2)^{1/2}$ were analyzed based on the chemical shift values perturbed by adding increasing amounts of the peptides.

Fig. 3-39 NS5A-D2 interacts with NS5B MK-17 peptide up to 5 mM

A) Chemical shift perturbations of the ^{15}N -labeled NS5A-D2 were monitored on a 2D ^1H - ^{15}N HSQC upon the addition of the unlabeled NS5B MK-17 peptide. The spectrum of free NS5A-D2 is shown in red while the spectrum of NS5A-D2 with the addition of NS5B MK-17 peptide is shown in blue. Concentrations of ^{15}N -labeled NS5A-D2 and NS5B MK-17 peptide was 0.1 mM and 5 mM, respectively. B) Section of the 2D ^1H - ^{15}N HSQC of ^{15}N -labeled NS5A-D2 titrated with unlabeled NS5B MK-17 peptide. Amino acid Y95 chemical shifts were changed upon the addition of increasing amount of MK-17 from 0 to 1.75 mM as displayed. Amino acid R68 chemical shifts were changed upon the addition of increasing amount of MK-17 from 0 to 4 mM.

To quantify the binding between NS5A-D2 and MK17 peptide, the equilibrium dissociation constant (K_D) was calculated based on NMR titration experiment. The calculated binding affinity of MK17 peptide to NS5A-D2 was 0.7185 ± 0084 mM. As shown in the following Fig. 3-40, NMR dissociation constant values are based on chemical shift perturbations of 4 residues.

Fig. 3-40 The relationship between chemical shift changes and the molar ratio of ligand to protein (L/P)

The chemical shift change was calculated according to the equation $\delta = ((\Delta\delta^1\text{H})^2 + (0.2 * (\Delta\delta^{15}\text{N})^2)^{0.5}$. L/P represents the molar ratio of ligand (MK17 peptide) to protein (NS5A-D2). The fitting curves were drawn using xcrvfit 3.0 (Boyko).

3.3.7.2. Secondary structure analysis of NS5A-D2 upon binding MK-17 peptide by CD

To examine the secondary structure change of NS5A-D2 upon NS5B MK-17 binding, circular dichroism spectra were collected including both free and various peptide bound NS5A-D2. To eliminate the influence of MK-17 on the spectrum of the mixture, the spectrum of MK-17 itself (unfolded) corresponding to each concentration was subtracted from each spectrum of the mixture with different ratio of MK-17 to NS5A-D2. As shown in Fig. 3-41, with the increase in the ratio of MK-17 to NS5A-D2, the minimum of NS5A-D2 shifted from 201 nm to 207 nm, the positive perpendicular around 192 nm appeared and increased, the ellipticity at 222 nm becomes slightly deeper. For the ellipticity at 222 nm is well correlated to the native α -helical content of proteins (Chen *et al*, 1972), the change of circular dichroism spectra of NS5A-D2 upon MK-17 binding gives some clues of its possible structural changes toward ordered conformation. Unfortunately, no evident ordered conformations in the average secondary structure upon the binding were detected.

Fig. 3-41 Analysis of NS5A-D2 upon NS5B MK-17 binding by CD at 25 °C

CD spectra of 100 μM free NS5A-D2 (blue line), 100 μM NS5A-D2 with 400 μM MK-17 (pink line), 100 μM NS5A-D2 with 600 μM MK-17 (yellow line) and 100 μM NS5A-D2 with 800 μM MK-17 (green line) at pH 6.5.

3.4. Anti-apoptosis function of NS5A *in vivo*

Apoptosis is essential for the viral clearance. The induced apoptosis is somewhat a host defense against viral infection and initiates the death of the infected cells. However, in the liver infected with Hepatitis C Virus, the persistence of the virus and the enhanced apoptosis in the host hepatocytes are observed simultaneously. The function of NS5A in apoptosis is not yet well determined. NS5A is predicted

as a homology to the Bcl-2 family proteins and hampers the pro-apoptotic action of Bax in hepatoma cells (Chung *et al*, 2003). NS5A binds to FKBP38, a chaperon protein reported to target Bcl-2 into mitochondria and inhibit apoptosis (Shirane & Nakayama, 2003), and takes effect of anti-apoptosis (Okamoto *et al*, 2006; Wang *et al*, 2006). The direct inhibition of pro-apoptotic bin1, a tumor suppressor protein, has also been detected in hepatoma cells (Nanda *et al*, 2006). Anti-apoptotic effects of NS5A are also reported to be involved in cytoplasmatic sequestering of p53 (Lan *et al*, 2002), activation of STAT3 with elevation in expression level of Bcl-X_L and p21 (Sarcar *et al*, 2004), activation of NFkB (Gong *et al*, 2001), and activation of PI3-kinase-Akt dependent pathway (Street *et al*, 2004). By contrast, a direct NS5A induced apoptosis has also been shown in dendritic cells (Siavoshian *et al*, 2005).

Validating the biological role of HCV NS5A protein in apoptotic signaling network will be helpful to understand the persistence of HCV infection and probe into the potential promising therapy. It was previously shown that BH domains are conserved between NS5A genotype 1b and 1a (Chung *et al*, 2003). In our study, HCV-1a NS5A was used for the transient transfection studies and HCV-1b NS5A was used for 293T stable cell lines. Sequence alignment showed that the BH domains were conserved between these two genotypes (**Error! Reference source not found.**). In our studies, anti-apoptosis effects were observed in NS5A derived from the two different genotypes, suggesting the anti-apoptotic properties of NS5A is not genotype-specific.

Fig. 3-42 Amino acids sequence alignment of BH domains in NS5A derived from HCV-1b and HCV-1a

The identical amino acids are shown in yellow, the less conserved amino acids are shown in green and the non-similar amino acids are shown in white.

3.4.1. Anti-serum deprivation effect of NS5A in HepG2 cells and 293T cells

Serum deprivation can induce apoptosis and lead to cell death finally. So the survival cells represent the cells with higher anti-apoptotic ability. The eukaryotic expression plasmid of full-length NS5A-pcDNA3.1 (+) was first introduced into the human hepatoma cell line HepG2. The HepG2 cells transfected with pcDNA3.1 (+) vector only were used as negative control. Then the cells were treated in serum-free DMEM. The expression of HCV NS5A protein in HepG2 cells after transfection was confirmed by western blot assay, while no expression was

observed in the HepG2 cells introduced with empty pcDNA3.1 (+) (Fig. 3-43).

Fig. 3-43 Western blot assay for the over-expression of NS5A in human hepatoma cell HepG2 48hr post-transfection, NS5A overexpression in HepG2-NS5A-pcDNA3.1 (+) was probed by a commercially available HCV NS5A mouse monoclonal antibody (1:1000 diluted).

In the cells transfected with the empty vector, treatment with serum-free culture medium induced obvious cell death, whereas the HepG2-NS5A cells show higher viability. As shown in Fig. 3-44, after serum-deprivation treatment of 72 h, the survival ratio of HepG2-NS5A-pcDNA3.1 (+) is obviously higher than that of HepG2-pcDNA3.1 (+). Therefore, the protection conferred by HCV NS5A suggests that the NS5A protein might induce an anti-apoptosis effect.

Fig. 3-44 Anti-serum deprivation effect of NS5A in human hepatoma cells HepG2

HepG2 cells seeded in a 96-well plate (5×10^3 cells/well) were treated with serum free culture medium. The cell proliferation at different time points, 0 h, 24 h, 48 h and 72 h was analyzed by MTT assay. Absorbances at 490 nm were measured by microplate reader (Bio Rad). Shown are cell proliferations expressed as percentages of those of the control cultures treated with 10% fetal bovine serum (C). The data shown represent the averages of the results of three independent experiments, and the error bars indicate the experimental standard deviations.

Two stable 293T cell lines expressing flag-tagged NS5A (293T-flag-NS5A) and flag-tagged NS5B (293T-flag-NS5B), respectively, under a Tet-On promoter (Zeng *et al*, 1998) were kindly supplied by Dr. Phuay-Yee Goh (Choi *et al*, 2004). The

induced flag-NS5A protein and flag-NS5B protein were detected by anti-flag antibody, respectively (Fig. 3-45).

Fig. 3-45 Two stable cell lines expressing flag-NS5A or flag-NS5B upon tetracycline (4 μ g/ml)

A) 293T-flag-NS5A stable cell line. B) 293T-flag-NS5B stable cell line.

Anti-serum deprivation effect of NS5A was also observed in 293T-flag-NS5A stable cell line, represented by higher cell proliferation after treatment with serum free medium (Fig. 3-46). By contrast, non-transfected 293T cells and 293T-flag-NS5B cells showed apparently sensitive to serum deprivation, with inhibited cell proliferation. NS5B is the viral RNA-dependent RNA polymerase.

Till now there have been no studies demonstrating a role of NS5B in apoptosis of hepatocytes or hepatoma cells. So 293T-flag-NS5B cell line was chosen as a control except for non-transfected 293T cells. The protective role of flag-NS5A protein to serum deprivation in 293T-flag-NS5A stable cell line further supports that the NS5A protein might exert an anti-apoptosis function in cells.

Fig. 3-46 Anti-serum deprivation effect of NS5A in 293T-flag-NS5A stable cell line

293T cells seeded in a 96-well plate (5×10^3 cells/well) were treated with serum free culture medium. The cell proliferation at different time points, 0 h, 24 h, 48 h and 72 h was analyzed by MTT assay. Absorbance at 490 nm was measured by microplate reader (Bio Rad). Shown are cell proliferations expressed as percentages of those of the control cultures treated with 10% fetal bovine serum (C) at each time point. The data shown represent the averages of the results of three independent experiments, and the error bars indicate the experimental standard deviations.

3.4.2. NS5A is associated with endoplasmic reticulum (ER) and mitochondria

The subcellular localization of NS5A protein has been reported associated with ER (Brass *et al*, 2002). Colocalization of NS5A protein and chaperon protein FKBP38 at ER and mitochondria has also been described (Wang *et al*, 2006). Here, immunostaining was used to investigate subcellular localization of over-expressed NS5A protein in 293T-flag-NS5A cells and HepG2 cells. Alexa Fluor® 488 goat anti-mouse IgG (Molecular Probes) was used to introduce green fluorescence after binding with the Hepatitis C NS5A monoclonal antibody (ID labs), which interacts with the expressed NS5A protein in cells. R6 (Rhodamine B hexyl ester perchlorate; Molecular Probes) staining, which is specific for endoplasmic reticulum (ER), was used here to overlap with the green fusion proteins signal. Mito tracker, specific for mitochondria, was used to determine the location of mitochondria. As shown in the green channel in Fig. 3-47A, the green signals of NS5A protein were strong enough, distributing to the nuclear membrane, emphasized in the perinuclear region, and extended in a reticular fashion through the cytoplasm. No staining of the nucleus or plasma membrane was observed. In majority, NS5A protein colocalized with R6, the marker for ER in both 293T and HepG2 cells. However, as shown in Fig. 3-47B, NS5A protein is partially colocalized with Mito tracker. This suggests that NS5A protein mainly localizes to endoplasmic reticulum (ER) or an ER derived modified compartment, but partially to mitochondria.

Fig. 3-47 Subcellular localization of NS5A in 293T cells and HepG2 cells

Cells with NS5A expression were analyzed by immunostaining with monoclonal mouse against NS5A. Bound primary antibodies were revealed with Alexa Fluor[®] 488 goat anti-mouse secondary antibody. Slides were analyzed by confocal laser scanning microscopy as described in “Materials and Methods”. Horizontal sections taken through the center of the nuclei are shown. Images recorded in green and red channels are presented separately on the left and the middle, respectively, and the merged images are shown in the right. A) Cells were stained with anti-NS5A antibody (green) and ER Tracker (red). The superimposed image is also shown; B) cells were stained with anti-NS5A antibody (green) and Mito Tracker (red). The superimposed image is also shown.

3.4.3. Anti-apoptosis effect of NS5A protein on 293T cells treated with cisplatin

Cisplatin has a broad range of antitumor activities and is widely used in anticancer chemotherapy. During the treatment of hepatocellular cancer (HCC), cisplatin is frequently applied systemically or chemo-embolization via transartery. The activity of cisplatin is thought to crosslink inter- and intra-strand DNA. The DNA damage and subsequent induced apoptosis might be the primary cytotoxic mechanism of cisplatin (Fisher, 1994). However, in some cases, damages to cytoplasmic proteins may take place during cisplatin-induced apoptosis (Perez, 1998). In hepatoma cells, apoptosis induced by cisplatin was also described as p53-independent, and associated with up-regulation of pro-apoptotic genes, cell cycle regulators, and genes involved in signal transduction (Qin *et al*, 2002).

To evaluate the effect of NS5A protein on cisplatin-induced apoptosis, cell proliferations of 293T cells were analyzed after treatments with different doses of cisplatin or at different time points. As shown in Fig. 3-48A, 293T cells, 293T-flag-NS5A cells and 293T-flag-NS5B cells were treated with cisplatin at different concentrations (0 μ M, 40 μ M, 80 μ M, 120 μ M, 160 μ M, 200 μ M) for 24 h, respectively. Then the cell proliferation was measured by MTT assay. Compared to 293T cells or 293T-flag-NS5B cells, 293T-flag-NS5A cells showed higher cell proliferation after cisplatin treatment. Especially, when the concentration of cisplatin reached 200 μ M, the cell proliferation of 293T-flag-NS5A cells is about

50% higher than that of 293T cells or 293T-flag-NS5B cells. Later, 293T cells, 293T-flag-NS5A cells and 293T-NS5B cells were treated with 200 μ M cisplatin for 12 h, 24 h, 36 h, 48 h, 60 h, respectively. Shown in Fig. 3-48B, the cell viability of 293T-flag-NS5A cells is obviously higher than that of 293T cells or 293T-flag-NS5B cells at different time points. This suggests that NS5A protein might resist the cisplatin-induced apoptosis. Caspase-3 plays key role in the execution of apoptosis. During the early stage of apoptosis, the activation of caspase-3, represented by a 17 kDa cleavage fragment, contributes to the changes in cellular morphology and functions with apoptosis (Thornberry & Lazebnik, 1998). To investigate activation of caspase-3 in the cells treated by cisplatin and the anti-apoptosis effects of NS5A expression, the expression of caspase-3 was analyzed by western blot assay. Shown in Fig. 3-49, the cleaved 17 kDa fragment of caspase-3 was detected in the 293T cells treated with cisplatin. Both of the 293T-flag-NS5A cells and 293T-flag-NS5A cells treated with cisplatin did not show the appearance of the small proteolytic fragment in western blot assay. This suggests that NS5A protein might exert anti-apoptosis functions by disturbing caspase-3 pathway.

Fig. 3-48 Anti-apoptosis effect of NS5A protein on 293T cells treated with cisplatin

293T cells seeded in a 96-well plate (5×10^3 cells/well) were treated with cisplatin. A) The cell proliferation at the treatment of different doses of cisplatin, 0 μ M, 40 μ M, 80 μ M, 120 μ M, 160 μ M, and 200 μ M for 24 h, was analyzed by MTT assay. Absorbances at 490 nm were measured by microplate reader (Bio Rad). Shown are cell proliferations expressed as percentages of those of the control cultures without cisplatin treatment (C) (100%). B) The cell proliferation treated with 200 μ M cisplatin at different time points, 0 h, 12 h, 24 h, 36 h, 48 h and 60 h was analyzed by MTT assay.

Fig. 3-49 Western blot of caspase-3 protein

The upper strip shows the expression of caspase-3 protein in 293T cells, 293T cells treated with 200 μ M cisplatin, 293T-flag-NS5A cells and 293T-flag-NS5A cells treated with 200 μ M cisplatin for 12 h, respectively. Clear proteolytic 17 kDa product of caspase-3 could be detected only in the 293T cells treated with 200 μ M cisplatin. The middle strip shows the expression of NS5A protein. The lower strip shows the equal expression of GAPDH.

3.4.4. siNS5A inhibits anti-apoptosis function of NS5A

It has been reported that NS5A protein participates HCV antiviral resistance (Tan & Katze, 2001). The mutations within the IFN sensitivity determining region (ISDR)

in NS5A are correlated with the resistance of HCV to IFN (Enomoto *et al*, 1995; Schiappa *et al*, 2002). The observed anti-apoptosis effects of NS5A might also be responsible for HCV antiviral resistance.

Small interfering RNA (siRNA) is reported to specifically down regulate target gene expression (Elbashir *et al*, 2001; Lindenbach & Rice, 2002; Sharp, 2001; Zamore, 2001), by destroying its mRNA. siRNA becomes a promising vehicle for inducing intracellular immunity. Additionally, the potency of siRNA in HCV therapy strategy can not be ignored, due to its ability of inhibiting viral gene expression and replication (Sen *et al*, 2003; Seo *et al*, 2003; Wakita & Wands, 1994). It has been described that siRNAs can inhibit expression of HCV NS5A and NS5A-mediated interleukin-8 activation (Sen *et al*, 2003).

To demonstrate the anti-apoptosis effect of HCV NS5A and evaluate the potential clinical application of siRNAs in HCV therapy, siRNA-mediated RNA interference method was used in this study. The domain 1 and domain 2 of NS5A have important roles in NS5A mediated functions such as viral replication or anti-apoptosis. Two siRNAs were designed by targeting the different domains of HCV 1b NS5A cDNA. siNS5A D1-1 targets the domain 1 of NS5A (from +113 to +131) and siNS5A D2-1 targets the domain 2 of NS5A (from +812 to +830) (Fig. 3-50). The GC content in each siRNA is 47.37%. One scramble siRNA, HYSR4, was also used in the control experiment. No significant similarities were found for

the scramble siRNA, HYSR4, by blast searching within the human genome. The three siRNAs used in this study are listed in Fig. 3-50.

Fig. 3-50 HCV 1b NS5A cDNA sequence and the two siRNA targeting sites

siNS5A D1-1 is targeting the sequence of NS5A domain 1 from +113 to +131 nt, siNS5A D2-1 is targeting the sequence of NS5A domain 2 from +812 to +830 nt. Sequences of siNS5A D1-1, siNS5A D2-1 and the scramble siRNA (HYSR4) used in these experiments are shown.

3.4.4.1. siNS5A inhibits NS5A expression in 293T cells

Both of the two siRNAs were chemically synthesized (DHARMACON, USA).

