A structural and functional study on Cot (MAPKKK8)

Wu, Binhui

2008

Wu, B. (2008). A structural and functional study on Cot (MAPKKK8). Doctoral thesis,
Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/13576>

<https://doi.org/10.32657/10356/13576>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

A Structural and Functional Study on Cot (MAPKKK8)

WU BINHUI

SCHOOL OF BIOLOGICAL SCIENCES

2008

A Structural and Functional Study on Cot (MAPKKK8)

WU BINHUI

School of Biological Sciences

A thesis submitted to the Nanyang Technological University
in partial fulfillment of the requirement for the degree of
Doctor of Philosophy

2008

Table of Contents

Acknowledgements	7
Abbreviations	9
Summary	17
Chapter 1 Introduction	20
1.1 Signal transduction in mammalian cells	20
1.2 Control of gene expression in mammalian cells by the Rel/NF- κ B pathway	21
1.3 Overview of mammalian MAPK signal transduction pathways	24
1.4 MAPKKK family	27
1.5 Raf kinase	29
1.6 Identification of Cot in humans and rats	30
1.7 Expression of Cot in different tissues	32
1.8 Cot is a member of MAPKKK family	34
1.9 Role of Cot in T cells	36
1.10 Function of Cot	40
1.11 Cot and extracellular signals	45
1.12 Cot and NF- κ B	52
1.13 Inhibitors of Cot	59
Chapter 2 Aims of This Study	60
Chapter 3 Materials and Methods	61
3.1 Molecular cloning and expression	61

3.1.1 Molecular cloning and expression of 6×His-tagged Cot in <i>E. coli</i>	61
3.1.2 Expression of Cot in BL21-CodonPlus (DE3)-RIPL	64
3.1.3 Expression of GST-tagged Cot in <i>E. coli</i> BL21(DE3).....	64
3.1.4 Expression of selenomethionine-substituted protein.....	66
3.1.5 Molecular cloning of Cot for expression in insect cells.....	67
3.1.6 Culture of insect cells <i>Spodoptera frugiperda</i> sf9 and transfection	68
3.1.7 Molecular cloning of plasmids for expression in mammalian cells.....	69
3.1.8 Mammalian cell culture	70
3.1.9 Transfection and expression of Cot in HEK293 cells	71
3.1.10 Co-transfection and expression of Cot with Plk1	71
3.2 Purification.....	72
3.2.1 Purification of 6×His-tagged protein.....	72
3.2.2 Purification of 6×His-tagged protein under denaturing conditions	72
3.2.3 Purification of GST (glutathione S-transferase)-tagged protein	73
3.2.4 Cleavage of GST tag	73
3.2.5 Gel filtration chromatography	74
3.2.6 Anion exchange chromatography	74
3.2.7 Removal of contaminating <i>E. coli</i> chaperone	75
3.2.8 Alternative method for the purification of GST-tagged protein	75
3.2.9 Protein secondary structure measurement.....	76
3.3 Crystallization.....	77
3.3.1 Crystallization screen	77

3.3.2 Optimization of the crystallization screen	78
3.3.3 X-ray diffraction.....	78
3.4 2D proteomics	78
3.4.1 2D electrophoresis	78
3.4.2 Silver staining	79
3.4.3 Protein identification by mass spectrometry	80
3.5 Functional experiments	81
3.5.1 Immunoprecipitation	81
3.5.2 Western blotting	82
3.5.3 Kinase assay in vitro	83
3.5.4 Kinase assay in vivo	84
3.5.5 Sub-cellular localization of Cot in mammalian cells.....	85
3.5.6 Peptide library screen for Cot substrates	86
3.5.7 Phospho-peptide isolation and analysis by mass spectrometry.....	87
3.5.8 RNA interference	89
3.6 Computational analysis	89
3.6.1 Cot kinase domain structure predication	89
3.6.2 Proteins interaction modeling	91
3.6.3 Peptide 3D structure prediction and solvent accessible surface calculation	91
Chapter 4 Expression of Cot in <i>E. coli</i> and Insect Cells.....	93
4.1 Expression of Cot in <i>E. coli</i>	93
4.2 Expression in <i>E. coli</i> of different Cot constructs	94

4.3 Correction of <i>E. coli</i> codon bias	97
4.4 Expression of Cot in insect cells	98
4.5 Co-expression of NF- κ B and Cot in insect cells.....	102
4.6 Discussion	102
Chapter 5 Purification and Crystallization of Cot	106
5.1 Purification and crystallization of 6 $\times$ His-tagged Cot ₁₋₃₈₈	106
5.1.1 <i>Purification of Cot₁₋₃₈₈ under denaturing condition</i>	106
5.1.2 <i>Secondary structure of refolded protein Cot₁₋₃₈₈</i>	108
5.1.3 <i>Kinase assay of Cot₁₋₃₈₈</i>	109
5.1.4 <i>Screening for crystals of Cot₁₋₃₈₈</i>	111
5.2 Purification and crystallization of untagged Cot ₁₋₃₈₈	113
5.3 Purification and crystallization of GST-tagged GST-Cot ₁₋₃₈₈	118
5.3.1 <i>Purification and crystallization of GST-tagged Cot₁₋₃₈₈</i>	118
5.3.2 <i>Purification of selenomethionine-substituted GST-Cot₁₋₃₈₈</i>	123
5.3.3 <i>Removal of chaperone DnaK protein</i>	128
5.3.4 <i>Crystallization of selenomethionine-substituted GST-Cot₁₋₃₈₈</i>	129
5.3.5 <i>Alternative method to remove the chaperone during purification</i>	130
5.4 Purification of the Cot kinase domain	134
5.5 Purification of Cot ₁₋₁₃₇	137
5.6 Discussion	140
Chapter 6 Expression of Cot in Mammalian Cells	146
6.1 Expression of Cot in mammalian cells	146

6.2 Sub-cellular localization of Cot in mammalian cells	148
6.3 Expression of Cot constructs	149
6.4 Immunoprecipitation.....	152
6.5 Kinase assays <i>in vitro</i> and <i>in vivo</i>	154
6.6 Discussion	157
Chapter 7 Proteomics Analysis of Proteins Associated with Cot	160
7.1 2D gel electrophoresis.....	160
7.2 Interaction between Cot and Hsp90	164
7.3 Discussion	170
Chapter 8 Substrate Specificity of Cot Protein	175
8.1 Peptide library screening.....	175
8.2 Kinase assay (protein-protein) <i>in vitro</i> of Plk1	178
8.3 Kinase assay (peptide-protein) <i>in vitro</i> of Plk1	183
8.4 Peptide structure prediction and analysis.....	195
8.4.1 Cluster analysis results	195
8.4.2 Solvent accessible surface of the hydroxy group on Ser/Thr	196
8.5 <i>In vivo</i> phosphorylation of Plk1	198
8.6 Interaction between Cot and Plk1	200
8.7 RNA interference	201
8.8 Cot kinase domain structure predication and interaction modeling with Plk1	203
8.9 Discussion	207
Appendices	214

References..... 232

Publications..... 258

Acknowledgements

I am deeply indebted to my supervisor Prof. Rupert Charles Wilmouth whose abundant help, stimulating suggestions, scientific guidance, patience and encouragement helped me in all the time of research experimental works and writing of this thesis. This thesis would not appear in its present form without the kind assistance and support from Prof. Wilmouth.

Special thanks Prof. Alex Law, to act as my supervisor, after Prof. Wilmouth had left Singapore. I am very grateful for his guidance during my Ph.D. study and thesis amendment.

I would like to express my gratitude to those professors who gave me help during the 4-year study in the School of Biological Sciences, NTU. I would like to thank Prof. Yuguang Mu for his contribution in peptide tertiary structure prediction. I would like to thank Prof. Joe Yoon for his useful suggestions and help in the expression and purification of the kinase. I appreciate the help in the mass spectrometry analysis from Prof. Kai Tang and Prof. Newman Sze. I would like to thank Prof. Peter Cheung for the help in the kinase assay experiments and Prof. Kenneth Yu for the training of the confocal microscope. I also thank Prof. Alex Gong, for his kind encouragement and suggestions in this study.

Many thanks should go to Dr. Kelly Ou and Miss Suet Ying from Agenica Research Pte Ltd for their generous training in MS sample preparation. I thank my friends, Dr. Biukovic Goran, Low Hai Loon, Tay Li Jin Shannie, Feng Lin, Shi Minlong, Tai Seng Thong and Jiang Ping in our school, for sharing their experiences. I would like to thank our school secretaries, Tan Kim Yin, Loke Yoke Lan Geraldine, Chong Pui Ling for their sincere help with all the administrative work. Special thanks to Chong Chye Hong May for her prompt offering of a helping hand whenever needed.

A note of thanks to my former lab colleagues in Prof. Wilmouth's group. They are Liu Bin, Chin Sze Yun, Westley Kee, and Ong Yean Sin. Thank you for your contributions to this work.

I am very grateful to my parents and my wife. Thank you for your continuous support, encouragement and faith in me. Especially for my wife, it is important to know that you are always on my side in the journey of life.