Each is 21-nucleotide duplex with 19 base pairs, bearing two overhangs at 3' ends. siRNA universal buffer (20 mM KCl, 6 mM HEPES-KOH pH 7.5, 0.2 mM MgCl₂) was used to obtain 20 μ M solution of each siRNA. The dissolved siRNAs were stored at -20 °C. To disrupt high aggregation and guarantee siRNA silencing efficiency, the siRNA solution was heated at 90 °C for 1 min, and then incubated at 37 °C for 60 min before use. 5'-fluorescein-labelled scramble siRNA (FITC-siRNA) targeting FKBP38 protein was transfected into 293T-flag-NS5A cells with oligofectamine as described in the materials and methods to assess siRNA transfection efficiency in 293T cells. The transient transfection efficiency is around 70%. This suggests that the siRNAs can be successfully transfected into the majority of the cells.

To examine whether the siRNAs (siNS5A D1-1 and siNS5A D2-1) targeting to HCV NS5A sequence specifically inhibit the expression of this protein, the siNS5A D1-1 and siNS5A D2-1 were transfected into 293T-flag-NS5A cells, respectively. The scramble siRNA, HYSR4, with no significant homology to the target sequences, was used as a negative control. Cell lysates were prepared after 48 h of transfection with a series of concentrations of siNS5A D1-1 or siNS5A D2-1, and then analyzed for NS5A protein expression by Western blot. As shown in Fig. 3-51A and Fig. 3-51B, NS5A expression level was reduced by the treatment with specific siNS5A D1-1 or siNS5A D2-1 with the increase of siRNAs concentrations when compared with the untreated cells. The low level of NS5A protein expression

after transfection may results from cells either untransfected with siRNA or incompletely silenced. Scramble siRNA was also used as a negative control in this experiment and did not alter the expression level of NS5A (Fig. 3-51C). The silencing effects of the two siRNAs on NS5A protein expression are similar. Our data demonstrate about 65% reduction of viral protein expression after transient transfection with 100 nM siNS5As. When the samples were also probed with antibody to GAPDH, similar level of GAPDH expression was noted (Fig. 3-51), indicating inhibition was specific for NS5A protein. Time dependent effects of siNS5A D1-1 and siNS5A D2-1 revealed that the silencing effects of siNS5A targeted siRNAs were significant after 48 h and 72 h of transfection (Fig. 3-52).

Fig. 3-51 Inhibitions on NS5A protein expression in 293T-flag-NS5A cells treated with different concentrations of siNS5A D1-1 or siNS5A D2-1

A) 293T-flag-NS5A cells were treated with siNS5A D1-1 (0 nM, 10 nM 20 nM, 50 nM and 100 nM) for 48 h, respectively. NS5A expression was detected by anti-flag antibody. B) 293T-flag-NS5A cells were treated with siNS5A D2-1 (0 nM, 10 nM 20 nM, 50 nM and 100 nM) for 48 h, respectively. NS5A expression was detected by anti-flag antibody. C) 293T-flag-NS5A cells were treated with scramble siRNA HYRS4 (0 nM, 10 nM 20 nM, 50 nM and 100 nM) for 48 h, respectively. NS5A expression was detected by anti-flag antibody.

Fig. 3-52 Inhibitions on NS5A protein expression in 293T-flag-NS5A cells treated with 100 nM siNS5A D1-1 or siNS5A D2-1 after different time of transfection

A) 293T-flag-NS5A cells were treated with 100 nM siNS5A D1-1 for 0 h, 24 h, 48 h and 72 h, respectively. NS5A expression was detected by anti-flag antibody. B) 293T-flag-NS5A cells were treated with 100 nM siNS5A D2-1 for 0 h, 24 h, 48 h and 72 h, respectively. NS5A expression was detected by anti-flag antibody.

We also analyzed the effect of siRNAs on the expression of NS5A at the RNA level. Total RNA was isolated from 293T-flag-NS5A cells without transfection, or transfected with 100 nM siNS5A D1-1, siNS5A D2-1, HYSR4, respectively. The results of semi-quantitative RT-PCR analysis showed inhibition of NS5A expression at mRNA level compared to that of the cells without transfection or the cells transfected with scramble siRNA (Fig. 3-53). This result is consistent with the specific inhibition of NS5A expression by siNS5As at the protein level as we observed previously.

Fig. 3-53 Inhibition of NS5A expression at mRNA level

A) NS5A expression at mRNA level. Lane 1, total RNA was prepared from cells untreated; Lane 2, 100 nM of HYSR4 scramble siRNA treated; Lane 3, 100 nM of siNS5A D1-1 treated; Lane 4, 100 nM siNS5A D2-1 treated. The total RNAs extracted from cells were used as template for semi-quantitative RT-PCR to determine the expression level of NS5A by using specific primers for NS5A. B) Corresponding GAPDH expression at mRNA level also determined as an internal control.

3.4.4.2. siNS5A inhibits anti-apoptosis effects of NS5A in 293T cells

In our study, anti-cisplatin induced apoptosis effects of NS5A protein were observed in 293T-flag-NS5A cells. To investigate whether NS5A targeted siRNAs can inhibit anti-apoptosis effects of NS5A protein, the cells treated with siRNAs for 48 h were treated with 200 μ M cisplatin. 293T cells, 293T-flag-NS5A cells and 293T-flag-NS5A cells transfected with scramble siRNAs were treated with cisplatin either. Cell proliferations were analyzed by MTT assay. As shown in **Error! Reference source not found.**A, 293T-flag-NS5A cells and 293T-flag-NS5A cells treated with scramble siRNAs showed higher cell proliferations after cisplatin treatment, suggesting potential anti-apoptosis effects of NS5A protein. The survival rate of 293T cells treated with cisplatin is very low. Although the 293T-flag-NS5A cells first treated with siNS5A D1-1 or siNS5A D2-1 and then treated with cisplatin later showed less cell viability than that of 293T-flag-NS5A cells, they had higher survival rate compared to that of 293T cells. PARP participates in DNA repair and genomic maintenance. Proteolytic cleavage of PARP by caspases is a characteristic marker of apoptosis. Activated executed caspases, such as caspase-3, 6, 7 and 8 can cleave 116 kDa PARP into an 89 kDa fragment. After treatment with cisplatin, the 89 kDa apoptosis-related fragment of PARP was only detected in 293T cells, and the 293T-flag-NS5A cells first treated with siNS5A D1-1 or siNS5A D2-1 (Fig. 3-54B). These results suggest that the NS5A targeted siRNAs can inhibit the anti-apoptosis effect of NS5A protein at

some degree.

Fig. 3-54 siNS5A inhibits anti-apoptosis effects of NS5A in 293T cells

A) 293T-NS5A cells or 293T cells were treated with 100 nM scramble siRNA, siNS5A D1-1 or D2-1, respectively, then 200 μ M cisplatin was added. Cell proliferation was checked by MTT assay. Shown are cell proliferations expressed as percentages of those of the control cultures without cisplatin treatment (C) (100%). The data shown represent the averages of the results of three independent experiments, and the error bars indicate the experimental standard deviations. B) 293T-flag-NS5A cells were treated with 100 nM scramble siRNA, siNS5A D1-1 or D2-1 for 48 h, respectively, and then 200 μ M cisplatin was added for another 24 h. PARP expression was checked.

Discussion

4.1. Expression and purification of NS5A proteins

Obtaining a large quantity of recombinant HCV-1a NS5A expressed from *E. coli* has been difficult. Most current purifications for NS5A protein was focused on HCV-1b genotype, which is over 20% different from HCV-1a genotype used in our study at amino acids level (Huang *et al*, 2004; Shirota *et al*, 2002). In this study, we tried to use well-established pET expression system and GST fusion expression system in *E. coli*, but failed to get enough soluble HCV-1a NS5A protein for biochemical and structural analyses. By using the pET-ubiquitin expression system kindly provided by Dr. Graig E. Cameron (Huang *et al*, 2004), we were able to generate milligram quantities of soluble HCV-1a NS5A protein in *E. coli*. To express and purify full length NS5A and NS5A- Δ N, NP40 was reduced to 0.1% without significant decrease in the final products. However, we can not get stable and soluble full length NS5A or NS5A- Δ N without NP40 or using NP40 lower than its critical micellar concentration, CMC (0.3 mM), which is the total concentration of detergent corresponding to the maximum possible concentration of detergent monomer in solution. Crystallization may be favored by the detergent at the concentration lower than its CMC. Several other detergents such as octyl β -D-glucopyranoside (CMC, 25 mM) or n-Dodecyl α -D- Maltoside (CMC, 0.15

mM), *et al.*, were also used for the purification, but we could not purify enough amount of the full length NS5A or NS5A-ΔN by using other detergents than NP40. This prevented our further trial to study the structure of this protein by crystallography.

Based on the three domains structural model of NS5A protein proposed by Tellinghuisen *et al.* (Tellinghuisen *et al.*, 2004), we tried to express and purify each domain with truncated loop of low complexity region (Fig. 1-2) by using the pET-ubiquitin expression system. The three domains of NS5A (NS5A-D1, amino acids 28-213; NS5A-D2, amino acids 240-335; NS5A-D3, amino acids 356-447) could be purified without the presence of NP40, that means deletion of some potential loop regions may increase the protein solubility. However, the purified NS5A-D1 was prone to precipitated at high concentration. 1D ¹H initial NMR evaluation results show that the NS5A-D1 is highly aggregated. Because the zinc binding site is located within the NS5A-D1 (Tellinghuisen *et al.*, 2004; Tellinghuisen *et al.*, 2005), we added 1-100 μM ZnCl₂ at the time of induction to facilitate the folding of NS5A-D1. Unfortunately the aggregation was not removed. The structural analysis of NS5A-D1 failed due to the impossibility of obtaining enough soluble and stable NS5A-D1. The purified NS5A-D2 and NS5A-D3 are relatively stable and not prone to precipitation. The secondary structural analysis of purified NS5A-D2 and NS5A-D3 by CD spectra and 1D ¹H NMR showed that they are highly flexible and lack of globular folded conformation.

4.2. The domain 2 of NS5A protein

The domain 2 of HCV NS5A contributes to the multiple functions of NS5A in viral replication or other regulations (Fig. 1-2). Currently, the precise function and structure of this domain is still unclear. In our current study, to characterize the biochemical behavior of the NS5A and define its structural basis, we designed an over-expression system of the domain 2 of NS5A which allowed us to consistently obtain soluble and stable NS5A-D2 covering the PKR/NS5B binding site. Our gel-filtration study, CD, DLS, 1D ^1H NMR analyses, collectively, suggest that the NS5A-D2 appears to contain high amount of flexible and unstructured regions. The pattern of the 2D ^1H - ^{15}N HSQC spectrum of the free NS5A-D2 is similar to those found in unfolded proteins (Merutka *et al*, 1995; Peti *et al*, 2001; Wishart *et al*, 1995). The analysis of the backbone assignments by CSI (Wishart & Sykes, 1994) suggests that NS5A-D2 lacks ordered secondary structures. $^3J_{\text{HN}\alpha}$ coupling constants and 3D NOE data for NS5A-D2 do not appear to provide supporting evidence of any ordered secondary structural elements. All the resulting T_1 , T_2 and heteronuclear ^1H - ^{15}N NOE values of NS5A-D2 indicate unrestricted dynamics on the nanosecond to picosecond time scales. The measurements of both static and dynamic multinuclear NMR parameters indicate that the free NS5A-D2 exists predominately in a random coil conformation.

It has been shown that that the non-structured regions of proteins are essential for

protein functions (Bode *et al*, 1978). Recently, it is emerging that the occurrence of natively disordered proteins or intrinsically unstructured proteins in functional proteins is increasing. More than 100 proteins including Bcl-2, p53, and eIF1A have been reported to have intrinsically disordered regions (Dunker *et al*, 2001; Tompa, 2002; Uversky, 2002). An earlier study revealed that a half of the protein sequences in the Swiss Protein Database contained segments of low complexity that correspond to non-globular regions (Wootton, 1994). In their native states, these protein domains or regions are disordered or contain large unstructured area. The mean hydrophobicity *versus* mean net charge values suggest that the NS5A-D2 sequence has an overall low hydrophobicity and a high net positive charge, similar to those of natively unfolded proteins (Fig. 4-1). Generally, the formations of a hydrophobic core and rigid secondary structure are not favored with the low content of hydrophobic amino acid residues (Dyson & Wright, 2002; Tompa, 2002). The analysis of the charge distribution of the residues in NS5A shows three acidic domains located in the C-terminal region from amino acids 220 to 300 (Tanimoto *et al*, 1997), mainly part of NS5A-D2. Low content of hydrophobic amino acid residues surrounded by numerous acidic residues can also result in an extended conformation because of the electrostatic repulsion (Uversky, 2002).

Fig. 4-1 Charge-hydrophobicity phase space plotted for NS5A-D2

The diagram plots the correlation between the mean hydrophobicity $\langle H \rangle$ and the mean net charge $\langle R \rangle$ for a set of 90 natively unfolded proteins as indicated in diamond as previously described (Uversky, 2002) and NS5A-D2 is shown in red circle . The solid black line represents the boundary between natively unfolded and folded proteins (Uversky, 2002): $\langle R \rangle = 2.785 \langle H \rangle - 1.151$.

Compared to folded proteins, one advantage that unfolded proteins might have is that they provide a much larger surface area, which would allow multiple molecular interactions (Gunasekaran *et al*, 2003). Unstructured proteins are inherently flexible. Unfolded proteins may have advantages to respond to environmental factors and their local and global structures can be easily adapted in cellular environments. The intrinsic structural flexibility of the unstructured

proteins could permit a single protein to recognize a large number of biological targets without sacrificing specificity (Wright & Dyson, 1999). The most common types of intrinsically unstructured protein are DNA-binding domains of transcription factors (Spolar & Record, 1994), transcriptional activation domains of transcription regulators (Donaldson & Capone, 1992; Kussie *et al*, 1996; Uesugi *et al*, 1997) and kinases (Nakayama *et al*, 2001). The unfolded structure of NS5A-D2 determined by our current NMR studies possibly provides a molecular basis to its multiple functions such as binding with different biological partners of NS5A including NS5B, PKR, TRAF2 and Grb2, participating the activation of transcription, IRES suppression and anti-apoptosis.

Compared to water, TFE is a slightly stronger proton donor, but a much weaker proton acceptor (Llinas, 1975). In an aqueous solution TFE can strengthen the intramolecular hydrogen bonds to stabilize the α -helical conformation (Nelson & Kallenbach, 1986). The effects of TFE have also been found to be sequence-dependent (Sonnichsen *et al*, 1992) and appear to be correlated with the propensity of α -helix formation (Shiraki *et al*, 1995). Our results indicate that in the presence of 40% or 60% TFE, NS5A-D2 demonstrated a cooperative transition from random coil to α -helix conformation, which is indicated by broaden amide proton line width, less crowded signals, and the chemical shift perturbations in ^1H and ^{15}N favoring a transition towards α -helix (Wang & Jardetzky, 2002) in 2D ^1H - ^{15}N HSQC spectrum. The results of CD spectrum also supply strong evidence

of transition from unfolded conformation to typical α -helix at secondary structural level. This indicates that NS5A-D2 has a possible transition structure with propensity to form helix.

High temperature favors the un-coverage of hydrophobic regions in the protein and lead to more chance of hydrophobic interactions, which may lead to disordered conformation and prone to aggregation. Some natively unfolded proteins may undergo conformation changes to ordered status partially with detectable residual structure at low temperature (Benison *et al*, 2006). We also tried to analyze the structural features of NS5A-D2 at low temperature by NMR spectroscopy. According to the data of $^3J_{\text{HN}\alpha}$ coupling constants and 3D NOEs of NS5A-D2 at 278 K, some helical trend was observed. Additionally, the mean steady-state ^1H - ^{15}N heteronuclear NOE value is a little higher than that of 298 K, indicating less flexible and dynamic characters of this protein at this low temperature. Even though it still remains unfolded conformation, the NMR data at 278 K suggest that NS5A-D2 is more stable at low temperature, indicating the presence of environment-sensitive conformation of NS5A-D2.

Natively unfolded proteins undergo partial or full folding processes upon binding to biological partners (Fink, 2005; Uversky, 2002). In unfolded proteins, the regions with residual structural elements are critical for biological recognition or regulating and folding the initial compact binding site of interaction with a

biological binding partner (Uversky, 2002). It was shown that HCV NS5A has a modulating role in the HCV replication, through regulating NS5B activity (Qin *et al*, 2001; Shiota *et al*, 2002). More recent analysis addressed the molecular interaction of NS5A with NS5B during HCV RNA replication in a HCV replicon model system. The deletions of the regions within NS5A essential to the interaction with NS5B (amino acids 105-162, amino acids 277-334) can cause the subgenomic replicon non-functional, whereas the one with deletion having no effect on NS5B binding was replication competent (Shimakami *et al.*, 2004). This strongly suggests that the interaction between NS5A and NS5B is critical for the HCV RNA replication. In our NMR titration study of NS5A-D2 with the MK-17 peptide, noticeable chemical shift perturbations were observed, suggesting the molecular interaction between these two molecules. Additionally, we have shown that NS5A-D2 of HCV also binds another biological partner PKR (Liang *et al*, 2006). Taken together, our results suggest that NS5A-D2 is natively in an unfolded state but can interact with its biological partners like full length NS5A does. This is consistent with the previous studies indicating that NS5A-D2 plays an important role in regulating virus replication and resisting IFN effects.

During our studies using NMR spectroscopy, we observed that when the amount of peptide is lower than 2 mM (20 fold excess compared to that of NS5A-D2), although some perturbation in the chemical shifts were detected, no great changes happened to the overall HSQC spectrum. At this stage, the signal perturbations of

NS5A-D2 residues upon adding its ligand do not induce global structural changes on this domain. One possible explanation is that the MK17 peptide only covers one part of the binding sites of NS5A on NS5B protein and is not sufficient for the fully folding of NS5A-D2. Full length of NS5B protein is probably preferable for later study. Although the global structure of NS5A-D2 was not greatly changed by binding to NS5B peptide, two regions (L48-V57 and L86-E96) of NS5A-D2 were major affected by adding NS5B MK-17 peptide. The heteronuclear NOE values also indicate less flexible regions in NS5A-D2 (I41-F70, A73-Y95) relatively to the rest of NS5A-D2. Because an interfacial surface region is less flexible than the rest of the protein surface (Cole & Warwicker, 2002), the more rigid regions in the unbound state of NS5A-D2 indicates that it might include some interface residues. This is consistent with our NMR titration results.