Wu Binhui

18-Feb-2008

Abbreviations

2D	<u>2</u> <u>d</u> imensional
4E-BP1	eIF- <u>4E</u> -binding protein <u>1</u>
ABIN-2	<u>A</u> 20-binding inhibitor of <u>NF-κB</u> <u>2</u>
ACN	<u>a</u> ceton <u>i</u> trile
AP-1	<u>a</u> ctivating protein- <u>1</u>
AKT	acutely transforming retrovirus <u>A</u> <u>K</u> <u>T</u> 8 in rodent T cell lymphoma
ASK	<u>a</u> poptosis <u>s</u> ignal-regulating <u>k</u> inase
ATP	<u>a</u> denosine <u>t</u> riphosphate
Bad	<u>B</u> cl-2- <u>a</u> ntagonist of cell <u>d</u> eath protein
Bcl2	<u>B</u> - <u>c</u> ell <u>l</u> eukemia/lymphoma <u>2</u>
BP	<i>att</i> <u>B</u> and <i>att</i> <u>P</u>
BSA	<u>b</u> ovine <u>s</u> erum <u>a</u> lbumin
βTrCP	<u>β</u> -transducin repeat- <u>c</u> ontaining protein
CAT	<u>c</u> hloramphenicol <u>a</u> cetyl <u>t</u> ransferase
CAML	<u>c</u> alcium- <u>m</u> odulating cyclophilin <u>l</u> igand
CsA	<u>c</u> yclo <u>s</u> porine <u>A</u>
CBB	<u>C</u> oomassie <u>b</u> rilliant <u>b</u> lue
CD	<u>c</u> ommon <u>d</u> ocking site <i>or</i> <u>c</u> ircular <u>d</u> ichroism
Cdc	<u>c</u> ell <u>d</u> ivision <u>c</u> ycle
Cdk	<u>c</u> yclin- <u>d</u> ependent <u>k</u> inase
CHAPS	3-[(3- <u>c</u> holamidopropyl)dimeth <u>y</u> lammonio]-1-propanesulfonate
CHCA	<u>α</u> - <u>c</u> yano-4- <u>h</u> ydroxycinnamic <u>a</u> cid
Chk	<u>c</u> heckpoint <u>k</u> inase

CNK	<u>c</u> on <u>n</u> ector- <u>e</u> nhancer of <u>K</u> SR
Cot	<u>c</u> ancer <u>O</u> saka <u>t</u> hyroid
ConA	<u>c</u> on <u>c</u> anavalin <u>A</u>
COX-2	<u>c</u> yclo <u>o</u> xygenase- <u>2</u>
CR	<u>c</u> onserved <u>r</u> egion
CRD	<u>c</u> ysteine- <u>r</u> ich <u>d</u> omain
CRE	<u>c</u> AMP <u>r</u> esponse <u>e</u> lement
CREB	<u>C</u> RE- <u>b</u> inding protein
CRIB	<u>C</u> dc42/ <u>R</u> ac <u>i</u> nteraction and <u>b</u> inding domain
CyP	<u>c</u> yclophilin
Da	<u>d</u> alton
DAPI	4',6- <u>d</u> ia <u>m</u> idino-2-phenyl <u>i</u> ndole
DD	<u>d</u> eath <u>d</u> omain
DLK	<u>d</u> ual <u>l</u> eucine zipper-bearing <u>k</u> inase
DMEM	<u>D</u> ulbecco's <u>M</u> odified <u>E</u> agle's <u>M</u> edium
DTT	1,4- <u>d</u> i <u>t</u> hi <u>o</u> threitol
ECL	<u>e</u> nhanced <u>c</u> hemil <u>u</u> minescence
ECM	<u>e</u> xtrac <u>e</u> llular <u>m</u> atrix
EDTA	<u>e</u> thylene <u>d</u> iamine <u>t</u> etraacetic <u>a</u> cid
EGF	<u>e</u> pidermal growth <u>f</u> actor
Egr-1	<u>e</u> arly growth <u>r</u> esponse factor 1
EGTA	<u>e</u> thylene glycol <u>t</u> etraacetic <u>a</u> cid
eIF4E	<u>e</u> karyotic <u>i</u> nitiation <u>f</u> actor <u>4E</u>
eIF4G	<u>e</u> karyotic <u>i</u> nitiation <u>f</u> actor <u>4G</u>
ER	<u>e</u> strogen <u>r</u> eceptor

ERK	extracellular signal-regulated protein kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FPLC	fast performance liquid chromatography
Gn-HCl	guanidine hydrochloride
GRP	glycine-arginine-proline
Grp78	glucose regulated protein 78
GST	glutathione-S-transferase
HBS	HEPES-buffered saline
HEK293	human embryonic kidney 293
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HRP	horseradish peroxidase
Hsp	heat shock protein
HTLV	human T cell leukemia virus
IEF	isoelectric focusing
IKK	I κ B kinase
IRAK	interleukin-1 receptor associated kinase
IL	interleukin
IMAC	immobilized metal ion affinity chromatography
IP	immunoprecipitation
IPG	immobilized pH gradient
IPTG	isopropyl- β -D-1-thiogalactopyranoside
IRAK	IL-1 receptor-associated kinase
ISH	<i>in situ</i> hybridization

JAK	<u>J</u> anus <u>k</u> inase
JNK	c- <u>J</u> un <u>N</u> -terminal <u>k</u> inase
kD	<u>k</u> ilo <u>d</u> alton
KSR	<u>k</u> inase <u>s</u> uppressor of <u>R</u> as
LB medium	<u>L</u> uria- <u>B</u> ertani <u>m</u> edium
LMP	<u>l</u> atent <u>m</u> embrane <u>p</u> rotien
LPS	<u>l</u> ipopolysaccharide
LR	<i>att</i> <u>L</u> and <i>att</i> <u>R</u>
LTR	<u>l</u> ong <u>t</u> erminal <u>r</u> epet
LZ	<u>l</u> eucine <u>z</u> ipper
MAD	<u>m</u> ultiwavelength <u>a</u> nomalous <u>d</u> iffraction
MALDI	<u>m</u> atrix- <u>a</u> ssisted <u>l</u> aser <u>d</u> esorption/ <u>i</u> onization
MAPK	<u>m</u> itogen- <u>a</u> ctivated <u>p</u> rotein <u>k</u> inase
MAPKAPK	<u>m</u> itogen- <u>a</u> ctivated <u>p</u> rotein <u>k</u> inase- <u>a</u> ctivated <u>p</u> rotein <u>k</u> inase
MAPKK	<u>m</u> itogen- <u>a</u> ctivated <u>p</u> rotein <u>k</u> inase <u>k</u> inase
MAPKKK	<u>m</u> itogen- <u>a</u> ctivated <u>p</u> rotein <u>k</u> inase <u>k</u> inase <u>k</u> inase
MBP	<u>m</u> altose <u>b</u> inding <u>p</u> rotein
MEK	<u>m</u> itogen <u>E</u> RK <u>k</u> inase
MEKK	<u>M</u> EK <u>k</u> inase
MEF	<u>m</u> ouse <u>e</u> mbryonic <u>f</u> ibroblast
MHC	<u>m</u> ajor <u>h</u> istocompatibility <u>c</u> omplex
MIP	<u>m</u> acrophage <u>i</u> nflammatory <u>p</u> rotein
MK2	<u>M</u> APKAPK <u>2</u>
MKK	<u>m</u> itogen- <u>a</u> ctivated <u>p</u> rotein <u>k</u> inase <u>k</u> inase
MLK	<u>m</u> ixed <u>l</u> ineage <u>k</u> inase

MLCK	<u>m</u> ynosin <u>l</u> ight <u>c</u> hain <u>k</u> inase
MMP	<u>m</u> atrix <u>m</u> etalloproteinase
MMTV	<u>m</u> ouse <u>m</u> ammary <u>t</u> umor <u>v</u> irus
MOPS	3-[N- <u>m</u> orpho <u>l</u> ino] <u>p</u> ropane <u>s</u> ulfonic acid
MORG	<u>M</u> APK <u>o</u> rganizer
MP	<u>M</u> EK partner
MS	<u>m</u> ass spectrometry
MSK	<u>m</u> itogen- and <u>s</u> tress-activated protein <u>k</u> inase
NEMO	<u>N</u> F- κ B <u>e</u> ssential <u>m</u> odulator
NFAT	<u>n</u> uclear <u>f</u> actor of <u>a</u> ctivated <u>T</u> cells
NIK	<u>N</u> F- κ B <u>i</u> nducing <u>k</u> inase
Ni-NTA	<u>n</u> ickel <u>n</u> itrilo <u>t</u> riacetic <u>a</u> cid
OD	<u>o</u> ptical <u>d</u> ensity
p-	<u>p</u> hospho-
p90RSK	<u>p</u> 90 <u>r</u> ibosomal <u>S</u> 6 <u>k</u> inase
Pap1p	<u>p</u> oly(<u>A</u>) <u>p</u> olymerase <u>1</u> p
PBD	<u>P</u> olo- <u>b</u> ox <u>d</u> omain
PBS	<u>p</u> hosphate- <u>b</u> uffered <u>s</u> aline
PCR	<u>p</u> olymerase <u>c</u> hain <u>r</u> eaction
PDB	<u>p</u> rotein <u>d</u> ata <u>b</u> ank
PDGF	<u>p</u> latelet <u>d</u> erived <u>g</u> rowth <u>f</u> actor
PDI	<u>p</u> rotein <u>d</u> isulphide <u>i</u> somerase
PEG	<u>p</u> oly <u>e</u> thylene <u>g</u> lycol
PEST	proline (<u>P</u>)-glutamic acid (<u>E</u>)-serine (<u>S</u>)-threonine (<u>T</u>)
PH	<u>p</u> leckstrin <u>h</u> omology

PHA	phytohemagglutinin
pI	isoelectric point
PIP ₂	phosphatidylinositol diphosphate
PKA	protein kinase A
PLC	phospholipase C
Plk	polo-like kinase
Plx1	<i>Xenopus</i> polo-like kinase 1
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PP1	4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
PPAR	peroxisome proliferator activated receptors
PVDF	polyvinylidene fluoride
Raf	rapidly accelerated fibrosarcoma
RBD	Ras binding domain
RBS	ribosome binding site
RHD	Rel-homology domain
RIP	receptor interacting protein
RMSD	root mean squared deviation
RNAP II	RNA polymerase II
RSK	ribosomal S6 kinase
SAPK	stress-activated protein kinase
SAS	solvent accessible surface
SCF	Skp1, Cul1 and F-box
SD	Shine-Delgarno
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEK	<u>s</u> tress-activated protein kinase/ <u>e</u> xtracellular signal-regulated <u>k</u> inase
SH	<u>S</u> rc <u>h</u> omology
siRNA	<u>s</u> mall <u>i</u> nterfering <u>R</u> NA
SNR	<u>s</u> ignal: <u>n</u> oise <u>r</u> atio
STAT	<u>s</u> ignal <u>t</u> ransducers and <u>a</u> ctivators of <u>t</u> ranscription
Sur	<u>s</u> uppressor of <u>R</u> as
Syk	<u>s</u> pleen tyrosine <u>k</u> inase
TAB	<u>T</u> AK1- <u>b</u> inding protein
TAK	<u>T</u> GF β - <u>a</u> ctivated <u>k</u> inase
TBST	<u>T</u> ris- <u>b</u> uffered <u>s</u> aline <u>T</u> ween-20
TD	<u>t</u> argeting <u>d</u> omain
TFA	<u>t</u> ri <u>f</u> luoroacetic <u>a</u> cid
TIR	<u>T</u> oll/ <u>i</u> nterleukin-1 <u>r</u> eceptor
TIRAP	<u>T</u> IR domain-containing <u>a</u> daptor <u>p</u> rotein
TLR	<u>T</u> oll- <u>l</u> ike <u>r</u> eceptor
TNF	<u>t</u> umor <u>n</u> ecrosis <u>f</u> actor
TNFR	<u>t</u> umor <u>n</u> ecrosis <u>f</u> actor <u>r</u> eceptor
TOF	<u>t</u> ime- <u>o</u> f- <u>f</u> light
Tpl-2	<u>t</u> umor <u>p</u> rogression <u>l</u> ocus <u>2</u>
TRADD	<u>T</u> NF <u>R</u> SF1A- <u>a</u> ssociated via <u>d</u> eath <u>d</u> omain
TRAF	<u>t</u> umor necrosis factor <u>r</u> eceptor- <u>a</u> ssociated <u>f</u> actor
TRAM	<u>T</u> RIF- <u>r</u> elated <u>a</u> daptor <u>m</u> olecule
TRIF	<u>T</u> oll/ <u>I</u> L-1 <u>r</u> eceptor domain-containing adaptor <u>i</u> nducing <u>I</u> FN- β
TRIS	<u>t</u> ris(hydroxymethyl)aminomethane
Ub	<u>u</u> biquitin

WB	<u>w</u> estern <u>b</u> lot
xPlkk1	<u>X</u> enopus <u>P</u> olo- <u>l</u> ike <u>k</u> inase <u>k</u> inase <u>1</u>

Summary

Cot (cancer Osaka thyroid, also known as Tpl-2), a proto-oncogenic kinase, is a member of the MAPKKK (mitogen-activated protein kinase kinase kinase) family and plays a key role in the regulation of the immune response to pro-inflammatory stimuli such as LPS (lipopolysaccharide) and TNF- α (tumor necrosis factor- α). After LPS-stimulation, Cot activates the MEK (mitogen ERK kinase) 1/2-ERK1/2 pathway, which is parallel to Raf, to regulate gene transcription, cell growth, differentiation and survival.

In this study, the *cot* gene was cloned and expressed in *E. coli*, insect cells and mammalian cells. Cot constructs which contained the last 30 bp of *cot*_{full-length} did not express well in *E. coli*. However, C-terminally truncated constructs of Cot can express well. The 6 $\times$ His tag could not be used for purification as it was inaccessible. The GST tag was useful for purification, but cleavage between GST and the Cot protein was difficult. Purified Cot proteins were used for crystallization screening. Several crystals were observed with Cot₁₋₃₈₈ (refolded protein), GST-Cot₁₋₃₈₈ and GST-Cot₁₋₃₈₈ (selenomethionine-substituted). However, the quality of the crystals was inadequate to obtain good X-ray diffraction.

To investigate potential binding partners of Cot, a series of Cot constructs with an N-terminal 6 $\times$ His tag were transiently expressed in HEK293 cells: Cot₁₃₀₋₃₉₉ (kinase

domain), Cot₁₋₃₈₈ (N-terminal and kinase domains), Cot₁₋₄₁₃, Cot₁₋₄₃₈ (containing a putative PEST sequence which is a region rich in proline, glutamic acid, serine, and threonine), Cot₁₋₄₅₇ (containing both PEST motif and degron sequence which is a target signal peptide for ubiquitin conjugation and proteolysis) and Cot₁₋₄₆₇ (full-length protein). These Cot proteins were precipitated using an anti-6×His antibody and separated by 2D electrophoresis. The gels were silver-stained and 21 proteins were detected that did not appear, or had substantially reduced intensity, in the control sample. Three of these were identified by MS (mass spectrometry) and MS/MS analysis as Hsp90 (heat shock protein 90), Hsp70 and Grp78 (glucose regulated protein 78). Hsp90 appeared to bind to the kinase domain of Cot and the interaction was confirmed by co-immunoprecipitation.

As Cot is involved in a diverse range of cellular pathways, a peptide-based screening assay was employed to identify further possible target proteins of Cot. This assay revealed that Cot was able to phosphorylate Plk (polo-like kinase) 1, a human kinase with key roles in multiple stages of the cell cycle, at Ser137. This was supported by *in vitro* kinase assays and further confirmed by mass spectrometric experiments which examined the phosphorylation of intact Plk1 and an 11 residue peptide identical to the sequence surrounding Ser137. A similar peptide matching the sequence surrounding Thr210 was not phosphorylated. In addition, S137T mutant peptide could not be phosphorylated by Cot. The result was further confirmed by 3D peptide structure prediction and protein-protein interaction modeling. *In vivo* experiments demonstrated

that wild-type Cot, but not kinase dead mutant (K167R), was able to phosphorylate Plk1 at Ser137 in HEK293 cells. In addition, siRNA (small interfering RNA) knock-down of endogenous Cot in Hela cells showed a reduction in the level of phosphorylation of Plk1 at Ser137. These results imply that Cot might be an upstream kinase of Plk1, and suggest a new mechanism for the phosphorylation of Plk1 during the cell cycle.

In conclusion, this study demonstrates for the first time that Hsp90 is a binding partner of Cot and that Cot can phosphorylate Plk1 at Ser137 not at Thr210.

Chapter 1

Introduction

1.1 Signal transduction in mammalian cells

Mammalian cells can recognize and properly respond to particular stimuli in the extracellular environment. Generally, there are eight major signaling pathways that deliver the proper and activated transcription factors to the nucleus in the response to the cell surface receptor bound by the extracellular ligand (Figure 1-1) [1]: (a) SMAD pathway, activated by phosphorylation on the serine in the C-terminal domain after binding with TGF- β [2]; (b) STAT (signal transducers and activators of transcription) pathway, activated by phosphorylation on a specific tyrosine residue on STAT cytoplasmic tail after binding with several cytokines and growth factors [3]; (c) Rel/NF- κ B pathway, activated by several extracellular ligands, for instance, LPS, IL-1 (interleukin-1) and TNF- α , and involves different intracellular kinases, including the MAPK pathway, in different cell types [4]; (d) Ci/Gli pathway, or hedgehog pathway, activated by the Hh family of ligands [5]; (e) Wnt pathway, activated by the Wnt/Wg ligands [6]; (f) Notch pathway, activated by membrane-associated ligands [7]; (g) NFAT (nuclear factor of activated T cells) pathway, NFAT is imported into the nucleus after dephosphorylation [8]; (h) PLC (phospholipase C) pathway, activated PLC will cleave PIP₂ (phosphatidylinositol diphosphate) subsequently with Tubby released from PIP₂, and the transcription factor Tubby can translocate into the nucleus [9-11].

Figure 1-1 Eight major signaling pathways in mammals that deliver an active transcription factor to nucleus in response to cell surface receptor binding by an extracellular ligand (adapted from reference [1])

1.2 Control of gene expression in mammalian cells by the Rel/NF-κB pathway

NF-κB was first discovered as a transcription factor binding the enhancer of the immunoglobulin κ light-chain inactivated B cells in 1986 [12]. The NF-κB family of homodimeric and heterodimeric transcription factors consist of Rel-A (p65), Rel-B, c-Rel, NF-κB1 (p50) and NF-κB2 (p52) (Figure 1-2) and NF-κB1 (p50) is produced from p105 by proteasome-mediated processing of the C-terminus [13-16] [the precursor of NF-κB2 (p52) is p100].

NF- κ B/Rel proteins

Figure 1-2 Structure of the NF- κ B family (adapted from reference [17])

The p105 precursor acts as an inhibitor by retaining p50, c-Rel and Rel-A in the cytoplasm through an interaction with its C-terminus [18, 19]. Following cell stimulation (e.g. by TNF- α), p105 is rapidly degraded and the Rel subunits translocate into the nucleus. The IKK (I κ B kinase) complex phosphorylates p105 at Ser927 and Ser932 which leads to p105 proteolysis [20-22]. IKK also phosphorylates I κ B, targeting this inhibitor for ubiquitination and proteasome-mediated degradation, which allows the released NF- κ B (p65 and p50) to move to the nucleus and exert their trans-activation function [23]. p65 is also regulated by phosphorylation of Ser276 in the Rel homology domain [24-27]. This phosphorylation, which significantly enhances the transcriptional activity of NF- κ B, can probably be mediated by several kinases including PKA (protein kinase A) and MSK1 (mitogen and stress-activated kinase 1), which are known to phosphorylate p65 at this site in response to different signals [25, 28].