At the point when the peptide concentration reached 2 mM, a heavy precipitation appeared. Interestingly, as we increased the MK-17 peptide concentration from 2 mM to 5 mM, the precipitation gradually disappeared and the solution finally became transparent again at the point of 5 mM peptide concentration (50 fold excess of the NS5A-D2). Both the line-widths and dispersion of the HSQC spectrum of NS5A-D2 with 5 mM MK-17 were affected, similar to the spectrum of NS5A-D2 in the presence of 60% TFE to a certain degree. This indicates that the interaction between the domain 2 and the MK-17 peptide results in some conformation changes. The accumulations of folding intermediates tending to

aggregate are commonly observed during the course of protein folding (Brems *et al*, 1986; Cleland & Wang, 1990; Yeh & Rousseau, 1998). It might be possible that the MK-17 peptide at 2 mM induces conformational change of NS5A-D2 resulting in a folding intermediate that might causes a heavy aggregation. From folding energy landscape, without sufficient energetic interactions natively unfolded proteins appear to have no ability compensate conformational entropy to form a globular state. Therefore, interactions with their ligands or oligomerization might contribute to achieving structural stability of the unfolded proteins (Garbuzynskiy *et al*, 2004). This principle could explain the phenomenon that was seen with the increasing of the peptide from 2 mM to 5 mM, showing that the aggregation of NS5A-D2 gradually disappeared and finally reached a more stable conformation. The requirement of the higher concentration of the MK-17 peptide might be due to the faster dissociation of the peptide from the binding site on NS5A-D2. Alternatively, the ligand concentration-dependent conformational changes might involve a potential cooperativity in the structural transition of the NS5A-D2.

In summary, our results suggest that NS5A-D2 exists intrinsically unfolded, but it still maintains in its functional state. The NMR parameters reflecting backbone dynamics of NS5A-D2, the difference in 2D ^1H - ^{15}N HSQC spectrum between the native NS5A-D2 and the chemical denaturant-induced NS5A-D2, and the more stable status of NS5A-D2 at low temperature are indicative of existence of a potential transition between ordered and disordered structures. The molecular

interaction between the NS5A-D2 and the MK-17 peptide appears to induce some conformation change under the condition used in this study, which is shown as wider amide proton line width and better dispersion in 2D ^1H - ^{15}N HSQC spectrum. However, upon binding the side-chain signals of asparagine still accumulate at about 7.4 ppm / 6.85 ppm, which resonate at the chemical shifts indicative of short unstructured peptides (Merutka *et al*, 1995). The chemical shifts perturbations induced by longer range specific contacts in natively folded protein will prevent this accumulation of side chain signals in NMR spectrum. From the 2D ^1H - ^{15}N HSQC spectrum of the complex, we can only assume that partially ordered or residual structure, but no global conformational changes from disordered to ordered state, may be induced to NS5A-D2 upon adding the peptide. It remains to be further explored that the molecular interaction of NS5A-D2 with physiological ligands such as full-length NS5B, PKR or other partners could induce a global conformational change of the intrinsically disordered NS5A-D2 to a more stable folded state.

NMR titration experiments were performed to check whether NS5Bt could induce fully conformation changes in NS5A-D2. ^{15}N -labeled NS5A-D2 (100 μM) was titrated with NS5Bt with the molar ratio of 1:0.5, 1:1, 1:2 and 1:4. Unfortunately, the complex showed a heavy precipitation at the ratio of 1:2 and remained unchanged at the ratio of 1:4. Because of the large size of NS5Bt (62 kD), it is very difficult to get enough protein for further titration. Thus it remains elusive whether

adding more NS5Bt could reverse the precipitation.

4.3. Methods for analyzing unstructured proteins

Crystallography can be used to differentiate disordered regions from structured proteins by introducing missing electron density that corresponds to the unfolded parts. The scattering signals from the coiled region are noncoherent and unobservable (Muchmore *et al*, 1996; Worbs *et al*, 2000). However, it is difficult to apply standard method of crystallography to study natively unfolded proteins which are essentially disordered under physiological conditions and lack of compact structured conformation. In recent years, the introduce of ultra-high magnetic field instruments and the heteronuclear multidimensional experiments make NMR spectroscopy become a powerful tool for detecting structural characters of unfolded and partially folded intermediates of proteins by defining the constraints and preferences at backbone conformation, long-range interaction, and observing protein dynamics (Benison *et al*, 2006; Bhavesh *et al*, 2003; Brems *et al*, 1986; Dyson & Wright, 1998; Farrow *et al*, 1994; Redfield, 2004; Zhang & Forman-Kay, 1997). As previously introduced, several NMR studies of the natively unfolded proteins or the proteins in their unfolded states have been good examples, such as cytoplasmic dynein intermediate chain (Benison *et al*, 2006), HIV-1 Tat (Shojania & O'Neil, 2006), lysozyme (Evans *et al*, 1991; Schwalbe *et al*, 1997), FK506-binding protein (Logan *et al*, 1994), and SH3 domain (Zhang & Forman-Kay, 1997). Circular dichroism (CD) is sensitive for detecting variations in

protein conformation and properties of secondary structure in disordered proteins. DLS can be used to characterize natively unfolded proteins by measuring the average molecule diameters. In our study, the CD spectrum of NS5A-D2 lacks the evident property of α -helix or β -sheet but with the typical characteristic of random coil. This gave us initial evidence of disordered conformation of NS5A-D2 at secondary structural level. Additionally, detected by DLS, the average diameter of NS5A-D2 is greater than to that of Bcl-X_L, which is a larger well folded protein with compact globular conformation. This provides us the evidence about the extended and flexible characters of unfolded NS5A-D2. From the backbone preferences of C $_{\alpha}$, C $_{\beta}$, H $_{\alpha}$ and CO, and 3D NOE data we confirmed that NS5A-D2 is disordered. The high flexibility of NS5A-D2 was also observed by NMR dynamic experiments. The prior advantage of the study by NMR spectroscopy is that we can detect whether there are some conformation changes in NS5A-D2 after changing environmental temperature, adding chemical helical inducer TFE or interacting with its biological partners by analyzing the NMR spectrum, backbone constraints, and dynamic parameters. Although fully conformation changes of unfolded NS5A to folded state was not observed in our present conditions, some promising changes in NMR preferences of NS5A-D2 indicate that as a natively unfolded protein, NS5A-D2 is potentially ordered partially or totally under some environmental changes or interaction with its bio-partners. In summary, by the combination of alternative physical methods such as NMR spectroscopy, DLS or CD, we can yield credible insights into structural characters or changes of

intrinsically disordered NS5A-D2.

Single-molecule fluorescence resonance energy transfer (SM-FRET) has been another powerful method for analyzing the structure and dynamic conformations of natively unfolded proteins (Mukhopadhyay *et al*, 2007; Ying *et al*, 2003). In SM-FRET, the excitation energy from a donor fluorophore is nonradiatively transferred to an acceptor fluorophore. The transfer efficiency depends on the relative distance and orientations between the two fluorophores. Natively unfolded proteins experience dynamic conformation changes from disordered to ordered states, which can be detected by SM-FRET. The folding intermediates, structural distributions and dynamic changes could be directly observed and quantified from these SM-FRET experiments. Additionally, even proteins at nanomolar concentrations permit the study by SM-FRET. The chance of oligomerization or aggregation will be minimized at such low concentration. We have proved that NS5A-D2 is natively unfolded by CD, DLS and NMR spectroscopy. To quantify and investigate more tiny details in the folding states of NS5A-D2, SM-FRET could be another choice.

4.4. Anti-apoptosis of NS5A

Sequence alignment results showed that NS5A protein might be a potential Bcl-2 homologue protein (Chung *et al*, 2003). Different Bcl-2 homology (BH) domains have different functions, such as BH1 and BH2 have roles in death antagonizing

and heterodimerization with Bax for anti-apoptosis, whereas BH3 involves in the heterodimerization of death agonists (Bax, Bak) with Bcl-X_L and Bcl-2 to activate cell death (Cory & Adams, 2002). Like Bcl-2, NS5A can bind to FKBP38, probably mimic the anti-apoptotic functions of Bcl-2 or augment the anti-apoptotic action of Bcl-2 (Wang *et al*, 2006). NS5A also can inhibit the pro-apoptotic action of Bax or Bad in hepatoma cells (Chung *et al*, 2003; Street *et al*, 2004). Additionally, anti-apoptotic functions of NS5A can be mediated by cytoplasmatic sequestering of p53 in human hepatoma HepG2 cells (Majumder *et al*, 2001). This suggests that anti-apoptotic functions of NS5A might contribute to the development of hepatocarcinoma. In contrast, other studies showed that NS5A can induce apoptosis directly in mature dendritic cells (Siavoshian *et al*, 2005). Mature dendritic cells are involved in the induction of immune response and stimulation of lymphocytes (Banchereau & Steinman, 1998). Pro-apoptotic effects of NS5A on mature dendritic cells might facilitate viral escaping from host immune response. The discrepancy between the anti-apoptotic and pro-apoptotic properties of NS5A is probably due to the different cell types used.

In our serum deprivation experiments, the HepG2 cells with NS5A expression and 293T-flag-NS5A cells showed about 20% higher viability than that of the cells without NS5A expression. Additionally, the cell proliferation of 293T-flag-NS5A cells treated with apoptotic inducer cisplatin was about 40% higher than that of the 293T cells without NS5A expression. This suggests possible anti-apoptotic

functions of NS5A protein with respect to serum deprivation or apoptotic inducer. Our data is consistent with the earlier results of anti-apoptosis functions about NS5A protein (Chung *et al*, 2003), in which the cells encountered apoptosis induced by sodium phenylbutyrate (NaPB). Activated caspase-3 is executioner during the early stage of apoptosis. During our study, the cleaved 17 kDa fragment of activated caspase-3 was detected in the 293T cells without NS5A expression and treated with cisplatin. However, the 293T-flag-NS5A cells showed no evidence of activation of caspase-3 by western blot analysis. Cleaved PARP is another specific marker of apoptosis. Full-length PARP or poly (ADP-ribose) polymerase is 116 kDa and participates in the repair of DNA, cell differentiation and formation of chromatin structure. During apoptosis PARP is cleaved by caspase-3, or other caspases, into a fragment about 89 kDa (Lazebnik *et al*, 1994). The cleavage of PARP was observed in the 293T cells without NS5A expression treated with cisplatin. However, no cleaved PARP was detected from 293T-flag-NS5A cells. These results suggest that NS5A protein might inhibit the activation of caspases pathway and results in the anti-apoptosis. However, in our present conditions, we did not detect obvious augments or decrease in the expression levels of Bcl-2 family proteins including anti-apoptotic Bcl-2 and Bcl-X_L or pro-apoptotic Bax. Cellular location studies of NS5A showed that this protein is mostly located at ER but partially localize at mitochondria. The localization of Bcl-2 family proteins on mitochondria is important for their anti-apoptotic or pro-apoptotic effects. We assume that NS5A probably mimic the anti-apoptotic action of Bcl-2 but not

changes the total expression levels of Bcl-2 family proteins. The preferable way of NS5A protein to exert anti-apoptotic actions is probably by interacting with pro-apoptotic proteins and translocating these proteins from mitochondria to nucleus or cytoplasm. The anti-apoptotic functions of NS5A protein could be inhibited by specific siRNAs in our present study conditions. Additionally, the 293T-flag-NS5B cells showed the same cell viability as that of 293T cells under serum deprivation conditions or cisplatin treatment. This suggests that NS5B polymerase probably has no role in disturbing the apoptosis pathway. This is consistent with the current studies about the functions of NS5B polymerase.

4.5. Effects of siNS5As

Recently, siRNAs have been used in protecting cells from virus infections. For instance, siRNAs targeted to HIV related genes or receptors have been reported to be effective in the inhibition of early and late steps of HIV-1 (Coburn & Cullen, 2002; Jacque *et al*, 2002). Hepatitis C Virus is single-stranded RNA replicating in the cytoplasm, thereby seems to be a proper target for siRNA. Several initial trials of utilizing siRNAs in HCV therapy have been reported (Benitec, 2005; Kapadia *et al*, 2003; Kronke *et al*, 2004). According to these studies, siRNA could inhibit HCV replication by targeting different HCV proteins or IRES sequences in the HCV replicons. The expression level of NS5A can also be inhibited by introducing specific targeting siRNAs, resulting in the perturbation on the activation of IL-8 promoter or HCV replication (Kanda *et al*, 2007; Sen *et al*, 2003).

In this study, we utilized two siNS5As specifically targeting the domain1 and the domain 2 of NS5A protein, respectively. We demonstrated that both of the two siRNAs against NS5A resulted in the specific inhibition of NS5A protein expression in 293T-flag-NS5A stable cell line. The silencing effects were about 65%. At the same time, the inhibition on anti-apoptosis effects of NS5A protein by the siRNAs was observed. Compared to the viability of the 293T-flag-NS5A cells treated with cisplatin, the cell proliferation of the cells pre-treated with siNS5As decreased obviously after further treatment with apoptotic inducer cisplatin. Furthermore, the cleaved PARP was also detected from the 293T-flag-NS5A cells pre-treated with siNS5As and subsequently treated with cisplatin. This indicates that specific siNS5As have the ability of reversing the anti-apoptotic actions of NS5A and assisting the pro-apoptotic effects of chemical drugs. However, the inhibition efficiency of siNS5As is relatively limited. In our study, although after cisplatin treatment, the cell proliferation of the 293T-flag-NS5A cells pre-treated with siNS5As was obviously lesser than that of 293T-flag-NS5A cells without treatment, it was still about 20% higher than that of 293T cells. The cleavage of full length PARP in the 293T-flag-NS5A cells pre-treated with siNS5As was not completely as that in 293T cells. This is probably due to the non-transfected cells or incompletely silenced cells. The assisting effects of siNS5As on cisplatin treatment observed in this study suggest that siRNAs could be a compliment in combination with traditional therapy.

Host pathogen-targeting immune responses are required for the clearance of infectious virus in the body. The interferon system is thought to be the most important immune response to viral spread and induction of viral death. In mammal cells, the antiviral response mediated by interferon could be initiated by dsRNAs longer than 30 base pairs, resulting in the global repression on mRNA translation. This is the reason that Interferon (IFN)-based therapies have become the current major therapies for hepatitis C in clinics. However, NS5A carries the biological signals determining the ability of resistance to the IFN therapy in the infected patients. The introduction of siRNAs specific targeting NS5A resulted in the inhibition on the NS5A protein expression, thereby probably assisting IFN therapy and reducing the chance of IFN resistance.

Conclusion

In this thesis, pET-ubiquitin system was used to express and purify full length NS5A protein HCV-1a and truncated domains of NS5A protein in *E. coli*. Functional analysis by GST pull down assay showed that the purified NS5A protein was active and capable of interacting with its biological partners, such as NS5B polymerase and PKR. Secondary structural analysis by CD showed that NS5A protein is mainly β -sheet at secondary structural level. The domain 1 of NS5A protein was reported to be zinc metalloprotein with β -sheet as its mainly structural element. Our results of CD and 1D ^1H NMR analysis showed that the domain 3 of NS5A protein (NS5A-D3) is mostly in random coil conformation.

Because the domain 2 has been reported to be involved in variety of biological functions of NS5A protein, we focused to study the structural and functional characters of the domain 2. Our results suggest that NS5A-D2 is intrinsically unfolded but functionally active. The results of CD, 1D ^1H NMR and DLS revealed the disorder and flexible natures of NS5A-D2. The analysis of the backbone ^1H , ^{13}C , and ^{15}N resonances, $^3J_{\text{HN}\alpha}$ coupling constants and 3D NOE data suggests that NS5A-D2 lacks ordered formations at secondary structural level. However, dynamics of NS5A-D2 analyzed by NMR experiments, the difference

showed in 2D ^1H - ^{15}N HSQC and CD spectra of native NS5A-D2 compared to the chemical denaturant-induced NS5A-D2, the more stable status of NS5A-D2 at low temperature, and the helical transition under the presence of TFE, are clues of a potential transition to folded structures from disordered status. Although some conformation change under our study conditions seemed to be observed on NS5A-D2 upon the interaction with NS5B MK-17 peptide, no global conformational changes was detected. The global conformational change of the natively unfolded NS5A-D2 to folded state upon interacting with its biological partners such as full-length NS5B, PKR or others is still remained to be answer.

The anti-apoptosis functions of NS5A protein were observed in hepatoma HepG2 cells and 293T cells. Specific siRNAs targeting NS5A protein were capable of suppressing the expression of NS5A protein. Furthermore, the siNS5As could disturb and reverse the anti-apoptosis effects of NS5A protein at some degree. Our results confirm the anti-apoptotic actions of NS5A protein and supply more evidences for the feasibility of RNA interference in HCV treatments.

Author's publications

1. Liang, Y., Ye, H., Kang, CB., and Yoon, HS. (2007) Domain 2 of Non-structural Protein 5A (NS5A) of hepatitis C virus is natively unfolded. *Biochemistry*. 46 (41), 11550-11558.
2. Liang, Y., Kang, CB., and Yoon, HS. (2006) Molecular and structural characterization of the domain 2 of Hepatitis C virus non-structural protein 5A. *Mol Cells*, 22, 13-20.

Conference papers

Structural and biochemical analysis of the domain 2 of Hepatitis C Virus non-structural Protein 5A, AOHUPO and Structural Biology conference, Singapore (December, 2006).