NF- κ B family is normally located in the cytoplasm as inactive precursors by the physical binding of the inhibitor such as I κ B. Also, the inactive precursors act as an inhibitor to retain associated NF- κ B subunits in the cytoplasm. Once activated by the cellular stimuli, the IKK complex will phosphorylate I κ B at the N-terminal two serine residues. The phosphorylated serine residues will create a binding site for β TrCP (β -transducin repeat-containing protein). After the binding, this complex is the receptor for the SCF (Skp1, Cul1 and F-box) ubiquitin E3 ligase complex. This new complex will catalyze the rapid ubiquitination of I κ B on the two adjacent lysine residues, targeting I κ B for degradation by the 26S proteasome [20, 29]. NF- κ B will be released by hydrolysis followed by translocation to the nucleus. The IKK complex comprises IKK1, IKK2 and a structural subunit NEMO (NF- κ B essential modulator, IKK γ). NEMO does not show any kinase activity. However, it is required to couple the IKK complex to the upstream signal [30]. Interestingly, between the IKK1 and IKK2, only the phosphorylation of IKK2 is essential for the signaling pathway triggered by IL-1 and TNF. Furthermore, activation of IKK2 by IL-1 and TNF also requires the MAPKKs, which may phosphorylate the activation loop of IKK2 [31]. Rel-A, c-Rel both have C-terminal transactivation domains in their RHD (Rel-homology domain) and can strongly activate transcription from the NF- κ B binding site in the target gene. Rel-B also contains the transactivation domain in its RHD and can act as an activator of NF- κ B when forming the complex with p50 or p52. The complex of Rel-A and Rel-B is inactivated because the complex cannot bind the DNA substrate. Activated NF- κ B (p50 and p52) does not have a transactivation domain [17]. They can promote gene

transcription only when they form the heterodimer with a transactivational Rel subunit [17].

1.3 Overview of mammalian MAPK signal transduction pathways

Mammalian mitogen-activated protein kinase (MAPK) signal transduction pathways, which comprise a group of protein serine/threonine and tyrosine kinases, are key mechanisms in eukaryotic cell regulation when cells are treated with extracellular stimulus or stress [32]. Stimuli include hormones and growth factors such as insulin, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), interleukin-1 (IL-1), and tumor necrosis factor (TNF). Stress factors include osmotic shock, ionizing radiation, LPS, and ischemic injury. MAPK pathways can regulate gene transcription, protein synthesis, cell proliferation, apoptosis, differentiation through phosphorylation at conserved serine/threonine or tyrosine residues [33]. All MAPK pathways can be schematically represented by a central three-tiered core signaling module [33, 34] (Figure 1-3).

Figure 1-3 Schematic representation of the MAPK pathway (adapted from references [33, 34])

In general, the components of MAPK pathways have three characteristics. First, MAPKs are proline-directed kinases. They need at least one proline residue at the C-terminal side of the phosphoacceptor site. Secondly, activation of MAPK requires phosphorylation of conserved serine/threonine residues by a MAPK kinase (MAPKK), which in turn is activated via phosphorylation of two serine residues by the upstream MAPKKK. Phosphorylation of some MAPKs result in their translocation to the nucleus where they activate specific transcription factors by phosphorylation. Kinases in MAPK pathways always have more than one biological function under different stimuli. Thirdly, these kinases are organized by scaffolding proteins [35]. When a stimulus or stress is presented, these proteins can be specifically integrated by scaffolding proteins and efficiently activated to provide a response. Regulation of MAPKKKs is the entry point of MAPK pathways. According to the stimulus or stress

event, a specific MAPKKK is activated, including recruitment to the membrane by G-proteins and phosphorylation by RTKs (receptor tyrosine kinases) or MAP4Ks, homoligomerization together with other regulatory proteins. There are four major MAPK pathways [33, 36, 37] (Figure 1-4), one is the ERK1/2 pathway, which is mostly activated by growth factors via the Ras-Raf-MEK phosphorylation pathway [38]. However, Cot is obligatory for the activation of MEK1/2-ERK1/2 when the cell is stimulated by LPS via TLR (Toll-like receptor) 4. The second is the c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) pathway, which together with the third pathway, p38 pathway, and the fourth pathway, ERK5, is activated by cellular stress and stimuli [33, 39].

Figure 1-4 Four major MAPK pathways in mammalian cells

1.4 MAPKKK family

There are at least 14 known MAPKKKs (Table 1-1) in mammals. All the Raf (rapidly accelerated fibrosarcoma) proteins are composed of three conserved regions: CR1, CR2, and CR3. CR1 has a Ras binding domain (RBD) and a cysteine rich domain. CR2 has unknown function and no obvious motifs. CR3 is the kinase domain. Raf is ubiquitously expressed and well-studied, but still poorly understood [34]. It is known that there are seven phosphorylation sites (S259, S338, Y341, S471, T491, S494, S621) in Raf [40, 41]. S259 lies in the CR2; S338 lies near the N-terminus of the kinase domain; Y340/341 also lies near the N-terminus of the kinase domain, but is activated by Src and JAK (Janus kinase). S491 and S494 lie in the activation loop of the kinase domain. Mutation of all phosphorylation sites to acidic residues results in full function of the kinase [42]. S621 lies in the C-terminus of CR3. MEKK (MEK kinase) 1 is a 160 kD (kilodalton) protein that contains a C-terminal kinase domain (1221-1493) and an N-terminal domain (1-1220). There are several binding or regulatory sites in the N-terminal domain, such as a binding site for 14-3-3 protein and cleavage sites for caspase [43]. MEKK2 and MEKK3 are very closely related with their kinase domain having almost 90% similarity. They both have a C-terminal catalytic domain (362-619 for MEKK2, 368-626 for MEKK3) [44]. Their N-terminal domains are also similar. However, unlike MEKK1, there is no known motif for function and regulation in this domain. MEKK4 can activate both SAPK and p38. It also has a C-terminal domain (1337-1597) and an N-terminal domain which includes a polyproline SH (Src homology) 3 docking site, a PH (pleckstrin homology) domain, and a Cdc42/Rac

interaction and binding domain [45]. ASK1 is a 150 kD protein with a central kinase domain (677-936). Its N-terminal (1-676) and C-terminal (937-1375) domain also have TNFR (tumor necrosis factor receptor)-associated factor binding sites. TAK (TGF β -activated kinase) 1 is a 60 kD protein with a N-terminal domain (30-294), a small regulatory domain (1-22), and a C-terminal domain (295-557) [46]. ASK1 and TAK1 both can activate the SAPK and p38 pathway. MAPKKK8 is a 52 kD protein with an N-terminal domain of unknown function, a central catalytic domain (149-388), and a C-terminal regulatory domain (389-467). It activates the ERK pathway in parallel to Raf. When overexpressed, it activates ERK, JNK, p38 MAPK, ERK5, NF- κ B, and NFTA. MLK (mixed lineage kinase) 2, MLK3, DLK (dual leucine zipper-bearing kinase) belongs to a small family of protein Ser/Thr kinase MLKs (mixed lineage kinases). They all have a similar structure, an N-terminal domain followed by one or two leucine zippers, a Cdc42/Rac interaction and binding domain (CRIB), and a C-terminal proline-rich domain. They are entirely specific to the SAPK pathway [47]. Tao is a protein of 1001 amino acid Ser/Thr kinases which appear to be specific activators of the p38 pathway. This implies that Tao may have a specific binding site for MEK3 or MEK6 [48].

Table 1-1 Members of the MAPKKK family

Name	Alternate Names	Substrates/Effectors
Raf		MEK1, MEK2
A-Raf		MEK1, MEK2
B-Raf		MEK1, MEK2
MEKK1		SEK1, MKK7
MEKK2		SEK1, MEK1
MEKK3		SEK1, MEK1
MEKK4	MTK1	SEK1, MKK3, MKK6
ASK	MAPKKK5	SEK1, MKK3, MKK6
TAK1		SEK1, MKK3, MKK6
MAPKKK8	Cot (in humans), Tpl-2 (in rats)	MEK1, SEK1/MKK4
MLK2	MST	SEK1, MKK7
MLK3	SPRK, PTK1	SEK1, MKK7
DLK	MUK, ZPK	SEK1, MKK7
Tao1/2	PSK	MKK3, MKK7

1.5 Raf kinase

The receptor tyrosine kinase effector, Raf was discovered in 1983 as the transforming oncogene of the murine retrovirus 3611-MSV, and it was shown that it had an important role in the MAPK pathway [49]. Ras was demonstrated to be upstream of Raf, and the substrate of Raf was shown to be MEK [50]. Raf has been implicated in cell proliferation, differentiation, cell invasion, cell cycle progression and cell survival. Its regulation involves various post-translation modifications, phosphorylation, protein-protein interactions (oligomerization, binding with adapter protein and binding with scaffold protein), cross-talk and feedback mechanisms [41]. Raf knock-out mice displayed embryonic lethality and poor development of the placenta, liver, and

haematopoietic organs [51, 52].

The activation of Raf required the binding of GTP-Ras to the N-terminal Ras binding domain (RBD) and cysteine-rich domain (CRD). After binding, S259 was phosphorylated by Akt [53]. 14-3-3 then bound to this site [54]. S621 is critical for its activation [55]. The activation of Raf also required the phosphorylation of Y341 by Src and S338 by PAK [56, 57]. The phosphorylation of S471 is important for the binding of MEK. Furthermore, the activation of Raf required the phosphorylation of T491 and S494, these two phosphorylation sites were located in the kinase activation loop [58].

Several scaffold proteins, heat-shock proteins and adapter proteins have been linked to Raf activation. Kinase suppressor of Ras (KSR) binds Raf and MEK and presents MEK to Raf [59]. The connector-enhancer of KSR (CNK) links different mitogens to Raf activation and can enhance its activation [60, 61]. Raf is assembled into a complex with Hsp90 and Cdc37, and these two proteins are required for the activation of Raf protein [62].

1.6 Identification of Cot in humans and rats

Cot (cancer Osaka thyroid) was first identified as a rearranged form during transfection assays with SHOK cells [63, 64]. Cot was homologous to members of the MAPKKK family and has been implicated in cellular activation of ERK1/2, c-Jun N-

terminal kinase, ERK5 pathways and upregulation of the activity of NF- κ B and AP-1 transcription factors [65, 66]. The Cot protein has 415 amino acid residues and a molecular weight of 52 kD. The Cot protein with a truncated and substituted C-terminus (the last 44 amino acids are replaced by 10 intron-encoded amino acids) could transform NIH 3T3 and hamster SHOK cells [67]. Subsequently, two protein products of the *cot* gene were identified with molecular weights of 52 and 58 kD [67] due to the alternative initiation from two AUGs [68]. The 52 kD protein, which is truncated by 29 amino acids at the N-terminus, had a shorter half-life in the cytoplasm suggesting the importance of the N-terminal domain in regulating the stability of Cot protein. However, the 58 kD protein (467 amino acids) had a stronger transforming ability than the 52 kD protein, indicating that the N-terminal domain may be necessary for cellular transformation. Both proteins displayed autophosphorylation activity at serine residues and appeared to be predominantly located in the cytoplasm.

Two rat provirus insertions were cloned from Moloney leukemia virus-induced rat thymoma 2769 and named Tpl-1 (tumor progression locus 1) and Cot/Tpl-2 [69]. Provirus insertion in the Cot/Tpl-2 locus gave rise to activation of a gene coding for a Ser/Thr protein kinase. This activation was characterized by the enhanced expression of a truncated RNA transcription that encoded a protein with an altered C-terminal domain (the last 43 amino acids are replaced by 7 intron-encoded amino acids). The biological outcome of this alteration and/or enhanced expression appeared to be the tumor (rodent T-cell lymphoma) progression via the promotion of cellular

proliferation and/or the prevention of apoptosis. It was also suggested that the Cot/Tpl-2 protein was involved in the transmission of mitotic signals regulating the transition of normal lymphocytes from a quiescent state to the G1 phase of the cell cycle. The *cot/tpl-2* gene, which is homologous to *cot*, maps to rat chromosome 17, mouse chromosome 18 and human chromosome 10p11. It consists of eight exons and spans a 35 kb genomic DNA region [70]. Provirus insertion (8.8 kb) targets reproducibly the last *cot/tpl-2* intron (1 kb) which gives rise to truncated RNA transcriptions that terminate in the 5' proviral long terminal repeat (LTR). Similarly, the mouse mammary tumor virus (MMTV) was found to integrate between exons 7 and 8 giving rise to a truncated Cot/Tpl-2 product, suggesting that Cot/Tpl-2 may play a role in mammary gland epithelial tumor formation [71]. Cot/Tpl2 is transcribed from two alternating promoters, P₁ and P₂, with the P₂ promoter utilized primarily in tumors carrying a provirus in this locus.

1.7 Expression of Cot in different tissues

Cot was found to be expressed primarily in spleen, thymus and lung tissue of rats [70] and in many tissues of fetal, newborn and adult mice, with particularly high expression in adult submandibular, thymus, spleen and newborn digestive tract [72]. Ohara *et al.* identified cell types expressing *cot* proto-oncogene mRNA using *in situ* hybridization (ISH) [64]. From a variety of adult mouse tissues that were examined, four types of glandular cells expressing the *cot* gene were identified: granular duct cells in the submandibular gland and sublingual glands; serous cells in the parotid

gland; peptic cells in gastric glands; and goblet cells in colonic glands. Furthermore, Cot was only expressed in those tissues that were morphologically differentiated and functionally activated. Expression of the Cot gene was found in most cell lines derived from colonic and gastric adenocarcinomas [64]. The involvement of Cot in 35 human breast tumor specimens was examined. In 14 of them, Cot was found to be overexpressed with an increased number of the copies of the *cot* gene while the rest were at normal expression level. Overexpression of *cot* was not significantly associated with a particular histological subtype, tumor grade or with the age of the patients. However significant association was found between the overexpression of *cot* and stage I of the tumors, which may suggest that overexpression of *cot* was an early event in the development of tumors [73].

It was also shown that Cot was overexpressed in four patients with large granular lymphocyte proliferate disorders, but not in eight patients with other T cell neoplasias. This suggests that Cot regulation may be a defining molecular event for the development of certain type of neoplasias. Normally, Cot activates the transcription factors NFAT and NF- κ B in T-cells. These two transcription factors will enhance the transcription of several cytokine genes like IL-2 and TNF- α . However, in all the patients that displayed Cot overexpression, the serum TNF- α , but not IL-2, levels were elevated. This may indicate that activation of transcription of cytokine by Cot is cell dependent [74].

Finally, Cot was also found to be expressed in primary biopsies from patients with nasopharyngeal carcinoma and Hodgkin's disease [75, 76]. A different truncated form of Cot was found in primary lung tumor samples, which contain the first 421 amino acids of wild-type Cot followed by eight amino acids [76]. The transforming activity of this mutant was substantially higher than the wild-type protein. However, it was suggested that this particular mutational activation of the gene is a rare event in lung cancer and need further confirmation. Cot and JNK2 also both appear to promote the development of intestinal inflammation in TNF-induced Chrohn's like inflammatory bowel disease, while MK2 (MAPKAPK2) inhibits it [77].

1.8 Cot is a member of MAPKKK family

Overexpression of Cot appeared to induce MAPK activation in COS-1 and NIH 3T3 cells. This induced MAPK activation could be inhibited by Ras N17 (dominant negative mutant) or the Raf dominant negative mutant (S621A) [78], which suggested that Cot may act upstream of Ras and Raf. However, the kinase-negative mutant of Cot (K167M or K167R) partially blocked MAPK activation induced by v-Ras, indicating Cot functions downstream of Ras and Raf. It was suggested that these apparently contradictory results may be possible if Cot, Ras and Raf formed a complex that phosphorylated MEK1. Due to the high expression of Cot in the spleen but low expression in other tissues, Cot may be involved in the transduction of mitogenic signals in T lymphocytes, whereas related kinases may transfer similar signals in other cell types. Transformation of NIH 3T3 cells with Cot resulted in a

hyperphosphorylated form of Raf which is typical of the active kinase; this suggested that there may be a functional interaction between Cot and Raf [79].

The kinase domain of Cot was found to be homologous to the *S. cerevisiae* gene product STE11, a MAPKKK which regulates the responses to pheromones, suggesting that Cot is a MAPKKK [80]. Immunoprecipitated Cot activated the human MAPKKs, such as MEK1 and SEK (stress-activated protein kinase/extracellular signal-regulated kinase)-1, *in vitro*. Transfection of Cot into COS-1 or Jurkat T cells, activated the MAPKs, such as ERK1 and SAPK (JNK). In contrast, Raf is only able to activate ERK1 and not SAPK. Similarly, transfection of Cot into HEK293 cells was found to stimulate ERK via phosphorylation and activation of MEK, and to stimulate SAPK (JNK) via the phosphorylation of SEK [81]. The phosphorylation of MEK1 by C-terminally truncated Cot (which mimics the oncogenic form) was greater than that by wild-type Cot (full-length).

Cot protein was the fourth oncogene product with Ser/Thr kinase activity to be identified [67]. The other three are Raf, Mos, and Pim-1, which were reported to be Ser/Thr kinases before. The protein sequence of Cot was analyzed by the website PredictProtein (<http://www.predictprotein.org/>) (Figure 1-5). Although Cot has 87% protein sequence similarity with the Tpl-2 protein (rat homolog), the similarity between Cot and other kinases is low (30-45%). This indicates that the structure and the function of Cot may be different from other human kinases. Furthermore, the

activation of Cot is not inhibited by staurosporine which is a non-specific kinase inhibitor. Also, Cot is the only human kinase that has a proline instead of a conserved glycine in the glycine-rich ATP (adenosine triphosphate) binding loop [82]. Therefore, Cot is likely to have different structural features [83].

1.9 Role of Cot in T cells

It was shown that an increase in the expression of wild-type and truncated Cot correlated with an increase in IL-2 production in anti-CD3-treated Jurkat T cells [84]. Truncated Cot expression also cooperated with PHA (phytohemagglutinin) or a phorbol ester and a calcium ionophore in the production of IL-2 from Jurkat cells. Expression of a dominant negative form of Cot inhibited transcription directed by the IL-2 promoter following stimulation by the phorbol ester or the calcium ionophore. Similarly, it was found that Cot expression was induced within 30-60 min after ConA (concanavalin A) stimulation of rat splenocytes, which suggested that Cot may contribute to the induction of IL-2 expression during T cell activation [85]. T cell activation is induced via the interaction of antigenic peptides bound to MHC (major histocompatibility complex) molecules on antigen-presenting cells with the T cell receptor/CD3 complex. This leads to the activation of multiple pathways leading to IL-2 transcription within 2-5 hr. This depends on the concerted action of several transcription factors, including the NFAT family (NFATp, NFATc, NFAT3, NFATx), AP-1 and NF- κ B. It was shown (in Jurkat, NIH 3T3 and HEK293 cells) that Cot

Figure 1-5 Alignment for Cot with other kinases. Colored by the sequence consensus (red highlights indicate basic residues; dark blue highlights indicate acidic residues; light blue and purple highlights indicate hydrophilic residues; yellow highlights indicate cysteine residues; green highlights indicate hydrophobic residues).

activates NFATp but not NFATx, and NFATc only minimally. Furthermore, Cot only induces IL-2 expression in some T cell lymphoma lines but not in normal T cells. Thus, Cot-induced IL-2 expression is dependent on the activity of other complementary pathways. In addition, the dominant negative mutants of Ras, Raf1, MEK1 and NFATc blocked Cot-mediated induction of IL-2 [86]. This suggested that activation of the IL-2 promoter by Cot depends on the convergence of the MAPK and the calcineurin/NFAT pathways. However, the MEK1 dominant negative mutant did not block Cot-mediated activation of NFAT. Also, Cot can activate NF- κ B and this is also blocked by dominant negative mutants of Ras, Raf1, and MEK1. Thus, Cot activates NF- κ B in a MEK1-dependent and NFAT in a MEK1-independent manner (Figure 1-6).