Reference

- Adler AJ, Greenfield NJ, Fasman GD (1973) Circular dichroism and optical rotatory dispersion of proteins and polypeptides. *Methods Enzymol* **27**: 675-735
- Ago H, Adachi T, Yoshida A, Yamamoto M, Habuka N, Yatsunami K, Miyano M (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Structure* **7**: 1417-26
- Arima N, Kao CY, Licht T, Padmanabhan R, Sasaguri Y (2001) Modulation of cell growth by the hepatitis C virus nonstructural protein NS5A. *J Biol Chem* **276**: 12675-84
- Aus dem Siepen M, Lohmann V, Wiese M, Ross S, Roggendorf M, Viazov S (2005) Nonstructural protein 5A does not contribute to the resistance of hepatitis C virus replication to interferon alpha in cell culture. *Virology* **336**: 131-6
- Baek KH, Park HY, Kang CM, Kim SJ, Jeong SJ, Hong EK, Park JW, Sung YC, Suzuki T, Kim CM, Lee CW (2006) Overexpression of hepatitis C virus NS5A protein induces chromosome instability via mitotic cell cycle dysregulation. *J Mol Biol* **359**: 22-34
- Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* **392**: 245-52
- Bassett SE, Guerra B, Brasky K, Miskovsky E, Houghton M, Klimpel GR, Lanford RE (2001) Protective immune response to hepatitis C virus in chimpanzees rechallenged following clearance of primary infection. *Hepatology* **33**: 1479-87
- Bayer E, Goettsch S, Mueller JW, Griewel B, Guiberman E, Mayr LM, Bayer P (2003) Structural

analysis of the mitotic regulator hPin1 in solution: insights into domain architecture and substrate binding. *J Biol Chem* **278**: 26183-93

Behrens SE, Tomei L, De Francesco R (1996) Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *Embo J* **15**: 12-22

Behrens SE, Tomei L, De Francesco R (1996) Identification and properties of the RNA-dependent RNA polymerase of hepatitis C virus. *Embo J* **15**: 12-22

Benedict C, Norris, P. & Ware, C. (2002) To kill or be killed: viral evasion of apoptosis. . *Nat Immu* **3**: 1013-1018

Benison G, Nyarko A, Barbar E (2006) Heteronuclear NMR identifies a nascent helix in intrinsically disordered dynein intermediate chain: implications for folding and dimerization. *J Mol Biol* **362**: 1082-93

Benitec (2005) Benitec announces clinical candidate for treatment of hepatitis C.

<http://www.benitec.com/PRDownloads/Hepatitis%20C%20Clinical%20Candidate%20050905%20.pdf> (2005).

Berg JM, Shi Y (1996) The galvanization of biology: a growing appreciation for the roles of zinc. *Science* **271**: 1081-5

Berne B, Percora R (1974) Dynamic light scattering with applications to chemistry, biology and physics.

Bhavesh NS, Sinha R, Mohan PM, Hosur RV (2003) NMR elucidation of early folding hierarchy in HIV-1 protease. *J Biol Chem* **278**: 19980-5

Biswal BK, Cherney MM, Wang M, Chan L, Yannopoulos CG, Bilimoria D, Nicolas O, Bedard J,

- James MN (2005) Crystal structures of the RNA-dependent RNA polymerase genotype 2a of hepatitis C virus reveal two conformations and suggest mechanisms of inhibition by non-nucleoside inhibitors. *J Biol Chem* **280**: 18202-10
- Blight KJ, Kolykhalov AA, Rice CM (2000) Efficient initiation of HCV RNA replication in cell culture. *Science* **290**: 1972-4
- Bode W, Schwager P, Huber R (1978) The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding. The refined crystal structures of the bovine trypsinogen-pancreatic trypsin inhibitor complex and of its ternary complex with Ile-Val at 1.9 Å resolution. *J Mol Biol* **118**: 99-112
- Bottomley MJ, Macias MJ, Liu Z, Sattler M (1999) A novel NMR experiment for the sequential assignment of proline residues and proline stretches in ¹³C/¹⁵N-labeled proteins. *J Biomol NMR* **13**: 381-5
- Boyko R, Sykes, B.D. xcrvfit: a graphical X-windows program for binding curve studies and NMR spectroscopic analysis.
- Bradley DW (2000) Studies of non-A, non-B hepatitis and characterization of the hepatitis C virus in chimpanzees. *Curr Top Microbiol Immunol* **242**: 1-23
- Brass V, Bieck E, Montserret R, Wolk B, Hellings JA, Blum HE, Penin F, Moradpour D (2002) An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J Biol Chem* **277**: 8130-9
- Brass V, Bieck E, Montserret R, Wolk B, Hellings JA, Blum HE, Penin F, Moradpour D (2002) An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J Biol Chem* **277**: 8130-9

- Breiman A, Camus I (2002) The involvement of mammalian and plant FK506-binding proteins (FKBPs) in development. *Transgenic Res* **11**: 321-35
- Brems DN, Plaisted SM, Kauffman EW, Havel HA (1986) Characterization of an associated equilibrium folding intermediate of bovine growth hormone. *Biochemistry* **25**: 6539-43
- Bressanelli S, Tomei L, Roussel A, Incitti I, Vitale RL, Mathieu M, De Francesco R, Rey FA (1999) Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc Natl Acad Sci U S A* **96**: 13034-9
- Brillet R, Penin F, Hezode C, Chouteau P, Dhumeaux D, Pawlotsky JM (2007) The nonstructural 5A protein of hepatitis C virus genotype 1b does not contain an interferon sensitivity-determining region. *J Infect Dis* **195**: 432-41
- Brown RS, Jr., Gaglio PJ (2003) Scope of worldwide hepatitis C problem. *Liver Transpl* **9**: S10-3
- Bukh J, Miller RH, Purcell RH (1995) Biology and genetic heterogeneity of hepatitis C virus. *Clin Exp Rheumatol* **13 Suppl 13**: S3-7
- Bukh J, Miller RH, Purcell RH (1995) Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. *Semin Liver Dis* **15**: 41-63
- Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**: 37-40
- Chen S-Hea (2005) P1 and P1' optimization of [3,4]-bicycloproline P2 incorporated tetrapeptidyl -ketoamide based HCV protease inhibitors. *Drug Des Disc* **2**: 118-123
- Chen YH, Yang JT, Martinez HM (1972) Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* **11**: 4120-31
- Cho NJ, Cheong KH, Lee C, Frank CW, Glenn JS (2007) Binding dynamics of hepatitis C virus'

- NS5A amphipathic peptide to cell and model membranes. *J Virol* **81**: 6682-9
- Choi J, Lee KJ, Zheng Y, Yamaga AK, Lai MM, Ou JH (2004) Reactive oxygen species suppress hepatitis C virus RNA replication in human hepatoma cells. *Hepatology* **39**: 81-9
- Choi YW, Tan YJ, Lim SG, Hong W, Goh PY (2004) Proteomic approach identifies HSP27 as an interacting partner of the hepatitis C virus NS5A protein. *Biochem Biophys Res Commun* **318**: 514-9
- Choo QL, Kuo G, Ralston R, Weiner A, Chien D, Van Nest G, Han J, Berger K, Thudium K, Kuo C, et al. (1994) Vaccination of chimpanzees against infection by the hepatitis C virus. *Proc Natl Acad Sci U S A* **91**: 1294-8
- Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**: 359-62
- Chung KM, Song OK, Jang SK (1997) Hepatitis C virus nonstructural protein 5A contains potential transcriptional activator domains. *Mol Cells* **7**: 661-7
- Chung YL, Sheu ML, Yen SH (2003) Hepatitis C virus NS5A as a potential viral Bcl-2 homologue interacts with Bax and inhibits apoptosis in hepatocellular carcinoma. *Int J Cancer* **107**: 65-73
- Chung YL, Sheu ML, Yen SH (2003) Hepatitis C virus NS5A as a potential viral Bcl-2 homologue interacts with Bax and inhibits apoptosis in hepatocellular carcinoma. *Int J Cancer* **107**: 65-73
- Cleland JL, Wang DI (1990) Refolding and aggregation of bovine carbonic anhydrase B: quasi-elastic light scattering analysis. *Biochemistry* **29**: 11072-8
- Coburn GA, Cullen BR (2002) Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J Virol* **76**: 9225-31
- Cole C, Warwicker J (2002) Side-chain conformational entropy at protein-protein interfaces. *Protein*

Sci **11**: 2860-70

Coley (2005) Coley reports results from phase I studies of Actilon™ for hepatitis C.

http://www.coleypharma.com/coley/pr_1105025921 (2005). *Med*. **9**, 347–351 (2003).

Cory S, Adams JM (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**: 647-56

Davis GL, Nelson DR, Reyes GR (1999) Future options for the management of hepatitis C. *Semin Liver Dis* **19 Suppl 1**: 103-12

DeLano WL (2002) The PyMOL Molecular Graphics System.

Dimitrova M, Imbert I, Kieny MP, Schuster C (2003) Protein-protein interactions between hepatitis C virus nonstructural proteins. *J Virol* **77**: 5401-14

Dobson CM (1992) Unfolded proteins, compact states and molten globules. *Curr Opin Struct Biol* **2**: 6-12

Donaldson L, Capone JP (1992) Purification and characterization of the carboxyl-terminal transactivation domain of Vmw65 from herpes simplex virus type 1. *J Biol Chem* **267**: 1411-4

Dunker AK, Lawson JD, Brown CJ, Williams RM, Romero P, Oh JS, Oldfield CJ, Campen AM, Ratliff CM, Hipps KW, Ausio J, Nissen MS, Reeves R, Kang C, Kissinger CR, Bailey RW, Griswold MD, Chiu W, Garner EC, Obradovic Z (2001) Intrinsically disordered protein. *J Mol Graph Model* **19**: 26-59

Dyson HJ, Wright PE (1998) Equilibrium NMR studies of unfolded and partially folded proteins. *Nat Struct Biol* **5 Suppl**: 499-503

Dyson HJ, Wright PE (2002) Coupling of folding and binding for unstructured proteins. *Curr Opin*

Struct Biol **12**: 54-60

Elazar M, Cheong KH, Liu P, Greenberg HB, Rice CM, Glenn JS (2003) Amphipathic helix-dependent localization of NS5A mediates hepatitis C virus RNA replication. *J Virol* **77**: 6055-61

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**: 494-8

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C (1995) Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J Clin Invest* **96**: 224-30

Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C (1996) Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* **334**: 77-81

Evans MJ, Rice CM, Goff SP (2004) Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci U S A* **101**: 13038-43

Evans PA, Topping KD, Woolfson DN, Dobson CM (1991) Hydrophobic clustering in nonnative states of a protein: interpretation of chemical shifts in NMR spectra of denatured states of lysozyme. *Proteins* **9**: 248-66

Failla C, Tomei L, De Francesco R (1994) Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J Virol* **68**: 3753-60

Farrow NA, Muhandiram R, Singer AU, Pascal SM, Kay CM, Gish G, Shoelson SE, Pawson T,

- Forman-Kay JD, Kay LE (1994) Backbone dynamics of a free and phosphopeptide-complexed Src homology 2 domain studied by ^{15}N NMR relaxation. *Biochemistry* **33**: 5984-6003
- Feld JJ, Hoofnagle JH (2005) Mechanism of action of interferon and ribavirin in treatment of hepatitis C. *Nature* **436**: 967-72
- Ferrari E, Wright-Minogue J, Fang JW, Baroudy BM, Lau JY, Hong Z (1999) Characterization of soluble hepatitis C virus RNA-dependent RNA polymerase expressed in *Escherichia coli*. *J Virol* **73**: 1649-54
- Fink AL (2005) Natively unfolded proteins. *Curr Opin Struct Biol* **15**: 35-41
- Fisher DE (1994) Apoptosis in cancer therapy: crossing the threshold. *Cell* **78**: 539-42
- Forgues M, Difilippantonio MJ, Linke SP, Ried T, Nagashima K, Feden J, Valerie K, Fukasawa K, Wang XW (2003) Involvement of Crml in hepatitis B virus X protein-induced aberrant centriole replication and abnormal mitotic spindles. *Mol Cell Biol* **23**: 5282-92
- Fretz H, Furet P, Garcia-Echeverria C, Schoepfer J, Rahuel J (2000) Structure-based design of compounds inhibiting Grb2-SH2 mediated protein-protein interactions in signal transduction pathways. *Curr Pharm Des* **6**: 1777-96
- Fukuma T, Enomoto N, Marumo F, Sato C (1998) Mutations in the interferon-sensitivity determining region of hepatitis C virus and transcriptional activity of the nonstructural region 5A protein. *Hepatology* **28**: 1147-53
- Gale M, Jr., Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG (1998) Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. *Mol Cell Biol* **18**: 5208-18

- Gale M, Jr., Kwieciszewski B, Dossett M, Nakao H, Katze MG (1999) Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. *J Virol* **73**: 6506-16
- Gale MJ, Jr., Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG (1997) Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* **230**: 217-27
- Gao L, Aizaki H, He JW, Lai MM (2004) Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. *J Virol* **78**: 3480-8
- Garbuzynskiy SO, Lobanov MY, Galzitskaya OV (2004) To be folded or to be unfolded? *Protein Sci* **13**: 2871-7
- Gaur U, Aggarwal BB (2003) Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. *Biochem Pharmacol* **66**: 1403-8
- Georgopoulou U, Caravokiri K, Mavromara P (2003) Suppression of the ERK1/2 signaling pathway from HCV NS5A protein expressed by herpes simplex recombinant viruses. *Arch Virol* **148**: 237-51
- Ghosh AK, Majumder M, Steele R, Yaciuk P, Chrivia J, Ray R, Ray RB (2000) Hepatitis C virus NS5A protein modulates transcription through a novel cellular transcription factor SRCAP. *J Biol Chem* **275**: 7184-8
- Girard S, Shalhoub P, Lescure P, Sabile A, Misek DE, Hanash S, Brechot C, Beretta L (2002) An altered cellular response to interferon and up-regulation of interleukin-8 induced by the hepatitis C viral protein NS5A uncovered by microarray analysis. *Virology* **295**: 272-83
- Gong G, Waris G, Tanveer R, Siddiqui A (2001) Human hepatitis C virus NS5A protein alters

intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proc Natl Acad Sci USA* **98**: 9599-604

Gong G, Waris G, Tanveer R, Siddiqui A (2001) Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proc Natl Acad Sci U S A* **98**: 9599-604

Gong GZ, Cao J, Jiang YF, Zhou Y, Liu B (2007) Hepatitis C virus non-structural 5A abrogates signal transducer and activator of transcription-1 nuclear translocation induced by IFN-alpha through dephosphorylation. *World J Gastroenterol* **13**: 4080-4

Gunasekaran K, Tsai CJ, Kumar S, Zanuy D, Nussinov R (2003) Extended disordered proteins: targeting function with less scaffold. *Trends Biochem Sci* **28**: 81-5

Hardwick JM, Bellows DS (2003) Viral versus cellular BCL-2 proteins. *Cell Death Differ* **10 Suppl 1**: S68-76

Harris MA, Wise MS, Bonington A, Slevin NJ (2004) Case report: radical radiotherapy for early laryngeal cancer in a patient with human immunodeficiency virus: no evidence of increased toxicity. *Br J Radiol* **77**: 519-20

Havel HA, Kauffman EW, Plaisted SM, Brems DN (1986) Reversible self-association of bovine growth hormone during equilibrium unfolding. *Biochemistry* **25**: 6533-8

He J, OJ, Kasahara N. & Lai M. C. C. (2001) Characterization of hepatitis C virus polymerase expressed in mammalian cells: phosphorylation is essential for its polymerase activity. *20th ASV Annual Meeting, Madison, Wisc*

He Y, Tan SL, Tareen SU, Vijaysri S, Langland JO, Jacobs BL, Katze MG (2001) Regulation of mRNA translation and cellular signaling by hepatitis C virus nonstructural protein NS5A. *J Virol* **75**:

5090-8

Hershey JW (1991) Translational control in mammalian cells. *Annu Rev Biochem* **60**: 717-55

Hong Z, Beaudet-Miller M, Lanford RE, Guerra B, Wright-Minogue J, Skelton A, Baroudy BM, Reyes GR, Lau JY (1999) Generation of transmissible hepatitis C virions from a molecular clone in chimpanzees. *Virology* **256**: 36-44

Horsmans Yea (2004) Isatoribine, a Toll-like receptor 7 agonist, significantly reduced plasma viral load in a clinical proof-of-concept study in patients with chronic hepatitis C virus. *Hepatology* **40** (Suppl. 1): 282A

Hu Y, Macinnis JM, Cherayil BJ, Fleming GR, Freed KF, and Perico A (1990) Polypeptide dynamics: experimental tests of an optimized Rouse-Zimm type model. *J Chem Phys* **93**: 822-836

Huang L, Sineva EV, Hargittai MR, Sharma SD, Suthar M, Raney KD, Cameron CE (2004) Purification and characterization of hepatitis C virus non-structural protein 5A expressed in *Escherichia coli*. *Protein Expr Purif* **37**: 144-53

Huang Y, Staschke K, De Francesco R, Tan SL (2007) Phosphorylation of hepatitis C virus NS5A nonstructural protein: a new paradigm for phosphorylation-dependent viral RNA replication? *Virology* **364**: 1-9

Hwang SB, Park KJ, Kim YS, Sung YC, Lai MM (1997) Hepatitis C virus NS5B protein is a membrane-associated phosphoprotein with a predominantly perinuclear localization. *Virology* **227**: 439-46

Iacovacci S, Manzin A, Barca S, Sargiacomo M, Serafino A, Valli MB, Macioce G, Hassan HJ, Ponzetto A, Clementi M, Peschle C, Carloni G (1997) Molecular characterization and dynamics of hepatitis C virus replication in human fetal hepatocytes infected in vitro. *Hepatology* **26**: 1328-37

- Ide Y, Tanimoto A, Sasaguri Y, Padmanabhan R (1997) Hepatitis C virus NS5A protein is phosphorylated in vitro by a stably bound protein kinase from HeLa cells and by cAMP-dependent protein kinase A-alpha catalytic subunit. *Gene* **201**: 151-8
- Ide Y, Zhang L, Chen M, Inchauspe G, Bahl C, Sasaguri Y, Padmanabhan R (1996) Characterization of the nuclear localization signal and subcellular distribution of hepatitis C virus nonstructural protein NS5A. *Gene* **182**: 203-11
- Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S, Sameshima M, Hase A, Seto Y, Nagata S (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell* **66**: 233-43
- Jacque JM, Triques K, Stevenson M (2002) Modulation of HIV-1 replication by RNA interference. *Nature* **418**: 435-8
- Jiang D, Srinivasan A, Lozano G, Robbins PD (1993) SV40 T antigen abrogates p53-mediated transcriptional activity. *Oncogene* **8**: 2805-12
- Jin DY, Spencer F, Jeang KT (1998) Human T cell leukemia virus type 1 oncoprotein Tax targets the human mitotic checkpoint protein MAD1. *Cell* **93**: 81-91
- Johnson BAB, R.A (1994) NMR View - A Computer-Program for the Visualization and Analysis of NMR Data. *J Biomol NMR* **4**: 603-614
- Johnston H, Kneer J, Chackalaparampil I, Yaciuk P, Chrivia J (1999) Identification of a novel SNF2/SWI2 protein family member, SRCAP, which interacts with CREB-binding protein. *J Biol Chem* **274**: 16370-6
- Kalamvoki M, Georgopoulou U, Mavromara P (2006) The NS5A protein of the hepatitis C virus genotype 1a is cleaved by caspases to produce C-terminal-truncated forms of the protein that reside

mainly in the cytosol. *J Biol Chem* **281**: 13449-62

Kalliampakou KI, Kalamvoki M, Mavromara P (2005) Hepatitis C virus (HCV) NS5A protein downregulates HCV IRES-dependent translation. *J Gen Virol* **86**: 1015-25