Figure 1-6 Role of Cot in T cells

Separately it was shown that transfection of Cot in Jurkat T cells promoted TNF- α expression (via induction of AP-1) and hence enhanced TNF- α secretion [87]. This kind of activation of TNF- α expression was at a similar level to that induced by a phorbol ester or a calcium ionophore. Expression of a dominant negative mutant of Cot inhibited TNF- α promoter activation by the same phorbol ester or calcium ionophore (with/without anti-CD3 or anti-CD28). It was also shown that T-cell activation (by a phorbol ester, anti-CD3 or a calcium ionophore) increased the transcriptional activity of the human *cot* gene promoter via the JNK/SAPK signal transduction pathway [88]. Similar results were obtained by Lin *et al.* which showed that ectopic expression of Cot mimics CD3/CD28 stimulation of T cells leading to activation of the CD28RE/AP-1 composite element of the IL-2 promoter [89]. The activation of this element was dependent on NF- κ B and required activation of both IKK1 and IKK2. Expression of a Cot kinase-dead mutant selectively impaired CD3/CD28 induction of NF- κ B but did not interfere with TNF- α activation of NF- κ B.

AKT (acutely transforming retrovirus AKT8 in rodent T cell lymphoma) is a Ser/Thr kinase capable of inducing transformation [90, 91] and contributes to T cell NF- κ B induction [92-95] in a manner similar to that of the CD28 coreceptor [96]. It was demonstrated that the N-terminus of Cot and the kinase and/or C-terminal domains of AKT can physically interact. This interaction is apparently required for the Cot-mediated activation of NF- κ B. AKT phosphorylates the C-terminal domain of Cot at two serine residues, Ser400 and Ser413. Mutating one or both of these residues to

alanine had no effect on the kinase activity of Cot, but the S400A mutant lost the ability to activate both NF- κ B and IKK1/2. This suggested that the failure of the S400A mutant to activate NF- κ B may be due to an inability to interact with the IKK complex [97].

CTLL-2 T cells are dependent on IL-2 for the G1 to S phase progression. When these cells were transfected by a dominant negative mutant of Cot, DNA synthesis induced by IL-2 was partially inhibited [98]. The contribution of Cot to cell cycle progression was demonstrated when the transient expression of Cot or truncated Cot decreased the number of cells in subG1, but increased the number of cells in G1, S and G2-M phases of the cell cycle. IL-2 increases the expression of Bcl-X_L (anti-apoptosis) and decreases the expression of p27^{kip} cyclin-dependent kinase inhibitor that arrests cell cycle progression. After expression of Cot (or truncated Cot), p27^{kip} could not be detected; the levels of Bcl-X_L were unaffected. Cot also enhanced the activity of E2F, a transcription factor whose activity is crucial for G1 to S phase transition.

1.10 Function of Cot

At present, the molecular mechanisms responsible for the oncogenic potential of Cot are still poorly defined. Although the *cot* gene appears to be expressed in a number of tissues such as spleen, thymus, liver, breast, lung, it was difficult to obtain significant expression of recombinant Cot in various mammalian cells such as Jurkat, cos-7, HeLa and various vectors such as pcINEO and pEFBOS [99]. Regulation of proto-

oncogenic Cot kinase by extracellular signals implies that the Cot kinase activity may be involved in apoptosis [100]. When overexpressed in immortalised non-transformed cells, Cot induces apoptosis by promoting the activation of caspase-3 via a caspase-9-dependent mechanism [101]. Tvl-1, a small protein that binds to (via its C-terminal ankyrin repeat domain) and is phosphorylated by Raf-1 [102], enhances Cot-induced apoptosis. The hypothesis is that Cot may associate with Tvl-1 and Apaf-1 as part of an apoptosome. Procaspase-9 would then associate with this complex, be phosphorylated and then undergo autocatalytic cleavage giving rise to active caspase-9. This larger complex would then bind and activate procaspase-3, thus producing active caspase-3 which would then initiate apoptosis.

Overexpression of the *cot* proto-oncogene was sufficient to stimulate the expression of *c-jun* and the activity of c-Jun, in turn, was required for Cot-induced transformation [100]. Cot-mediated stimulation of *c-jun* was through enhancement of the activity of JNK, p38 (ERK6) and ERK5 via stimulation of their respective MAKs, SEK, MKK (mitogen-activated protein kinase kinase) 6 and MEK5, respectively (Figure 1-7). Activity of these MAPKKs was necessary for full stimulation of the *c-jun* promoter by Cot and for Cot-induced neoplastic transformation.

Figure 1-7 Function of Cot (adapted from reference [100])

Macrophages and B-cells from the Cot knock-out mouse have a restricted defect in LPS, TNF- α and anti-CD40 stimulation and limited to the activation of ERK, JNK and NF- κ B. This indicates that, Cot regulates both innate and adaptive immunity and may play a critical role in the response to inflammatory signals. When the macrophages of the Cot knock-out mouse were exposed to LPS, they secreted low levels of TNF- α , which indicates these macrophages are resistant to LPS-induced endotoxin shock. Furthermore, Cot knock-out protects mice from TNF- α induced inflammatory bowel disease. However, signaling role of Cot is different due to the different cell type and specificity of stimulus. In the MEF (mouse embryo fibroblasts), Cot was required for the transduction of TNF- α signals which is induced via ligation of TNFR1. The binding of TNF- α and TNFR1 will release the receptor binding silencer of death domain and recruit the death domain-containing adaptor protein

TRADD (TNF RSF1A-associated via death domain). The binding of TNFR1 and TRADD will act as the assembly place for the binding of TNF-receptor-associated factor 2 (TRAF2). Although, there is no direct evidence to show that TRAF2 is critical in the signaling of TNF- α , TRAF2 contributes to the engagement of ERK in response to TNF stimulation. RIP (receptor interacting protein) 1 is essential for the TNF- α -induced NF- κ B and p38 MAPK pathway. RIP1 recruits MEKK3 and I κ B kinase (IKK) into a signaling complex after the stimulation. Phosphorylation of I κ B results in its degradation via the proteasome. Degradation of I κ B will release NF- κ B from the trimeric complex p65-p50-I κ B. Although TRAF2 and RIP1 are essential for the activation of the Cot-MEK-ERK axis by TNFR1, their overexpression is not sufficient to activate Cot and ERK.

The cell lines that lack or express reduced levels of Syk (spleen tyrosine kinase) exhibited diminished activation of Cot and ERK in response to TNF- α stimuli. Furthermore, knock-down of Syk will prevent the activation of IKK β . These results suggest that Syk functions upstream of IKK signaling complex to control the activation of Cot via dissociation from NF- κ B [103]. Cot functions downstream of IKK- β since IKK- β is required for the activation of NF- κ B and Cot involved in the activation of NF- κ B [104]. Although p105 acts as the physiological partner for the stability and inhibition of Cot, the mechanism is unclear. Previous study has shown that Cot can phosphorylate NF- κ B at amino acids 542 to 613, which means the binding of p105 does not inhibit the intrinsic catalytic activity of Cot [105]. However,

binding of p105 will stop the phosphorylation of MEK1 by Cot, which may indicate that p105 acts as a competitive substrate of Cot and may have more efficient binding compared with MEK1.

MyD88 is a common adaptor protein and is associated with the IL-1 receptor and TLR (Toll-like receptor) through their TIR domain (Toll/interleukin-1 receptor domain). Once the complex forms, MyD88 recruits IRAK (interleukin-1 receptor associated kinase) to the complex, and IRAK is phosphorylated and activated. Once IRAK is activated, the complex will recruit TRAF6 in response to IL-1. TRAF6 will form a poly-ubiquitin chain with TAK by the conjugated heterodimeric ubiquitin-conjugated enzyme. Once TAK is activated, it will form a complex with several TABs (TAK1-binding proteins). This complex will phosphorylate IKK and activate p38 and JNK pathways. However, in the macrophages of Cot knock-out mice, p38 and JNK pathways remain unchanged upon the extracellular stimulus. Furthermore, it was found that in wild-type macrophages, Cot can be activated in response to CpG-DNA stimulation through TLR9 [106]. In B cells, Cot can be activated in response to anti-CD40 or TNF- α . In addition, the Src kinase activity is involved in the Cot-MEK-ERK pathway response to IL-1, and it is cell type independent. Cot will regulate the expression of IL-8 and MIP (macrophage inflammatory protein)-1 β , these two chemokines play important roles in the development of inflammation [107].

1.11 Cot and extracellular signals

LPS is the predominant endotoxin produced by Gram negative bacteria and it can activate various cells including T cells and macrophages. The binding of LPS to Toll-like receptors (TLR) is probably the best-studied model of innate immunity [108]. Activation of TLR4 by LPS, probably by the binding of LPS to CD14, leads to the recruitment of MyD88 which contains two domains: a C-terminal Toll homology domain that interacts with the Toll homology domain on the receptor and an N-terminal death domain [109]. This death domain then interacts with the death domain on a Ser/Thr protein kinase known as IRAK (IL-1 receptor-associated kinase) leading to the autophosphorylation of IRAK. Phosphorylated IRAK then binds to TRAF6. TRAF6 is known to activate TAK-1 which leads to the activation of the I κ B kinases [110]. This results in the production of the proinflammatory cytokine TNF- α (Figure 1-8). Excessive production of TNF- α can lead to neurodegenerative disorders and sepsis [111] as well as a number of autoimmune diseases. Cot knock-out mice did not exhibit obvious phenotypic defects but produced low levels of TNF- α when exposed to LPS [112]. LPS-stimulation of this mouse did not activate MEK1, ERK1 and ERK2, but did activate JNK, p38 and NF- κ B. It was shown that the inactivation of Cot specifically blocks the activation of ERK1 and ERK2. Blocking ERK1 and ERK2 activity through a specific MEK inhibitor (PD98059) also blocked the induction of TNF- α by LPS. However, Cot was not required for LPS-induced activation of NF- κ B. Thus, Cot is obligatory only for the activation of ERK1 and ERK2 by LPS.

Figure 1-8 LPS-mediated Cot activation (adapted from reference [113])

Addition of LPS to RAW264 macrophages induced a 10-fold increase in endogenous Cot activity [114]. Taxol, but not PMA (phorbol 12-myristate 13-acetate), induced a similar activity of Cot. 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂, a natural ligand of PPAR (peroxisome proliferator activated receptors), a family of nuclear receptors that function in ligand-activated transcription), blocked LPS-mediated Cot activation. 15d-PGJ₂ also inhibited the LPS-induced Cot *in vitro*, but it did not inhibit MEK1 or ERK1/2 activation/phosphorylation induced by PMA or mediated by Raf. Inhibition of LPS-induced Cot may be a mechanism by which 15d-PGJ₂ acts as an anti-inflammatory agent [114].

It was found that the T290D but not the T290A mutant of Cot transduced LPS signals

in Cot^{-/-} macrophages [113]. In the absence of LPS stimulation, the phosphomimetic T290D mutant did not activate ERK, suggesting that phosphorylation at Thr290 is required but not sufficient for activation of Cot. A polyclonal antibody specific to phospho-Thr290-Cot did not detect the Cot (52 kD), suggesting that only the Cot (58 kD) undergoes phosphorylation. The same antibody showed that Cot becomes phosphorylated in RAW macrophages following LPS stimulation. Treatment of these cells with the IKK inhibitor PS-1145 or IKK siRNA blocked phosphorylation of Cot at Thr290, suggesting that this phosphorylation was dependent on the activity of IKK.

In wild-type MEFs, TNF- α stimulates ERK, JNK and p38, but in Cot^{-/-} MEFs the phosphorylation of ERK and JNK was impaired [104]. However, in the case of IL-1 stimulation of Cot^{-/-} MEFs and TNF- α stimulation of Cot^{-/-} macrophages, only the phosphorylation of ERK was impaired, the activation of JNK, p38 and NF- κ B was normal. This suggested that the Cot signaling defect was both cell-type and stimulus specific. It was also found that TNF- α -mediated activation of NF- κ B was weaker in Cot^{-/-} MEFs. Cot can be precipitated from TNF- α -stimulated TRAF2^{-/-} and RIP1^{-/-} fibroblasts but was unable to phosphorylate MEK1, suggesting that both TRAF2 and RIP1 are required for Cot activation in MEFs. The activation of JNK/SAPK in MEFs following TNF- α -stimulation required MKK7 and MKK4. MKK7 was obligatory, while MKK4 was required only for optimal JNK activation [115-117]. As JNK activation in TNF- α -treated Cot^{-/-} MEFs was reduced but not abolished, it was suggested that Cot may be required for activation of MKK4 (Figure 1-9). This would

indicate that MKK4 may be another substrate of Cot. It was also demonstrated that IKK signaling (phosphorylation of I κ B) and nuclear translocation of p50 and p65 remained intact in Cot^{-/-} MEFs. However, phosphorylation of p65 at Ser276, but not Ser536, was impaired, suggesting that this was due to reduced activation of MSK1. Phosphorylation of p65 at this site affected NF- κ B transactivation potential but not DNA binding, therefore some other pathway would appear to be responsible for Cot-mediated regulation of the DNA binding activity of NF- κ B.

Figure 1-9 TNF- α -mediated Cot activation (adapted from reference [104])

A weak association between RIP1 and Cot was found with co-immunoprecipitation which increased following TNF- α stimulation [103]. Furthermore, endogenous RIP1 also associated with NF- κ B1. The binding of TNF- α to TNFR1 promotes the release of the receptor-bound silencer of death domains and the recruitment of the death

domain-containing adaptor protein TRADD [118]. The binding of TRADD to TNFR1 then allows the binding of TRAF2 and the death domain-containing Ser/Thr kinase RIP1 [119]. RIP1 is essential for TNF- α -induced activation of the NF- κ B and p38 pathways but not the JNK pathway [120-123]. It was also shown that TRAF2 was co-immunoprecipitated with Cot [75]. Overexpression of TRAF2 or RIP1 in HEK293 cells leads to an increase in activation of JNK but not ERK and TRAF2 is obligatory for JNK activation [124]. The overexpression of Cot leads to activation of both pathways. This suggested that RIP1 and TRAF2 were necessary but not sufficient for activation of the Cot/ERK cascade in response to TNF- α stimulation; it seemed that an additional TNF- α -induced signal was necessary for Cot activation. A broad spectrum tyrosine kinase inhibitor herbimycin-A substantially reduced MEK and ERK activation in TNF- α -stimulated macrophages, which suggested that a tyrosine kinase might be responsible. Piceatannol, a Syk-selective inhibitor, had a similar effect to herbimycin-A in Cot^{+/+} but not Cot^{-/-} macrophages. Piceatannol also inhibited the release of Cot_L from NF- κ B1 and the phosphorylation of I κ B by IKK1 upon TNF- α treatment. This suggested Syk activated the Cot pathway by functioning upstream of IKK1. It was confirmed that Syk was phosphorylated following TNF- α stimulation and that ERK activation and Cot activity was diminished in Jurkat T cells that lack expression of Syk. In addition, phosphorylation of ERK was substantially decreased in Syk knock-down MCF7 cells (which express Syk) stimulated with TNF- α , and enhanced in HEK293 cells (which express very low levels of endogenous Syk) transiently transfected with Syk and stimulated with TNF- α .

Stimulation of HeLa cells with IL-1 but not with PMA increased Cot activity by about 10-fold; co-transfection with Cot siRNA blocked IL-1-mediated phosphorylation of ERK [125]. Expression of IL-8 and MIP-1 after stimulation by IL-1 was also blocked by Cot knock-down. Expression of a dominant negative mutant of TRAF6 blocked ERK2 phosphorylation after stimulation by IL-1. HeLa cells were treated with herbimycin-A and their activation of ERK1/2 by IL-1 was impaired. PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) [126], a specific inhibitor of Src tyrosine kinase, had a similar effect. Pre-incubation with PP1 also blocked the activation of ERK1/2 induced by LPS in HMEC-1 cells and RAW macrophages. PP1 had no effect on the phosphorylation of p38 or JNK induced by IL-1 and did not block the degradation of I κ B or the release of Cot from p105. This suggests that activation of the Cot-MEK1-ERK1/2 pathway requires Src kinase in addition to dissociation of Cot from the Cot-p105 complex.

The ERK activation defect in LPS-treated *nfkb1*^{-/-} macrophages coincides with reduced levels of Cot [127]. Macrophages were treated with peptidoglycan, dsRNA, LPS, loxoribine and CpG DNA, which are ligands for TLR2, TLR3, TLR4, TLR7 and TLR9, respectively. All the TLR ligands activated ERK in wild-type but not *nfkb1*^{-/-} macrophages, whilst JNK1/2 and p38 activation was unaffected. In wild-type macrophages, all the TLR ligands gave rise to a modest increase in activated Cot level. It was found that the impaired phosphorylation of ERK in the *nfkb1*^{-/-} macrophages during LPS stimulation could be overcome with the co-expression of Raf. The

transcription of IL-10 is ERK-dependent and it was shown that LPS- and CpG-stimulated *nfkb1*^{-/-} macrophages produced significantly less IL-10. However, IL-10 expression also depends on TLR-induced ERK-independent signals, probably via NF- κ B1. B cells and keratinocytes also express TLRs. Following LPS stimulation, *nfkb1*^{-/-} B cells but not *nfkb1*^{-/-} keratinocytes displayed reduced activation of ERK. This result was in line with the observation that Cot_L (58 kD protein) and Cot_S (52 kD protein) are both detectable in macrophages and B cells, but not keratinocytes.

CD40, an integral membrane protein found on the surface of B lymphocytes, dendritic cells, follicular dendritic cells, hematopoietic progenitor cells, epithelial cells, and carcinomas, is a 45-50 kD glycoprotein of 277 aa, which is a member of the tumor necrosis factor receptor superfamily. CD40 binds to a ligand (CD40L). CD40 regulates growth and survival in a cell-type-dependent manner (e.g. CD40 is anti-apoptotic in normal B cells, but is apoptotic in lymphoma and carcinoma cell lines). Following stimulation with CD40L, the cytoplasmic C-terminus of CD40 recruits several members of the family of TNFR-associated factors (TRAF2, TRAF3 and TRAF6) (Figure 1-10); TRAF6 plays an important role in the ERK activation by CD40. B cells and macrophages from Cot knock-out mice were shown to be deficient in ERK activation following CD40 stimulation, but not in activation of NF- κ B. Cot was required for TRAF6-mediated ERK activation. Cot was also found to pass the signal from TNF- α binding to TNFR1 which again leads to the activation of ERK. CD40-induced proliferation of B cells was unaffected by the knock-out of Cot,

suggesting that this effect was not ERK-dependent. However the Cot^{-/-} B cells were partially deficient in the secretion of IgE stimulated by anti-CD40 and/or IL-4 [128].