Kanda T, Steele R, Ray R, Ray RB (2007) Small interfering RNA targeted to hepatitis C virus 5' nontranslated region exerts potent antiviral effect. *J Virol* **81**: 669-76

Kaneko T, Tanji Y, Satoh S, Hijikata M, Asabe S, Kimura K, Shimotohno K (1994) Production of two phosphoproteins from the NS5A region of the hepatitis C viral genome. *Biochem Biophys Res Commun* **205**: 320-6

Kang CB, Feng L, Chia J, Yoon HS (2005) Molecular characterization of FK-506 binding protein 38 and its potential regulatory role on the anti-apoptotic protein Bcl-2. *Biochem Biophys Res Commun* **337**: 30-8

Kang CB, Tai J, Chia J, Yoon HS (2005) The flexible loop of Bcl-2 is required for molecular interaction with immunosuppressant FK-506 binding protein 38 (FKBP38). *FEBS Lett* **579**: 1469-76

Kapadia SB, Brideau-Andersen A, Chisari FV (2003) Interference of hepatitis C virus RNA replication by short interfering RNAs. *Proc Natl Acad Sci U S A* **100**: 2014-8

Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Shimotohno K (1990) Sequence diversity of hepatitis C viral genomes. *Mol Biol Med* **7**: 495-501

Kato N, Ikeda M, Mizutani T, Sugiyama K, Noguchi M, Hirohashi S, Shimotohno K (1996) Replication of hepatitis C virus in cultured non-neoplastic human hepatocytes. *Jpn J Cancer Res* **87**: 787-92

- Kato N, Lan KH, Ono-Nita SK, Shiratori Y, Omata M (1997) Hepatitis C virus nonstructural region 5A protein is a potent transcriptional activator. *J Virol* **71**: 8856-9
- Kato N, Ootsuyama Y, Tanaka T, Nakagawa M, Nakazawa T, Muraio K, Ohkoshi S, Hijikata M, Shimotohno K (1992) Marked sequence diversity in the putative envelope proteins of hepatitis C viruses. *Virus Res* **22**: 107-23
- Katze MG, Kwieciszewski B, Goodlett DR, Blakely CM, Neddermann P, Tan SL, Aebersold R (2000) Ser(2194) is a highly conserved major phosphorylation site of the hepatitis C virus nonstructural protein NS5A. *Virology* **278**: 501-13
- Khabar KS, Al-Zoghaibi F, Al-Ahdal MN, Murayama T, Dhalla M, Mukaida N, Taha M, Al-Sedairy ST, Siddiqui Y, Kessie G, Matsushima K (1997) The alpha chemokine, interleukin 8, inhibits the antiviral action of interferon alpha. *J Exp Med* **186**: 1077-85
- Kim J, Lee D, Choe J (1999) Hepatitis C virus NS5A protein is phosphorylated by casein kinase II. *Biochem Biophys Res Commun* **257**: 777-81
- Kolykhalov AA, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM (1997) Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* **277**: 570-4
- Kretsovali A, Agalioti T, Spilianakis C, Tzortzakaki E, Merika M, Papamatheakis J (1998) Involvement of CREB binding protein in expression of major histocompatibility complex class II genes via interaction with the class II transactivator. *Mol Cell Biol* **18**: 6777-83
- Kronke J, Kittler R, Buchholz F, Windisch MP, Pietschmann T, Bartenschlager R, Frese M (2004) Alternative approaches for efficient inhibition of hepatitis C virus RNA replication by small interfering RNAs. *J Virol* **78**: 3436-46
- Kucharczak J, Simmons MJ, Fan Y, Gelinas C (2003) To be, or not to be: NF-kappaB is the

answer--role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene* **22**: 8961-82

Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ, Pavletich NP (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **274**: 948-53

Lam E, Martin M, Wiederrecht G (1995) Isolation of a cDNA encoding a novel human FK506-binding protein homolog containing leucine zipper and tetratricopeptide repeat motifs. *Gene* **160**: 297-302

Lamarre D, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P, Bos M, Cameron DR, Cartier M, Cordingley MG, Faucher AM, Goudreau N, Kawai SH, Kukulj G, Lagace L, LaPlante SR, Narjes H, Poupart MA, Rancourt J, Sentjens RE, St George R, Simoneau B, Steinmann G, Thibeault D, Tsantrizos YS, Weldon SM, Yong CL, Llinas-Brunet M (2003) An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* **426**: 186-9

Lan KH, Lan KL, Lee WP, Sheu ML, Chen MY, Lee YL, Yen SH, Chang FY, Lee SD (2007) HCV NS5A inhibits interferon-alpha signaling through suppression of STAT1 phosphorylation in hepatocyte-derived cell lines. *J Hepatol* **46**: 759-67

Lan KH, Sheu ML, Hwang SJ, Yen SH, Chen SY, Wu JC, Wang YJ, Kato N, Omata M, Chang FY, Lee SD (2002) HCV NS5A interacts with p53 and inhibits p53-mediated apoptosis. *Oncogene* **21**: 4801-11

Lane DP (1992) Cancer. p53, guardian of the genome. *Nature* **358**: 15-6

Lavanchy D (1999) Hepatitis C: public health strategies. *J Hepatol* **31 Suppl 1**: 146-51

Lavia P, Mileo AM, Giordano A, Paggi MG (2003) Emerging roles of DNA tumor viruses in cell proliferation: new insights into genomic instability. *Oncogene* **22**: 6508-16

- Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**: 346-7
- Lemon SM, Lerat, H., Weinman, S.A., Honda, M. (2000) A transgenic mouse model of steatosis and hepatocellular carcinoma associated with chronic hepatitis C virus infection in humans. *Trans Am Clin Climatol Assoc* **111**: 146-157
- Lesburg CA, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC (1999) Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol* **6**: 937-43
- Levine AJ (1997) p53, the cellular gatekeeper for growth and division. *Cell* **88**: 323-31
- Liang Y, Kang CB, Yoon HS (2006) Molecular and structural characterization of the domain 2 of hepatitis C virus non-structural protein 5A. *Mol Cells* **22**: 13-20
- Liang Y, Kang CB, Yoon HS (2006) Molecular and structural characterization of the domain 2 of hepatitis C virus non-structural protein 5A. *Mol Cells* **22**: 13-20
- Liang Y, Ye H, Kang CB, Yoon HS (2007) Domain 2 of nonstructural protein 5A (NS5A) of hepatitis C virus is natively unfolded. *Biochemistry* **46**: 11550-8
- Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM (2005) Complete replication of hepatitis C virus in cell culture. *Science* **309**: 623-6
- Lindenbach BD, Rice CM (2002) RNAi targeting an animal virus: news from the front. *Mol Cell* **9**: 925-7
- Lingel A, Simon B, Izaurralde E, Sattler M (2004) NMR assignment of the Drosophila Argonaute2

PAZ domain. *J Biomol NMR* **29**: 421-2

Llinas M, and Klein, M. P (1975) Charge relay at the peptide bond. A proton magnetic resonance study of solvation effects on the amide electron density distribution. *J Amer Chem Soc* **97**: 4731-4737

Logan TM, Theriault Y, Fesik SW (1994) Structural characterization of the FK506 binding protein unfolded in urea and guanidine hydrochloride. *J Mol Biol* **236**: 637-48

Lohmann V, Korner F, Dobierzewska A, Bartenschlager R (2001) Mutations in hepatitis C virus RNAs conferring cell culture adaptation. *J Virol* **75**: 1437-49

Lohmann V, Korner F, Herian U, Bartenschlager R (1997) Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J Virol* **71**: 8416-28

Lohmann V, Korner F, Herian U, Bartenschlager R (1997) Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J Virol* **71**: 8416-28

Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**: 110-3

Macdonald A, Crowder K, Street A, McCormick C, Harris M (2004) The hepatitis C virus NS5A protein binds to members of the Src family of tyrosine kinases and regulates kinase activity. *J Gen Virol* **85**: 721-9

Macdonald A, Crowder K, Street A, McCormick C, Harris M (2004) The hepatitis C virus NS5A protein binds to members of the Src family of tyrosine kinases and regulates kinase activity. *J Gen Virol* **85**: 721-9

- Majumder M, Ghosh AK, Steele R, Ray R, Ray RB (2001) Hepatitis C virus NS5A physically associates with p53 and regulates p21/waf1 gene expression in a p53-dependent manner. *J Virol* **75**: 1401-7
- Marion D, Driscoll PC, Kay LE, Wingfield PT, Bax A, Gronenborn AM, Clore GM (1989) Overcoming the overlap problem in the assignment of ¹H NMR spectra of larger proteins by use of three-dimensional heteronuclear ¹H-¹⁵N Hartmann-Hahn-multiple quantum coherence and nuclear Overhauser-multiple quantum coherence spectroscopy: application to interleukin 1 beta. *Biochemistry* **28**: 6150-6
- Mehta SH, Cox A, Hoover DR, Wang XH, Mao Q, Ray S, Strathdee SA, Vlahov D, Thomas DL (2002) Protection against persistence of hepatitis C. *Lancet* **359**: 1478-83
- Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, Addison WR, Fischer KP, Churchill TA, Lakey JR, Tyrrell DL, Kneteman NM (2001) Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**: 927-33
- Merutka G, Dyson HJ, Wright PE (1995) 'Random coil' ¹H chemical shifts obtained as a function of temperature and trifluoroethanol concentration for the peptide series GGXGG. *J Biomol NMR* **5**: 14-24
- Miller RH, Purcell RH (1990) Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. *Proc Natl Acad Sci USA* **87**: 2057-61
- Mottola G, Cardinali G, Ceccacci A, Trozzi C, Bartholomew L, Torrisi MR, Pedrazzini E, Bonatti S, Migliaccio G (2002) Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. *Virology* **293**: 31-43

- Muchmore SW, Sattler M, Liang H, Meadows RP, Harlan JE, Yoon HS, Nettlesheim D, Chang BS, Thompson CB, Wong SL, Ng SL, Fesik SW (1996) X-ray and NMR structure of human Bcl-xL, an inhibitor of programmed cell death. *Nature* **381**: 335-41
- Mukhopadhyay S, Krishnan R, Lemke EA, Lindquist S, Deniz AA (2007) A natively unfolded yeast prion monomer adopts an ensemble of collapsed and rapidly fluctuating structures. *Proc Natl Acad Sci U S A* **104**: 2649-54
- Myszka DG (1999) Improving biosensor analysis. *J Mol Recognit* **12**: 279-84
- Nakayama KI, Hatakeyama S, Nakayama K (2001) Regulation of the cell cycle at the G1-S transition by proteolysis of cyclin E and p27Kip1. *Biochem Biophys Res Commun* **282**: 853-60
- Nanda SK, Herion D, Liang TJ (2006) The SH3 binding motif of HCV [corrected] NS5A protein interacts with Bin1 and is important for apoptosis and infectivity. *Gastroenterology* **130**: 794-809
- Neddermann P, Clementi A, De Francesco R (1999) Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. *J Virol* **73**: 9984-91
- Neddermann P, Tomei L, Steinkuhler C, Gallinari P, Tramontano A, De Francesco R (1997) The nonstructural proteins of the hepatitis C virus: structure and functions. *Biol Chem* **378**: 469-76
- Nelson JW, Kallenbach NR (1986) Stabilization of the ribonuclease S-peptide alpha-helix by trifluoroethanol. *Proteins* **1**: 211-7
- Nicoli DF, Benedek GB (1976) Study of thermal denaturation of lysozyme and other globular proteins by light-scattering spectroscopy. *Biopolymers* **15**: 2421-37
- O'Shannessy DJ, Brigham-Burke M, Peck K (1992) Immobilization chemistries suitable for use in

the BIAcore surface plasmon resonance detector. *Anal Biochem* **205**: 132-6

Ohsawa M, Shingu N, Miwa H, Yoshihara H, Kubo M, Tsukuma H, Teshima H, Hashimoto M, Aozasa K (1999) Risk of non-Hodgkin's lymphoma in patients with hepatitis C virus infection. *Int J Cancer* **80**: 237-9

Okamoto T, Nishimura Y, Ichimura T, Suzuki K, Miyamura T, Suzuki T, Moriishi K, Matsuura Y (2006) Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90. *Embo J* **25**: 5015-25

Okuda K, Yokosuka O, Otake Y, Hayashi H, Yokozeki K, Kashima T, Kobayashi S, Sakuma K, Ohni T, Irie Y (1998) Cryoglobulinaemia among maintenance haemodialysis patients and its relation to hepatitis C infection. *J Gastroenterol Hepatol* **13**: 248-52

Page R, Peti W, Wilson IA, Stevens RC, Wuthrich K (2005) NMR screening and crystal quality of bacterially expressed prokaryotic and eukaryotic proteins in a structural genomics pipeline. *Proc Natl Acad Sci U S A* **102**: 1901-5

Park KJ, Choi SH, Choi DH, Park JM, Yie SW, Lee SY, Hwang SB (2003) Hepatitis C virus NS5A protein modulates c-Jun N-terminal kinase through interaction with tumor necrosis factor receptor-associated factor 2. *J Biol Chem* **278**: 30711-8

Park KJ, Choi SH, Lee SY, Hwang SB, Lai MM (2002) Nonstructural 5A protein of hepatitis C virus modulates tumor necrosis factor alpha-stimulated nuclear factor kappa B activation. *J Biol Chem* **277**: 13122-8

Park KJ, Choi SH, Lee SY, Hwang SB, Lai MM (2002) Nonstructural 5A protein of hepatitis C virus modulates tumor necrosis factor alpha-stimulated nuclear factor kappa B activation. *J Biol Chem* **277**: 13122-8

Pearse MJ, Drane D (2005) ISCOMATRIX adjuvant for antigen delivery. *Adv Drug Deliv Rev* **57**:

465-74

Penin F, Brass V, Appel N, Ramboarina S, Montserret R, Ficheux D, Blum HE, Bartenschlager R, Moradpour D (2004) Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. *J Biol Chem* **279**: 40835-43

Penin F, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM (2004) Structural biology of hepatitis C virus. *Hepatology* **39**: 5-19

Perez RP (1998) Cellular and molecular determinants of cisplatin resistance. *Eur J Cancer* **34**: 1535-42

Peti W, Smith LJ, Redfield C, Schwalbe H (2001) Chemical shifts in denatured proteins: resonance assignments for denatured ubiquitin and comparisons with other denatured proteins. *J Biomol NMR* **19**: 153-65

Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R (2001) Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. *J Virol* **75**: 1252-64

Polakos NK, Drane D, Cox J, Ng P, Selby MJ, Chien D, O'Hagan DT, Houghton M, Paliard X (2001) Characterization of hepatitis C virus core-specific immune responses primed in rhesus macaques by a nonclassical ISCOM vaccine. *J Immunol* **166**: 3589-98

Polyak SJ, Khabar KS, Paschal DM, Ezelle HJ, Duverlie G, Barber GN, Levy DE, Mukaida N, Gretch DR (2001) Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J Virol* **75**: 6095-106

Polyak SJ, Khabar KS, Rezeiq M, Gretch DR (2001) Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. *J Virol* **75**: 6209-11

- Qadri I, Iwahashi M, Capasso JM, Hopken MW, Flores S, Schaack J, Simon FR (2004) Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1. *Biochem J* **378**: 919-28
- Qin LF, Lee TK, Ng IO (2002) Gene expression profiling by cDNA array in human hepatoma cell line in response to cisplatin treatment. *Life Sci* **70**: 1677-90
- Qin W, Yamashita T, Shirota Y, Lin Y, Wei W, Murakami S (2001) Mutational analysis of the structure and functions of hepatitis C virus RNA-dependent RNA polymerase. *Hepatology* **33**: 728-37
- Quintavalle M, Sambucini S, Summa V, Orsatti L, Talamo F, De Francesco R, Neddermann P (2007) Hepatitis C virus NS5A is a direct substrate of casein kinase I-alpha, a cellular kinase identified by inhibitor affinity chromatography using specific NS5A hyperphosphorylation inhibitors. *J Biol Chem* **282**: 5536-44
- Ralston R, Thudium K, Berger K, Kuo C, Gervase B, Hall J, Selby M, Kuo G, Houghton M, Choo QL (1993) Characterization of hepatitis C virus envelope glycoprotein complexes expressed by recombinant vaccinia viruses. *J Virol* **67**: 6753-61
- Randall G, Panis M, Cooper JD, Tellinghuisen TL, Sukhodolets KE, Pfeffer S, Landthaler M, Landgraf P, Kan S, Lindenbach BD, Chien M, Weir DB, Russo JJ, Ju J, Brownstein MJ, Sheridan R, Sander C, Zavolan M, Tuschl T, Rice CM (2007) Cellular cofactors affecting hepatitis C virus infection and replication. *Proc Natl Acad Sci U S A* **104**: 12884-9
- Redfield C (2004) NMR studies of partially folded molten-globule states. *Methods Mol Biol* **278**: 233-54
- Reed JC (1999) Mechanisms of apoptosis avoidance in cancer. *Curr Opin Oncol* **11**: 68-75

- Reed KE, Xu J, Rice CM (1997) Phosphorylation of the hepatitis C virus NS5A protein in vitro and in vivo: properties of the NS5A-associated kinase. *J Virol* **71**: 7187-97
- Saito I, Miyamura T (1990) [Hepatitis C virus]. *Tanpakushitsu Kakusan Koso* **35**: 2117-27
- Saragovi HU, Rebai N, Di Guglielmo GM, Macleod R, Sheng J, Rubin DH, Greene MI (1999) A G1 cell cycle arrest induced by ligands of the reovirus type 3 receptor is secondary to inactivation of p21ras and mitogen-activated protein kinase. *DNA Cell Biol* **18**: 763-70
- Sarcar B, Ghosh AK, Steele R, Ray R, Ray RB (2004) Hepatitis C virus NS5A mediated STAT3 activation requires co-operation of Jak1 kinase. *Virology* **322**: 51-60
- Satoh S, Hirota M, Noguchi T, Hijikata M, Handa H, Shimotohno K (2000) Cleavage of hepatitis C virus nonstructural protein 5A by a caspase-like protease(s) in mammalian cells. *Virology* **270**: 476-87
- Schiappa DA, Mittal C, Brown JA, Mika BP (2002) Relationship of hepatitis C genotype 1 NS5A sequence mutations to early phase viral kinetics and interferon effectiveness. *J Infect Dis* **185**: 868-77
- Schmidt-Mende J, Bieck E, Hugle T, Penin F, Rice CM, Blum HE, Moradpour D (2001) Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* **276**: 44052-63
- Schwalbe H, Fiebig KM, Buck M, Jones JA, Grimshaw SB, Spencer A, Glaser SJ, Smith LJ, Dobson CM (1997) Structural and dynamical properties of a denatured protein. Heteronuclear 3D NMR experiments and theoretical simulations of lysozyme in 8 M urea. *Biochemistry* **36**: 8977-91
- Seipp S, Mueller HM, Pfaff E, Stremmel W, Theilmann L, Goeser T (1997) Establishment of persistent hepatitis C virus infection and replication in vitro. *J Gen Virol* **78** (Pt 10): 2467-76

- Sen A, Steele R, Ghosh AK, Basu A, Ray R, Ray RB (2003) Inhibition of hepatitis C virus protein expression by RNA interference. *Virus Res* **96**: 27-35
- Seo MY, Abrignani S, Houghton M, Han JH (2003) Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7. *J Virol* **77**: 810-2
- Sharp PA (2001) RNA interference--2001. *Genes Dev* **15**: 485-90
- Shimakami T, Hijikata M, Luo H, Ma YY, Kaneko S, Shimotohno K, Murakami S (2004) Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. *J Virol* **78**: 2738-48
- Shiraki K, Nishikawa K, Goto Y (1995) Trifluoroethanol-induced stabilization of the alpha-helical structure of beta-lactoglobulin: implication for non-hierarchical protein folding. *J Mol Biol* **245**: 180-94
- Shirane M, Nakayama KI (2003) Inherent calcineurin inhibitor FKBP38 targets Bcl-2 to mitochondria and inhibits apoptosis. *Nat Cell Biol* **5**: 28-37
- Shirota Y, Luo H, Qin W, Kaneko S, Yamashita T, Kobayashi K, Murakami S (2002) Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J Biol Chem* **277**: 11149-55
- Shirota Y, Luo H, Qin W, Kaneko S, Yamashita T, Kobayashi K, Murakami S (2002) Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J Biol Chem* **277**: 11149-55
- Shojania S, O'Neil JD (2006) HIV-1 Tat is a natively unfolded protein: the solution conformation and dynamics of reduced HIV-1 Tat-(1-72) by NMR spectroscopy. *J Biol Chem* **281**: 8347-56

- Siavoshian S, Abraham JD, Thumann C, Kieny MP, Schuster C (2005) Hepatitis C virus core, NS3, NS5A, NS5B proteins induce apoptosis in mature dendritic cells. *J Med Virol* **75**: 402-11
- Sklan EH, Staschke K, Oakes TM, Elazar M, Winters M, Aroeti B, Danieli T, Glenn JS (2007) A Rab-GAP TBC domain protein binds hepatitis C virus NS5A and mediates viral replication. *J Virol* **81**: 11096-105
- Smet C, Duckert JF, Wieruszeski JM, Landrieu I, Buee L, Lippens G, Deprez B (2005) Control of protein-protein interactions: structure-based discovery of low molecular weight inhibitors of the interactions between Pin1 WW domain and phosphopeptides. *J Med Chem* **48**: 4815-23
- Sonnichsen FD, Van Eyk JE, Hodges RS, Sykes BD (1992) Effect of trifluoroethanol on protein secondary structure: an NMR and CD study using a synthetic actin peptide. *Biochemistry* **31**: 8790-8
- Spolar RS, Record MT, Jr. (1994) Coupling of local folding to site-specific binding of proteins to DNA. *Science* **263**: 777-84
- Srivastava SP, Kumar KU, Kaufman RJ (1998) Phosphorylation of eukaryotic translation initiation factor 2 mediates apoptosis in response to activation of the double-stranded RNA-dependent protein kinase. *J Biol Chem* **273**: 2416-23
- Stefano R, Martina, G., Francesca, D. P., Marco, M., Manuela, Z. & Alfredo, A. (2002) Localisation of PKR and HCV-NS5A in living cells using fusion proteins with different mutants of GFP. *J Hepatol* **36**: 4
- Street A, Macdonald A, Crowder K, Harris M (2004) The Hepatitis C virus NS5A protein activates a phosphoinositide 3-kinase-dependent survival signaling cascade. *J Biol Chem* **279**: 12232-41
- Street A, Macdonald A, Crowder K, Harris M (2004) The Hepatitis C virus NS5A protein activates a

- phosphoinositide 3-kinase-dependent survival signaling cascade. *J Biol Chem* **279**: 12232-41
- Street A, Macdonald A, McCormick C, Harris M (2005) Hepatitis C virus NS5A-mediated activation of phosphoinositide 3-kinase results in stabilization of cellular beta-catenin and stimulation of beta-catenin-responsive transcription. *J Virol* **79**: 5006-16
- Sun Y (1990) Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radic Biol Med* **8**: 583-99
- Tagawa M, Kato N, Yokosuka O, Ishikawa T, Ohto M, Omata M (1995) Infection of human hepatocyte cell lines with hepatitis C virus in vitro. *J Gastroenterol Hepatol* **10**: 523-7
- Tagawa S, Okamoto T, Abe T, Mori Y, Suzuki T, Moriishi K, Matsuura Y (2008) Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication. *J Virol* **82**: 2631-41
- Tai DI, Tsai SL, Chen YM, Chuang YL, Peng CY, Sheen IS, Yeh CT, Chang KS, Huang SN, Kuo GC, Liaw YF (2000) Activation of nuclear factor kappaB in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis. *Hepatology* **31**: 656-64
- Talarmin H, Rescan C, Cariou S, Glaize D, Zanninelli G, Bilodeau M, Loyer P, Guguen-Guillouzo C, Baffet G (1999) The mitogen-activated protein kinase kinase/extracellular signal-regulated kinase cascade activation is a key signalling pathway involved in the regulation of G(1) phase progression in proliferating hepatocytes. *Mol Cell Biol* **19**: 6003-11
- Tan SL, Katze MG (2001) How hepatitis C virus counteracts the interferon response: the jury is still out on NS5A. *Virology* **284**: 1-12
- Tan SL, Nakao H, He Y, Vijaysri S, Neddermann P, Jacobs BL, Mayer BJ, Katze MG (1999) NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor

protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling.

Proc Natl Acad Sci USA **96**: 5533-8

Tan SL, Nakao H, He Y, Vijaysri S, Neddermann P, Jacobs BL, Mayer BJ, Katze MG (1999) NS5A, a nonstructural protein of hepatitis C virus, binds growth factor receptor-bound protein 2 adaptor protein in a Src homology 3 domain/ligand-dependent manner and perturbs mitogenic signaling.

Proc Natl Acad Sci U S A **96**: 5533-8

Tanimoto A, Ide Y, Arima N, Sasaguri Y, Padmanabhan R (1997) The amino terminal deletion mutants of hepatitis C virus nonstructural protein NS5A function as transcriptional activators in yeast. *Biochem Biophys Res Commun* **236**: 360-4

Tanimoto A, Ide Y, Arima N, Sasaguri Y, Padmanabhan R (1997) The amino terminal deletion mutants of hepatitis C virus nonstructural protein NS5A function as transcriptional activators in yeast. *Biochem Biophys Res Commun* **236**: 360-4

Tanji Y, Hijikata M, Satoh S, Kaneko T, Shimotohno K (1995) Hepatitis C virus-encoded nonstructural protein NS4A has versatile functions in viral protein processing. *J Virol* **69**: 1575-81

Tanji Y, Kaneko T, Satoh S, Shimotohno K (1995) Phosphorylation of hepatitis C virus-encoded nonstructural protein NS5A. *J Virol* **69**: 3980-6

Tellinghuisen TL, Foss KL, Treadaway JC, Rice CM (2008) Identification of residues required for RNA replication in domains II and III of the hepatitis C virus NS5A protein. *J Virol* **82**: 1073-83

Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM (2004) The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J Biol Chem* **279**: 48576-87

Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM (2004) The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J Biol Chem* **279**: 48576-87

- Tellinghuisen TL, Marcotrigiano J, Rice CM (2005) Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* **435**: 374-9
- Thompson CB (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**: 1456-62
- Thormann T, Soroka V, Nielbo S, Berezin V, Bock E, Poulsen FM (2004) Backbone dynamics of the first, second, and third immunoglobulin modules of the neural cell adhesion molecule (NCAM). *Biochemistry* **43**: 10364-9
- Thornberry NA, Lazebnik Y (1998) Caspases: enemies within. *Science* **281**: 1312-6
- Tobias JW, Varshavsky A (1991) Cloning and functional analysis of the ubiquitin-specific protease gene UBP1 of *Saccharomyces cerevisiae*. *J Biol Chem* **266**: 12021-8
- Tokita H, Okamoto H, Iizuka H, Kishimoto J, Tsuda F, Lesmana LA, Miyakawa Y, Mayumi M (1996) Hepatitis C virus variants from Jakarta, Indonesia classifiable into novel genotypes in the second (2e and 2f), tenth (10a) and eleventh (11a) genetic groups. *J Gen Virol* **77** (Pt 2): 293-301
- Tomba P (2002) Intrinsically unstructured proteins. *Trends Biochem Sci* **27**: 527-33
- Tomba P (2002) Intrinsically unstructured proteins. *Trends Biochem Sci* **27**: 527-33
- Trepo C, Berthillon P, Vitvitski L (1998) HCV and lymphoproliferative diseases. *Ann Oncol* **9**: 469-70
- Tschopp J, Thome M, Hofmann K, Meink E (1998) The fight of viruses against apoptosis. *Curr Opin Genet Dev* **8**: 82-7
- Uesugi M, Nyanguile O, Lu H, Levine AJ, Verdine GL (1997) Induced alpha helix in the VP16 activation domain upon binding to a human TAF. *Science* **277**: 1310-3

- Ulrich DL, Kojetin D, Bassler BL, Cavanagh J, Loria JP (2005) Solution structure and dynamics of LuxU from *Vibrio harveyi*, a phosphotransferase protein involved in bacterial quorum sensing. *J Mol Biol* **347**: 297-307
- Uversky VN (2002) Natively unfolded proteins: a point where biology waits for physics. *Protein Sci* **11**: 739-56
- Uversky VN (2002) Natively unfolded proteins: a point where biology waits for physics. *Protein Sci* **11**: 739-56
- Uversky VN (2002) What does it mean to be natively unfolded? *Eur J Biochem* **269**: 2-12
- Vistica J, Dam J, Balbo A, Yikilmaz E, Mariuzza RA, Rouault TA, Schuck P (2004) Sedimentation equilibrium analysis of protein interactions with global implicit mass conservation constraints and systematic noise decomposition. *Anal Biochem* **326**: 234-56
- Vojnic E, Simon B, Strahl BD, Sattler M, Cramer P (2006) Structure and carboxyl-terminal domain (CTD) binding of the Set2 SRI domain that couples histone H3 Lys36 methylation to transcription. *J Biol Chem* **281**: 13-5
- Vuister GW BA (1993) Quantitative J correlation: A new approach for measuring homonuclear three-bond J (HNH α) coupling constants in ¹⁵N-enriched proteins. *J Am Chem Soc* **115**: 7772-7777
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Krausslich HG, Mizokami M, Bartenschlager R, Liang TJ (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* **11**: 791-6
- Wakita T, Wands JR (1994) Specific inhibition of hepatitis C virus expression by antisense oligodeoxynucleotides. In vitro model for selection of target sequence. *J Biol Chem* **269**: 14205-10

- Wang J, Tong W, Zhang X, Chen L, Yi Z, Pan T, Hu Y, Xiang L, Yuan Z (2006) Hepatitis C virus non-structural protein NS5A interacts with FKBP38 and inhibits apoptosis in Huh7 hepatoma cells. *FEBS Lett* **580**: 4392-400
- Wang X, Flynn A, Waskiewicz AJ, Webb BL, Vries RG, Baines IA, Cooper JA, Proud CG (1998) The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. *J Biol Chem* **273**: 9373-7
- Wang Y, Jardetzky O (2002) Probability-based protein secondary structure identification using combined NMR chemical-shift data. *Protein Sci* **11**: 852-61
- Waris G, Livolsi A, Imbert V, Peyron JF, Siddiqui A (2003) Hepatitis C virus NS5A and subgenomic replicon activate NF-kappaB via tyrosine phosphorylation of IkappaBalpha and its degradation by calpain protease. *J Biol Chem* **278**: 40778-87
- Waris G, Sarker S, Siddiqui A (2004) Two-step affinity purification of the hepatitis C virus ribonucleoprotein complex. *Rna* **10**: 321-9
- Wasley A, Alter MJ (2000) Epidemiology of hepatitis C: geographic differences and temporal trends. *Semin Liver Dis* **20**: 1-16
- Watashi K, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, Shimotohno K (2005) Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* **19**: 111-22
- Williams BR (2001) Signal integration via PKR. *Sci STKE* **2001**: RE2
- Wishart DS, Bigam CG, Holm A, Hodges RS, Sykes BD (1995) ¹H, ¹³C and ¹⁵N random coil NMR chemical shifts of the common amino acids. I. Investigations of nearest-neighbor effects. *J Biomol NMR* **5**: 67-81

- Wishart DS, Case DA (2001) Use of chemical shifts in macromolecular structure determination. *Methods Enzymol* **338**: 3-34
- Wishart DS, Nip AM (1998) Protein chemical shift analysis: a practical guide. *Biochem Cell Biol* **76**: 153-63
- Wishart DS, Sykes BD (1994) The ¹³C chemical-shift index: a simple method for the identification of protein secondary structure using ¹³C chemical-shift data. *J Biomol NMR* **4**: 171-80
- Wishart DS, Sykes BD (1994) Chemical shifts as a tool for structure determination. *Methods Enzymol* **239**: 363-92
- Wishart DS, Sykes BD, Richards FM (1991) Relationship between nuclear magnetic resonance chemical shift and protein secondary structure. *J Mol Biol* **222**: 311-33
- Wootton JC (1994) Non-globular domains in protein sequences: automated segmentation using complexity measures. *Comput Chem* **18**: 269-85
- Worbs M, Huber R, Wahl MC (2000) Crystal structure of ribosomal protein L4 shows RNA-binding sites for ribosome incorporation and feedback control of the S10 operon. *Embo J* **19**: 807-18
- World Health Organization (2004) Communicable Disease Surveillance & Response: Hepatitis C. (<http://www.who.int/csr/disease/hepatitis/whocdscsrlyo2003/en/>)
- Wright PE, Dyson HJ (1999) Intrinsically unstructured proteins: re-assessing the protein structure-function paradigm. *J Mol Biol* **293**: 321-31
- Yamashita T, Kaneko S, Shirota Y, Qin W, Nomura T, Kobayashi K, Murakami S (1998) RNA-dependent RNA polymerase activity of the soluble recombinant hepatitis C virus NS5B protein truncated at the C-terminal region. *J Biol Chem* **273**: 15479-86

- Yeh SR, Rousseau DL (1998) Folding intermediates in cytochrome c. *Nat Struct Biol* **5**: 222-8
- Ying L, Green JJ, Li H, Klenerman D, Balasubramanian S (2003) Studies on the structure and dynamics of the human telomeric G quadruplex by single-molecule fluorescence resonance energy transfer. *Proc Natl Acad Sci U S A* **100**: 14629-34
- Yoshikawa H, Matsubara K, Qian GS, Jackson P, Groopman JD, Manning JE, Harris CC, Herman JG (2001) SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nat Genet* **28**: 29-35
- Yun C, Lee JH, Park H, Jin YM, Park S, Park K, Cho H (2000) Chemotherapeutic drug, adriamycin, restores the function of p53 protein in hepatitis B virus X (HBx) protein-expressing liver cells. *Oncogene* **19**: 5163-72
- Zamore PD (2001) RNA interference: listening to the sound of silence. *Nat Struct Biol* **8**: 746-50
- Zeng Q, Tan YH, Hong W (1998) A single plasmid vector (pSTAR) mediating efficient tetracycline-induced gene expression. *Anal Biochem* **259**: 187-94
- Zhang O, Forman-Kay JD (1997) NMR studies of unfolded states of an SH3 domain in aqueous solution and denaturing conditions. *Biochemistry* **36**: 3959-70
- Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV (2005) Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci U S A* **102**: 9294-9

Appendix

Table A. 1 The assignment of NS5A-D2

Amino acid					Chemical Shift					Amino acid					Chemical shift					Amino acid					Chemical shift																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																															
2	ALA	HA	H	4.408	7	ASN	H	H	8.287	11	PRO	HD2	H	3.676	2	ALA	HB	H	1.350	7	ASN	HA	H	4.533	11	PRO	HD3	H	3.676	2	ALA	C	C	176.901	7	ASN	HB2	H	2.760	11	PRO	C	C	176.108	2	ALA	CA	C	52.253	7	ASN	HB3	H	2.660	11	PRO	CA	C	63.277	2	ALA	CB	C	18.699	7	ASN	HD21	H	7.432	11	PRO	CB	C	31.621	3	THR	H	H	8.250	7	ASN	HD22	H	6.777	11	PRO	CG	C	26.780	3	THR	HA	H	4.267	7	ASN	C	C	175.670	11	PRO	CD	C	50.223	3	THR	HB	H	4.078	7	ASN	CA	C	52.501	12	ASP	H	H	8.199	3	THR	HG2	H	1.113	7	ASN	CB	C	38.430	12	ASP	HA	H	4.462	3	THR	C	C	173.519	7	ASN	N	N	117.738	12	ASP	HB2	H	2.617	3	THR	CA	C	61.714	7	ASN	ND2	N	111.962	12	ASP	HB3	H	2.494	3	THR	CB	C	69.369	8	HIS	H	H	8.133	12	ASP	C	C	175.047	3	THR	CG2	C	20.964	8	HIS	HA	H	4.255	12	ASP	CA	C	53.955	3	THR	N	N	114.285	8	HIS	HB2	H	3.155	12	ASP	CB	C	40.555	4	CYS	H	H	8.289	8	HIS	HB3	H	3.064	12	ASP	N	N	119.465	4	CYS	HA	H	4.325	8	HIS	C	C	175.710	13	ALA	H	H	7.945	4	CYS	HB2	H	3.041	8	HIS	CA	C	56.459	13	ALA	HA	H	4.223	4	CYS	HB3	H	2.971	8	HIS	CB	C	29.454	13	ALA	HB	H	1.312	4	CYS	HG	H	2.533	8	HIS	N	N	121.852	13	ALA	C	C	177.014	4	CYS	C	C	176.019	9	ASP	H	H	8.274	13	ALA	CA	C	51.947	4	CYS	CA	C	55.525	9	ASP	HA	H	4.472	13	ALA	CB	C	19.145	4	CYS	CB	C	30.241	9	ASP	HB2	H	2.55	13	ALA	N	N	123.649	4	CYS	N	N	124.313	9	ASP	HB3	H	2.55	14	GLU	H	H	8.297	5	THR	H	H	8.008	9	ASP	C	C	175.239	14	GLU	HA	H	4.167	5	THR	HA	H	4.257	9	ASP	CA	C	53.977	14	GLU	HB2	H	2.105	5	THR	HB	H	4.109	9	ASP	CB	C	40.874	14	GLU	HB3	H	2.105	5	THR	HG2	H	1.128	9	ASP	N	N	121.258	14	GLU	HG2	H	2.200	5	THR	C	C	174.822	10	SER	H	H	8.110	14	GLU	HG3	H	2.200	5	THR	CA	C	61.870	10	SER	HA	H	4.676	14	GLU	C	C	175.925	5	THR	CB	C	68.961	10	SER	HB2	H	3.812	14	GLU	CA	C	56.329	5	THR	CG2	C	19.668	10	SER	HB3	H	3.769	14	GLU	CB	C	29.688	5	THR	N	N	114.816	10	SER	CA	C	55.661	14	GLU	CG	C	33.067	6	ALA	H	H	8.270	10	SER	CB	C	63.136	14	GLU	N	N	120.395	6	ALA	HA	H	4.109	10	SER	N	N	116.477	15	LEU	H	H	8.007	6	ALA	HB	H	1.273	11	PRO	HA	H	4.387	15	LEU	HA	H	4.280	6	ALA	C	C	176.529	11	PRO	HB2	H	2.219	15	LEU	HB2	H	1.503	6	ALA	CA	C	52.200	11	PRO	HB3	H	2.219	15	LEU	HB3	H	1.503	6	ALA	CB	C	18.729	11	PRO	HG2	H	1.916	15	LEU	HG	H	1.284	6	ALA	N	N	126.372	11	PRO	HG3	H	1.916	15	LEU	HD1	H	0.789

Appendix

Amino acid				Chemical shift				Amino acid				Chemical shift				Amino acid				Chemical shift				
15	LEU	HD2	H	0.789	19	ASN	HD21	H	7.423	22	TRP	NE1	N	120.262	23	ARG	H	H	7.738	23	ARG	HA	H	3.995
15	LEU	C	C	175.24	19	ASN	HD22	H	6.764	23	ARG	HA	H	3.995	23	ARG	HB2	H	1.940	23	ARG	HB3	H	1.940
15	LEU	CA	C	56.603	19	ASN	C	C	174.663	23	ARG	HB2	H	1.940	23	ARG	HG2	H	1.584	23	ARG	HG3	H	1.567
15	LEU	CB	C	38.559	19	ASN	CA	C	52.817	23	ARG	HG2	H	1.584	23	ARG	HD2	H	3.152	23	ARG	HD3	H	3.152
15	LEU	CG	C	26.793	19	ASN	CB	C	37.745	23	ARG	C	C	175.660	23	ARG	CA	C	53.858	23	ARG	CB	C	28.615
15	LEU	CD1	C	24.447	19	ASN	N	N	116.677	23	ARG	CG	C	25.889	23	ARG	CD	C	42.154	24	GLN	H	H	8.450
15	LEU	CD2	C	24.447	19	ASN	ND2	N	113.147	23	ARG	N	N	121.457	24	GLN	HA	H	4.155	24	GLN	HB2	H	1.942
15	LEU	N	N	115.252	20	LEU	H	H	8.009	24	GLN	HB3	H	1.942	24	GLN	HG2	H	2.254	24	GLN	HG3	H	2.254
16	ILE	H	H	8.117	20	LEU	HA	H	4.150	24	GLN	C	C	176.058	24	GLN	CA	C	56.397	25	GLU	H	H	8.381
16	ILE	HA	H	4.175	20	LEU	HB2	H	1.562	24	GLN	CA	C	56.397	25	GLU	CB	C	29.189	25	GLU	HA	H	4.153
16	ILE	HB	H	1.756	20	LEU	HB3	H	1.562	24	GLN	CG	C	35.856	25	GLU	HB2	H	2.023	25	GLU	HB3	H	2.023
16	ILE	HG12	H	1.382	20	LEU	HG	H	1.328	24	GLN	N	N	121.299	25	GLU	HG2	H	2.187	25	GLU	HG3	H	2.187
16	ILE	HG13	H	1.382	20	LEU	HD1	H	0.788	25	GLU	C	C	175.660	25	GLU	CA	C	56.291	26	MET	H	H	8.226
16	ILE	HG2	H	0.814	20	LEU	HD2	H	0.788	25	GLU	CA	C	56.291	26	MET	HA	H	4.381	26	MET	HB2	H	1.983
16	ILE	HD1	H	0.814	20	LEU	C	C	176.722	25	GLU	CB	C	29.592	26	MET	HB3	H	1.913	26	MET	HG2	H	2.410
16	ILE	C	C	175.801	20	LEU	CA	C	55.574	25	GLU	CG	C	35.848	26	MET	HG3	H	2.410	26	MET	HE	H	2.014
16	ILE	CA	C	61.558	20	LEU	CB	C	41.712															
16	ILE	CB	C	38.765	20	LEU	CG	C	26.400															
16	ILE	CG1	C	27.089	20	LEU	CD1	C	23.060															
16	ILE	CG2	C	17.120	20	LEU	CD2	C	23.060															
16	ILE	CD1	C	12.676	20	LEU	N	N	121.723															
16	ILE	N	N	121.162	21	LEU	H	H	8.029															
17	GLU	H	H	8.414	21	LEU	HA	H	4.280															
17	GLU	HA	H	4.106	21	LEU	HB2	H	1.526															
17	GLU	HB2	H	1.845	21	LEU	HB3	H	1.526															
17	GLU	HB3	H	1.845	21	LEU	HG	H	1.445															
17	GLU	HG2	H	2.187	21	LEU	HD1	H	0.836															
17	GLU	HG3	H	2.187	21	LEU	HD2	H	0.836															
17	GLU	C	C	175.848	21	LEU	C	C	176.376															
17	GLU	CA	C	56.585	21	LEU	CA	C	57.343															
17	GLU	CB	C	29.274	21	LEU	CB	C	39.104															
17	GLU	CG	C	35.950	21	LEU	CG	C	28.308															
17	GLU	N	N	124.512	21	LEU	CD1	C	26.525															
18	ALA	H	H	8.210	21	LEU	CD2	C	26.525															
18	ALA	HA	H	4.064	21	LEU	N	N	119.598															
18	ALA	HB	H	1.310	22	TRP	H	H	7.828															
18	ALA	C	C	177.122	22	TRP	HA	H	3.972															
18	ALA	CA	C	52.870	22	TRP	HB2	H	3.215															
18	ALA	CB	C	18.661	22	TRP	HB3	H	3.215															
18	ALA	N	N	124.51	22	TRP	HE1	H	10.213															
19	ASN	H	H	8.241	22	TRP	C	C	175.615															
19	ASN	HA	H	4.524	22	TRP	CA	C	55.188															
19	ASN	HB2	H	2.723	22	TRP	CB	C	29.820															
19	ASN	HB3	H	2.723	22	TRP	N	N	124.379															

Amino acid	Chemical shift	Amino acid	Chemical shift	Amino acid	Chemical shift
26 MET C	C 176.339	31 THR HA	H 4.261	35 SER H	H 8.263
26 MET CA	C 55.375	31 THR HB	H 4.140	35 SER HA	H 4.305
26 MET CB	C 32.034	31 THR HG2	H 1.251	35 SER HB2	H 3.799
26 MET CG	C 32.068	31 THR C	C 174.796	35 SER HB3	H 3.799
26 MET CE	C 20.138	31 THR CA	C 61.524	35 SER C	C 175.926
26 MET N	N 120.262	31 THR CB	C 69.380	35 SER CA	C 57.581
27 GLY H	H 8.240	31 THR CG2	C 19.668	35 SER CB	C 63.402
27 GLY HA2	H 3.888	31 THR N	N 118.801	35 SER N	N 116.420
27 GLY HA3	H 3.888	32 ARG H	H 8.289	36 GLU H	H 8.391
27 GLY C	C 174.033	32 ARG HA	H 4.325	36 GLU HA	H 4.220
27 GLY CA	C 45.025	32 ARG HB2	H 1.688	36 GLU HB2	H 1.942
27 GLY N	N 109.304	32 ARG HB3	H 1.688	36 GLU HB3	H 1.942
28 GLY H	H 8.174	32 ARG HG2	H 1.489	36 GLU HG2	H 2.177
28 GLY HA2	H 3.876	32 ARG HG3	H 1.489	36 GLU HG3	H 2.177
28 GLY HA3	H 3.876	32 ARG HD2	H 3.080	36 GLU C	C 175.395
28 GLY C	C 176.346	32 ARG HD3	H 3.080	36 GLU CA	C 56.472
28 GLY CA	C 44.859	32 ARG C	C 175.125	36 GLU CB	C 29.542
28 GLY N	N 108.175	32 ARG CA	C 55.525	36 GLU CG	C 35.849
29 ASN H	H 8.281	32 ARG CB	C 30.241	36 GLU N	N 122.254
29 ASN HA	H 4.552	32 ARG CG	C 26.580	37 ASN H	H 8.332
29 ASN HB2	H 2.726	32 ARG CD	C 42.967	37 ASN HA	H 4.409
29 ASN HB3	H 2.646	32 ARG N	N 124.313	37 ASN HB2	H 2.748
29 ASN HD21	H 7.519	33 VAL H	H 8.194	37 ASN HB3	H 2.748
29 ASN HD22	H 6.840	33 VAL HA	H 4.003	37 ASN HD21	H 7.532
29 ASN C	C 174.767	33 VAL HB	H 1.756	37 ASN HD22	H 6.855
29 ASN CA	C 52.747	33 VAL HG1	H 0.814	37 ASN C	C 175.262
29 ASN CB	C 38.371	33 VAL HG2	H 0.814	37 ASN CA	C 52.452
29 ASN N	N 118.535	33 VAL C	C 175.290	37 ASN CB	C 38.108
29 ASN ND2	N 112.883	33 VAL CA	C 61.936	37 ASN N	N 119.357
30 ILE H	H 8.034	33 VAL CB	C 32.265	37 ASN ND2	N 112.883
30 ILE HA	H 4.100	33 VAL CG1	C 20.194	38 LYS H	H 8.133
30 ILE HB	H 1.813	33 VAL CG2	C 20.194	38 LYS HA	H 4.249
30 ILE HG12	H 1.365	33 VAL N	N 122.124	38 LYS HB2	H 1.719
30 ILE HG13	H 1.365	34 GLU H	H 8.444	38 LYS HB3	H 1.719
30 ILE HG2	H 1.100	34 GLU HA	H 4.106	38 LYS HG2	H 1.308
30 ILE HD1	H 0.809	34 GLU HB2	H 2.191	38 LYS HG3	H 1.308
30 ILE C	C 175.786	34 GLU HB3	H 2.191	38 LYS HD2	H 1.627
30 ILE CA	C 60.589	34 GLU HG2	H 2.532	38 LYS HD3	H 1.627
30 ILE CB	C 38.165	34 GLU HG3	H 2.532	38 LYS HE2	H 2.907
30 ILE CG1	C 26.739	34 GLU C	C 175.992	38 LYS HE3	H 2.907
30 ILE CG2	C 16.979	34 GLU CA	C 55.897	38 LYS C	C 175.461
30 ILE CD1	C 12.372	34 GLU CB	C 29.433	38 LYS CA	C 55.750
30 ILE N	N 120.527	34 GLU CG	C 35.567	38 LYS CB	C 32.377
31 THR H	H 8.220	34 GLU N	N 124.512	38 LYS CG	C 23.856

Amino acid	Chemical shift	Amino acid	Chemical shift	Amino acid	Chemical shift
38 LYS CD	C 28.225	42 LEU HD1	H 0.808	47 PRO HG3	H 1.878
38 LYS CE	C 42.404	42 LEU HD2	H 0.808	47 PRO HD2	H 3.441
38 LYS N	N 121.855	42 LEU C	C 176.325	47 PRO HD3	H 3.441
39 VAL H	H 8.089	42 LEU CA	C 55.592	47 PRO C	C 176.324
39 VAL HA	H 4.024	42 LEU CB	C 41.520	47 PRO CA	C 63.052
39 VAL HB	H 1.730	42 LEU CG	C 26.482	47 PRO CB	C 31.719
39 VAL HG1	H 0.813	42 LEU CD1	C 22.738	47 PRO CG	C 26.497
39 VAL HG2	H 0.813	42 LEU CD2	C 22.738	47 PRO CD	C 53.008
39 VAL C	C 175.249	42 LEU N	N 127.434	48 LEU H	H 8.256
39 VAL CA	C 61.840	43 ASP H	H 8.241	48 LEU HA	H 4.169
39 VAL CB	C 32.002	43 ASP HA	H 4.441	48 LEU HB2	H 2.005
39 VAL CG1	C 20.070	43 ASP HB2	H 2.532	48 LEU HB3	H 2.005
39 VAL CG2	C 20.070	43 ASP HB3	H 2.532	48 LEU HG	H 1.533
39 VAL N	N 122.121	43 ASP C	C 175.344	48 LEU HD1	H 0.814
40 VAL H	H 8.219	43 ASP CA	C 53.862	48 LEU HD2	H 0.814
40 VAL HA	H 4.018	43 ASP CB	C 40.564	48 LEU C	C 176.754
40 VAL HB	H 1.904	43 ASP N	N 121.536	48 LEU CA	C 55.363
40 VAL HG1	H 0.787	44 SER H	H 8.025	48 LEU CB	C 41.138
40 VAL HG2	H 0.787	44 SER HA	H 4.280	48 LEU CG	C 26.264
40 VAL C	C 176.149	44 SER HB2	H 3.691	48 LEU CD1	C 23.972
40 VAL CA	C 62.379	44 SER HB3	H 3.691	48 LEU CD2	C 23.972
40 VAL CB	C 30.294	44 SER C	C 172.368	48 LEU N	N 120.727
40 VAL CG1	C 19.436	44 SER CA	C 57.581	49 VAL H	H 7.963
40 VAL CG2	C 19.436	44 SER CB	C 63.402	49 VAL HA	H 3.944
40 VAL N	N 125.575	44 SER N	N 115.215	49 VAL HB	H 1.990
41 ILE H	H 8.274	45 PHE H	H 8.131	49 VAL HG1	H 0.843
41 ILE HA	H 4.068	45 PHE HA	H 4.037	49 VAL HG2	H 0.843
41 ILE HB	H 1.773	45 PHE HB2	H 2.817	49 VAL C	C 174.979
41 ILE HG12	H 1.378	45 PHE HB3	H 2.817	49 VAL CA	C 61.803
41 ILE HG13	H 1.126	45 PHE C	C 175.339	49 VAL CB	C 32.298
41 ILE HG2	H 0.808	45 PHE CA	C 60.843	49 VAL CG1	C 19.618
41 ILE HD1	H 0.808	45 PHE CB	C 37.527	49 VAL CG2	C 19.618
41 ILE C	C 176.779	45 PHE N	N 122.050	49 VAL N	N 120.992
41 ILE CA	C 61.030	46 ASP H	H 8.097	50 ALA H	H 8.164
41 ILE CB	C 38.285	46 ASP HA	H 4.792	50 ALA HA	H 4.253
41 ILE CG1	C 27.077	46 ASP HB2	H 2.612	50 ALA HB	H 1.286
41 ILE CG2	C 16.495	46 ASP HB3	H 2.612	50 ALA C	C 176.779
41 ILE CD1	C 11.757	46 ASP CA	C 54.183	50 ALA CA	C 51.994
41 ILE N	N 126.370	46 ASP CB	C 42.099	50 ALA CB	C 18.656
42 LEU H	H 8.329	46 ASP N	N 123.779	50 ALA N	N 127.442
42 LEU HA	H 4.307	47 PRO HA	H 4.255	51 GLU H	H 8.060
42 LEU HB2	H 1.531	47 PRO HB2	H 2.476	51 GLU HA	H 4.280
42 LEU HB3	H 1.531	47 PRO HB3	H 2.476	51 GLU HB2	H 2.114
42 LEU HG	H 1.316	47 PRO HG2	H 1.878	51 GLU HB3	H 2.114

Amino acid	Chemical shift	Amino acid	Chemical shift	Amino acid	Chemical shift
51 GLU HG2	H 2.186	55 ARG HD3	H 3.019	59 VAL CG1	C 20.332
51 GLU HG3	H 2.186	55 ARG C	C 175.786	59 VAL CG2	C 20.332
51 GLU C	C 175.726	55 ARG CA	C 56.065	59 VAL N	N 123.051
51 GLU CA	C 55.689	55 ARG CB	C 30.319	60 PRO HA	H 4.258
51 GLU CB	C 30.200	55 ARG CG	C 26.473	60 PRO HB2	H 2.286
51 GLU CG	C 35.772	55 ARG CD	C 42.691	60 PRO HB3	H 2.286
51 GLU N	N 119.598	55 ARG N	N 121.457	60 PRO HG2	H 1.940
52 GLU H	H 8.274	56 GLU H	H 8.107	60 PRO HG3	H 1.849
52 GLU HA	H 4.196	56 GLU HA	H 4.258	60 PRO HD2	H 3.695
52 GLU HB2	H 2.106	56 GLU HB2	H 2.192	60 PRO HD3	H 3.586
52 GLU HB3	H 2.106	56 GLU HB3	H 2.192	60 PRO C	C 176.703
52 GLU HG2	H 2.193	56 GLU HG2	H 2.253	60 PRO CA	C 63.585
52 GLU HG3	H 2.193	56 GLU HG3	H 2.253	60 PRO CB	C 31.714
52 GLU C	C 177.108	56 GLU C	C 175.789	60 PRO CG	C 26.743
52 GLU CA	C 57.017	56 GLU CA	C 55.727	60 PRO CD	C 51.152
52 GLU CB	C 29.899	56 GLU CB	C 29.342	61 ALA H	H 8.367
52 GLU CG	C 35.567	56 GLU CG	C 35.567	61 ALA HA	H 4.055
52 GLU N	N 125.508	56 GLU N	N 120.527	61 ALA HB	H 1.370
53 ASP H	H 8.239	57 VAL H	H 8.036	61 ALA C	C 178.332
53 ASP HA	H 4.526	57 VAL HA	H 4.085	61 ALA CA	C 53.893
53 ASP HB2	H 2.748	57 VAL HB	H 2.177	61 ALA CB	C 18.480
53 ASP HB3	H 2.659	57 VAL HG1	H 0.871	61 ALA N	N 123.649
53 ASP C	C 175.561	57 VAL HG2	H 0.871	62 GLU H	H 8.486
53 ASP CA	C 53.115	57 VAL C	C 175.527	62 GLU HA	H 4.080
53 ASP CB	C 38.021	57 VAL CA	C 61.512	62 GLU HB2	H 2.172
53 ASP N	N 116.543	57 VAL CB	C 32.150	62 GLU HB3	H 2.172
54 GLU H	H 8.134	57 VAL CG1	C 21.728	62 GLU HG2	H 2.183
54 GLU HA	H 4.051	57 VAL CG2	C 21.728	62 GLU HG3	H 2.183
54 GLU HB2	H 1.964	57 VAL N	N 120.527	62 GLU C	C 176.390
54 GLU HB3	H 1.964	58 SER H	H 8.314	62 GLU CA	C 57.378
54 GLU HG2	H 2.376	58 SER HA	H 4.430	62 GLU CB	C 29.160
54 GLU HG3	H 2.376	58 SER HB2	H 3.760	62 GLU CG	C 35.859
54 GLU C	C 175.745	58 SER HB3	H 3.760	62 GLU N	N 118.004
54 GLU CA	C 56.900	58 SER C	C 173.536	63 ILE H	H 8.179
54 GLU CB	C 29.119	58 SER CA	C 57.658	63 ILE HA	H 4.181
54 GLU CG	C 36.331	58 SER CB	C 63.137	63 ILE HB	H 1.815
54 GLU N	N 121.921	58 SER N	N 119.332	63 ILE HG12	H 1.284
55 ARG H	H 7.738	59 VAL H	H 8.053	63 ILE HG13	H 1.263
55 ARG HA	H 3.997	59 VAL HA	H 4.329	63 ILE HG2	H 0.808
55 ARG HB2	H 1.660	59 VAL HB	H 1.983	63 ILE HD1	H 0.761
55 ARG HB3	H 1.527	59 VAL HG1	H 0.892	63 ILE C	C 177.520
55 ARG HG2	H 1.324	59 VAL HG2	H 0.850	63 ILE CA	C 61.177
55 ARG HG3	H 1.324	59 VAL CA	C 60.043	63 ILE CB	C 38.257
55 ARG HD2	H 3.019	59 VAL CB	C 32.153	63 ILE CG1	C 26.502

Amino acid	Chemical shift	Amino acid	Chemical shift	Amino acid	Chemical shift
63 ILE CG2 C 17.083		66 LYS CG C 23.960		70 PHE C C 174.875	
63 ILE CD1 C 12.125		66 LYS CD C 28.321		70 PHE CA C 57.188	
63 ILE N N 120.793		66 LYS CE C 42.625		70 PHE CB C 39.105	
64 LEU H H 8.033		66 LYS N N 120.129		70 PHE N N 119.664	
64 LEU HA H 4.255		67 SER H H 8.136		71 ALA H H 8.058	
64 LEU HB2 H 1.624		67 SER HA H 4.495		71 ALA HA H 4.202	
64 LEU HB3 H 1.515		67 SER HB2 H 3.818		71 ALA HB H 1.278	
64 LEU HG H 1.499		67 SER HB3 H 3.818		71 ALA C C 175.701	
64 LEU HD1 H 0.810		67 SER C C 172.926		71 ALA CA C 52.212	
64 LEU HD2 H 0.810		67 SER CA C 57.633		71 ALA CB C 18.912	
64 LEU C C 176.789		67 SER CB C 63.365		71 ALA N N 124.778	
64 LEU CA C 55.375		67 SER N N 121.856		72 ARG H H 7.876	
64 LEU CB C 40.760		68 ARG H H 7.890		72 ARG HA H 4.262	
64 LEU CG C 26.385		68 ARG HA H 4.054		72 ARG HB2 H 1.626	
64 LEU CD1 C 23.452		68 ARG HB2 H 1.968		72 ARG HB3 H 1.626	
64 LEU CD2 C 23.452		68 ARG HB3 H 1.968		72 ARG HG2 H 1.505	
64 LEU N N 119.798		68 ARG HG2 H 1.785		72 ARG HG3 H 1.505	
65 ARG H H 7.828		68 ARG HG3 H 1.785		72 ARG HD2 H 2.835	
65 ARG HA H 4.496		68 ARG HD2 H 3.048		72 ARG HD3 H 2.835	
65 ARG HB2 H 1.569		68 ARG HD3 H 3.048		72 ARG C C 174.962	
65 ARG HB3 H 1.569		68 ARG C C 176.064		72 ARG CA C 53.954	
65 ARG HG2 H 1.345		68 ARG CA C 57.245		72 ARG CB C 30.464	
65 ARG HG3 H 1.345		68 ARG CB C 30.134		72 ARG CG C 26.487	
65 ARG HD2 H 3.005		68 ARG CG C 28.998		72 ARG CD C 41.363	
65 ARG HD3 H 3.005		68 ARG CD C 43.883		72 ARG N N 118.668	
65 ARG C C 176.555		68 ARG N N 127.434		73 ALA H H 7.684	
65 ARG CA C 57.318		69 ARG H H 8.382		73 ALA HA H 3.978	
65 ARG CB C 28.781		69 ARG HA H 4.148		73 ALA HB H 1.253	
65 ARG CG C 26.788		69 ARG HB2 H 1.562		73 ALA C C 176.572	
65 ARG CD C 42.734		69 ARG HB3 H 1.562		73 ALA CA C 51.228	
65 ARG N N 120.262		69 ARG HG2 H 1.300		73 ALA CB C 18.854	
66 LYS H H 7.968		69 ARG HG3 H 1.300		73 ALA N N 119.996	
66 LYS HA H 4.337		69 ARG HD2 H 3.007		74 LEU H H 8.002	
66 LYS HB2 H 1.718		69 ARG HD3 H 3.007		74 LEU HA H 4.383	
66 LYS HB3 H 1.718		69 ARG C C 177.622		74 LEU HB2 H 1.507	
66 LYS HG2 H 1.307		69 ARG CA C 56.282		74 LEU HB3 H 1.507	
66 LYS HG3 H 1.307		69 ARG CB C 29.482		74 LEU HG H 1.400	
66 LYS HD2 H 1.626		69 ARG CG C 26.462		74 LEU HD1 H 0.734	
66 LYS HD3 H 1.626		69 ARG CD C 42.671		74 LEU HD2 H 0.734	
66 LYS HE2 H 2.901		69 ARG N N 121.192		74 LEU CA C 52.149	
66 LYS HE3 H 2.901		70 PHE H H 8.028		74 LEU CB C 41.625	
66 LYS C C 174.067		70 PHE HA H 4.530		74 LEU CG C 26.545	
66 LYS CA C 55.995		70 PHE HB2 H 3.110		74 LEU CD1 C 23.776	
66 LYS CB C 32.342		70 PHE HB3 H 2.888		74 LEU CD2 C 23.776	

Amino acid	Chemical shift	Amino acid	Chemical shift	Amino acid	Chemical shift
74 LEU N	N 122.719	79 ARG HB3	H 1.780	83 ASN CA	C 50.711
75 PRO HA	H 4.405	79 ARG HG2	H 1.584	83 ASN CB	C 38.339
75 PRO HB2	H 2.218	79 ARG HG3	H 1.584	83 ASN N	N 123.782
75 PRO HB3	H 2.218	79 ARG HD2	H 2.887	85 PRO HA	H 4.190
75 PRO HG2	H 1.911	79 ARG HD3	H 2.887	85 PRO HB2	H 2.188
75 PRO HG3	H 1.911	79 ARG CA	C 54.016	85 PRO HB3	H 2.188
75 PRO HD2	H 3.688	79 ARG CB	C 29.073	85 PRO HG2	H 1.765
75 PRO HD3	H 3.688	79 ARG CG	C 28.756	85 PRO HG3	H 1.565
75 PRO C	C 176.839	79 ARG CD	C 41.815	85 PRO HD2	H 3.651
75 PRO CA	C 62.287	79 ARG N	N 121.657	85 PRO HD3	H 3.651
75 PRO CB	C 31.378	80 PRO HA	H 4.287	85 PRO C	C 176.412
75 PRO CG	C 26.605	80 PRO HB2	H 2.161	85 PRO CA	C 63.081
75 PRO CD	C 50.171	80 PRO HB3	H 2.161	85 PRO CB	C 31.585
76 VAL H	H 8.112	80 PRO HG2	H 1.919	85 PRO CG	C 26.461
76 VAL HA	H 3.681	80 PRO HG3	H 1.919	85 PRO CD	C 50.456
76 VAL HB	H 1.919	80 PRO HD2	H 3.847	86 LEU H	H 8.257
76 VAL HG1	H 0.842	80 PRO HD3	H 3.847	86 LEU HA	H 4.182
76 VAL HG2	H 0.842	80 PRO C	C 175.733	86 LEU HB2	H 1.625
76 VAL C	C 175.414	80 PRO CA	C 63.439	86 LEU HB3	H 1.504
76 VAL CA	C 63.346	80 PRO CB	C 31.152	86 LEU HG	H 1.504
76 VAL CB	C 31.033	80 PRO CG	C 26.592	86 LEU HD1	H 0.812
76 VAL CG1	C 19.564	80 PRO CD	C 51.193	86 LEU HD2	H 0.812
76 VAL CG2	C 19.564	81 ASP H	H 8.242	86 LEU C	C 177.032
76 VAL N	N 119.199	81 ASP HA	H 4.422	86 LEU CA	C 55.100
77 TRP H	H 7.135	81 ASP HB2	H 2.574	86 LEU CB	C 41.133
77 TRP HA	H 4.237	81 ASP HB3	H 2.574	86 LEU CG	C 26.489
77 TRP HB2	H 3.209	81 ASP C	C 175.124	86 LEU CD1	C 23.671
77 TRP HB3	H 3.048	81 ASP CA	C 53.356	86 LEU CD2	C 23.671
77 TRP HD1	H 10.105	81 ASP CB	C 39.875	86 LEU N	N 120.660
77 TRP C	C 175.147	81 ASP N	N 117.738	87 VAL H	H 7.702
77 TRP CA	C 55.771	82 TYR H	H 7.744	87 VAL HA	H 3.978
77 TRP CB	C 28.399	82 TYR HA	H 4.487	87 VAL HB	H 1.971
77 TRP N	N 118.602	82 TYR HB2	H 2.969	87 VAL HG1	H 0.848
77 TRP NE1	N 129.592	82 TYR HB3	H 2.969	87 VAL HG2	H 0.848
78 ALA H	H 7.498	82 TYR C	C 173.902	87 VAL C	C 175.671
78 ALA HA	H 4.244	82 TYR CA	C 57.557	87 VAL CA	C 61.965
78 ALA HB	H 1.307	82 TYR CB	C 38.593	87 VAL CB	C 32.217
78 ALA C	C 176.482	82 TYR N	N 120.527	87 VAL CG1	C 19.888
78 ALA CA	C 51.171	83 ASN H	H 8.105	87 VAL CG2	C 19.888
78 ALA CB	C 18.843	83 ASN HA	H 4.796	87 VAL N	N 120.063
78 ALA N	N 124.645	83 ASN HB2	H 2.579	88 GLU H	H 8.561
79 ARG H	H 8.241	83 ASN HB3	H 2.579	88 GLU HA	H 4.225
79 ARG HA	H 4.477	83 ASN HD21	H 7.423	88 GLU HB2	H 2.098
79 ARG HB2	H 1.788	83 ASN HD22	H 6.764	88 GLU HB3	H 2.098

Amino acid				Chemical shift				Amino acid				Chemical shift				Amino acid				Chemical shift			
88	GLU	HG2	H	2.532	91	LYS	HD2	H	1.438	93	PRO	HD2	H	3.537	93	PRO	HD3	H	3.537				
88	GLU	HG3	H	2.532	91	LYS	HD3	H	1.438	93	PRO	HD3	H	3.537	93	PRO	HD3	H	3.537				
88	GLU	C	C	175.776	91	LYS	HE2	H	2.809	93	PRO	C	C	175.822	93	PRO	C	C	175.822				
88	GLU	CA	C	56.231	91	LYS	HE3	H	2.809	93	PRO	CA	C	62.921	93	PRO	CA	C	62.921				
88	GLU	CB	C	29.557	91	LYS	C	C	174.798	93	PRO	CB	C	31.681	93	PRO	CB	C	31.681				
88	GLU	CG	C	35.830	91	LYS	CA	C	55.389	93	PRO	CG	C	26.818	93	PRO	CG	C	26.818				
88	GLU	N	N	124.778	91	LYS	CB	C	32.395	94	ASP	H	H	8.248	94	ASP	H	H	8.248				
89	THR	H	H	7.948	91	LYS	CG	C	24.002	94	ASP	HA	H	4.400	94	ASP	HA	H	4.400				
89	THR	HA	H	4.208	91	LYS	CD	C	28.664	94	ASP	HB2	H	2.533	94	ASP	HB2	H	2.533				
89	THR	HB	H	4.100	91	LYS	CE	C	41.659	94	ASP	HB3	H	2.533	94	ASP	HB3	H	2.533				
89	THR	HG2	H	1.067	91	LYS	N	N	123.250	94	ASP	C	C	175.560	94	ASP	C	C	175.560				
89	THR	C	C	173.643	92	LYS	H	H	8.035	94	ASP	CA	C	53.842	94	ASP	CA	C	53.842				
89	THR	CA	C	61.707	92	LYS	HA	H	4.213	94	ASP	CB	C	40.299	94	ASP	CB	C	40.299				
89	THR	CB	C	69.105	92	LYS	HB2	H	1.768	94	ASP	N	N	119.996	94	ASP	N	N	119.996				
89	THR	CG2	C	20.852	92	LYS	HB3	H	1.699	95	TYR	H	H	7.855	95	TYR	H	H	7.855				
89	THR	N	N	114.949	92	LYS	HG2	H	1.518	95	TYR	HA	H	4.378	95	TYR	HA	H	4.378				
90	TRP	H	H	7.847	92	LYS	HG3	H	1.518	95	TYR	HB2	H	2.748	95	TYR	HB2	H	2.748				
90	TRP	HA	H	4.492	92	LYS	HD2	H	1.757	95	TYR	HB3	H	2.659	95	TYR	HB3	H	2.659				
90	TRP	HB2	H	3.186	92	LYS	HD3	H	1.757	95	TYR	C	C	175.644	95	TYR	C	C	175.644				
90	TRP	HB3	H	3.186	92	LYS	HE2	H	3.076	95	TYR	CA	C	57.559	95	TYR	CA	C	57.559				
90	TRP	HD1	H	10.073	92	LYS	HE3	H	3.004	95	TYR	CB	C	38.269	95	TYR	CB	C	38.269				
90	TRP	C	C	176.036	92	LYS	CA	C	55.484	95	TYR	N	N	119.664	95	TYR	N	N	119.664				
90	TRP	CA	C	57.051	92	LYS	CB	C	29.868	96	GLU	H	H	8.148	96	GLU	H	H	8.148				
90	TRP	CB	C	28.841	92	LYS	CG	C	26.872	96	GLU	HA	H	4.037	96	GLU	HA	H	4.037				
90	TRP	N	N	122.121	92	LYS	CD	C	29.710	96	GLU	HB2	H	1.819	96	GLU	HB2	H	1.819				
90	TRP	NE1	N	129.367	92	LYS	CE	C	42.855	96	GLU	HB3	H	1.811	96	GLU	HB3	H	1.811				
91	LYS	H	H	7.676	92	LYS	N	N	124.114	96	GLU	HG2	H	2.182	96	GLU	HG2	H	2.182				
91	LYS	HA	H	4.084	93	PRO	HA	H	4.191	96	GLU	HG3	H	2.181	96	GLU	HG3	H	2.181				
91	LYS	HB2	H	1.544	93	PRO	HB2	H	2.189	96	GLU	CA	C	55.958	96	GLU	CA	C	55.958				
91	LYS	HB3	H	1.544	93	PRO	HB3	H	2.189	96	GLU	CB	C	29.503	96	GLU	CB	C	29.503				
91	LYS	HG2	H	1.036	93	PRO	HG2	H	1.881	96	GLU	CG	C	35.653	96	GLU	CG	C	35.653				
91	LYS	HG3	H	1.036	93	PRO	HG3	H	1.881	96	GLU	N	N	121.709	96	GLU	N	N	121.709				