Figure 1-10 Cot-mediated CD40 signal pathway

Kinase-dead Cot (K167M) was shown to block CD40-mediated NF-κB induction and IKK activation in HEK293 cells, but had no effect on phosphorylation of IκB or NF-κB activity induced by TNF [129]. Co-immunoprecipitation indicated that Cot associated with CD40 following stimulation with CD40L. Overexpression of TRAF2 and TRAF6 increased the level of Cot phosphorylation and the activity of IKK and NF-κB; these effects were diminished in the presence of kinase-dead Cot [129].

1.12 Cot and NF-κB

Expression of Cot in HEK293 or Jurkat T cells results in NF-κB activation [86]. Cot also interacts with NIK (NF-κB inducing kinase) and leads to its phosphorylation and

activation [89]. The C-terminal 70 amino acids of Cot formed a complex with the C-terminus (residues 459-969) of p105 [130]. Two regions within the C-terminus of p105 appeared to interact with Cot: the last 89 amino acids (residues 880-969 which contain a PEST sequence) and residues 545-777. Wild-type Cot activated a NF- κ B reporter gene by 140-fold, whereas C-terminally truncated Cot only activated it by 12-fold, suggesting that direct interaction with p105 is required for NF- κ B activation. Kinase-dead Cot did not activate NF- κ B and transient expression of the kinase-dead mutant partially blocked NF- κ B activation by TNF- α . However, it appeared that Cot did not phosphorylate p105 *in vitro*, thus suggesting that a kinase downstream of Cot phosphorylates p105 which then promotes ubiquitination and subsequent degradation by the proteasome.

LMP1 (latent membrane protein 1) is a pleiotropic protein encoded by the Epstein-Barr virus, a human herpes virus associated with several types of malignancy, which has effects on cell transformation and phenotype, growth and survival. LMP1 promotes NF- κ B activation through the recruitment of the adapter protein TRAF2 and the formation of a multiprotein complex that includes NIK, IKKs and their downstream targets, I κ Bs and p105. Inducible expression of LMP1 promoted the activation of Cot, and expression of a catalytically inactive Cot mutant suppressed LMP1 and TRAF2-induced NF- κ B activation [75]. The expression of the kinase-dead Cot did not affect LMP1 mediated Cdc42c (cell division cycle 42c) signaling, which occurs in a TRAF2-independent fashion. Cot is recruited in the TRAF2 signaling

complex and affects its NF- κ B -inducing properties; this interaction between Cot and TRAF2 may be indirect and may be mediated by NIK. NIK plays role upstream of IKK but downstream of Cot. Kinase-dead Cot also inhibited the ability of LMP1 to induce COX-2 (cyclooxygenase-2), suggesting that Cot is involved in LMP1-mediated COX-2 activation and hence LMP1-induced angiogenesis and metastasis.

LPS stimulation of normal macrophages leads to activation of ERK1/2, JNK1/2 and p38 and degradation of I κ B. However, LPS stimulation of NF- κ B1-deficient macrophages only leads to activation of JNK1/2 and p38 and degradation of I κ B, *i.e.* ERK signaling was severely attenuated [131]. Induction of Egr-1 (early growth response factor 1) is downstream of ERK, and accordingly LPS did not activate Egr-1 in NF- κ B1-deficient macrophages. MEK1 is upstream of ERK, and likewise LPS-stimulation of MEK1 activation was largely blocked in these mutant macrophages. However, activation of MEK1 and ERK and induction of Egr-1 by PMA was normal, suggesting the effect was LPS-specific. While the two isoforms of Cot were readily detectable in normal macrophages, they were hardly detected in the NF- κ B1-deficient macrophages since NF- κ B1 binds with Cot to prevent its digestion. Raf expression was unaffected and it responded efficiently to PMA in the mutant macrophages, suggesting the mitogen-responsive pathway was functioning normally. However, transfection with p105 restored Cot expression and LPS-stimulated ERK1/2 activation. As a control these experiments were repeated with NF- κ B2-deficient macrophages: Cot expression and LPS-stimulation of MEK1 and ERK1/2 was normal. Pulse-chase

experiments revealed that the low Cot expression was due to rapid degradation in the NF- κ B1-deficient macrophages, *i.e.* NF- κ B1 stabilizes Cot by blocking its degradation. Transfection with p105 (and N-terminal truncated p105) restored Cot stability: this is in accordance with the previous experiments which suggested an interaction between the C-terminus of Cot and the C-terminus of p105. Also in line with previous conclusions, the NF- κ B1-deficient macrophages did not exhibit LPS-stimulated COX-2 expression and MSK1, RSK (ribosomal S6 kinase) 1 and CREB (CRE-binding protein) activation. The p105-associated Cot is catalytically inactive and that dissociation from p105 is required for Cot activity. Upon LPS stimulation, catalytically active Cot is released from p105 and activates the MEK/ERK pathway before being rapidly degraded (Figure 1-11).

Figure 1-11 Cot and NF- κ B (Adapted from references [131, 132])

It was shown, in a refinement of the previous study, that the Cot kinase domain interacts with the p105 death domain (residues 808 to 892) and that the C-terminal domain of Cot interacts with residues 497 to 534 of p105 (just before the ankyrin repeat region) [133]. Both p105 binding sites appeared to be required for optimal Cot interaction, and it was demonstrated that this interaction regulated the metabolic stability of Cot. The binding of the p105 death domain to Cot inhibits its MEK kinase activity; this inhibitory effect was reduced with C-terminally truncated Cot. It was therefore suggested that p105 negatively regulates Cot kinase activity by preventing access to its substrate.

A20 is an inhibitor of NF- κ B activation and ABIN-2 (A20-binding inhibitor of NF- κ B 2) may be a downstream effector of A20 that mediates this inhibitory function [134, 135]. It was found that ABIN-2 specifically interacted with p105 and Cot in HeLa and HEK293 cells [136]. In macrophages, the majority of the endogenous ABIN-2 was associated with Cot-p105 complexes. It appeared that the C-terminal regions of p105 and Cot were interacting with ABIN-2 and that ABIN-2 was necessary to maintain the stability of Cot. ABIN-2/Cot/p105 ternary complex is proteolyzed by the proteasome in LPS-stimulated macrophages. However, unlike p105, ABIN-2 did not appear to function as an inhibitor of Cot kinase activity.

The selective IKK inhibitor, PS1145, blocked the LPS-mediated secretion of TNF- α and expression of COX-2 by macrophages [137]. LPS- and TNF- α - stimulation of

ERK1 and ERK2 but not JNKs and p38 was blocked by PS1145, but PS1145 had no effect on ERK activation induced by PMA. This suggested that IKK may function as an essential component of the LPS-responsive ERK signaling pathway. LPS- but not PMA-mediated activation of MEK1, and both TNF- α - and LPS-mediated activation of Cot were also blocked by PS1145. Kinetic studies showed that IKK was activated about 2.5 min after LPS-stimulation, whereas Cot was activated at about 5 min. After TNF- α stimulation, Cot was activated in IKK^{+/+} but not IKK^{-/-} MEFs (mouse embryonic fibroblasts). Also, dissociation of Cot from p105 was blocked in PS1145-treated macrophages and IKK^{-/-} MEFs. The serine residues of p105 which were normally phosphorylated by IKK were replaced with alanines. Interestingly, this mutant p105 was still able to stabilize Cot but was unable to respond to the LPS signal for degradation and was unable to release Cot_L. Therefore IKK appears to function upstream of Cot in the LPS-stimulated ERK pathway (Figure 1-11) by phosphorylating p105 and permitting active Cot to be liberated.

It was found that Cot_L was preferentially phosphorylated at Thr290 and released from p105 (Figure 1-11) [132]. The phosphorylation at Thr290 reduced the affinity of Cot for p105 binding. In LPS-treated macrophages, Cot was released from p105 even in the presence of the proteasome inhibitor MG132, which suggested the principal cause of the dissociation of Cot from p105 was due to the change of affinity upon phosphorylation of Cot rather than degradation of p105. Mutant Cot (T290A) did not dissociate from p105 in LPS-stimulated macrophages. The phosphomimetic mutant

T290D dissociated from p105, but did not undergo degradation suggesting phosphorylation at T290 was not necessary for targeting to the proteasome.

In line with previous experiments it was shown that Cot phosphorylates p105 and it was suggested that the region covering amino acids 542 to 613 of p105 (within the ankyrin repeats region) is critical for its phosphorylation by Cot [138]. It was confirmed that p105 inhibits the ability of Cot to phosphorylate MEK1. The autophosphorylation of Cot was also reduced but not completely blocked. This, together with the observation that Cot phosphorylates p105, suggests that p105 does not inhibit the intrinsic catalytic activity of Cot but functions as a competitive inhibitor.

Constitutive (*i.e.* unstimulated) Cot activity was noted in a range of HTLV-I (human T cell leukemia virus type I) transformed cells [139]. In the transformed cells and RAW macrophages, the expression level of Cot_S was approximately equal to that of Cot_L, but in untransformed T cells, except for fresh human T cells, the expression level of Cot_S was significantly lower. A weak increase in Cot activity was noted when these fresh human T cells were stimulated with either PMA or ionomycin. A larger increase in activity was noted for the HTLV-I transformed cells which correlated with a larger proportion of Cot that was not bound to p105. It was also found that although Cot_L was rapidly degraded in the HTLV-I transformed cells (via IKK-mediated phosphorylation of p105), Cot_S was much more stable. It was suggested that the

stability of Cot_s might be responsible for the observed constitutive activity. Tax is a protein encoded by HTLV-I and deregulates gene expression. Transfection of HeLa cells with Tax stimulated the activity of Cot via increased degradation of p105, probably through IKK-mediated phosphorylation. Expression of Tax in Jurkat T cells stimulates NF- κ B activity; this effect is enhanced by co-expression of Cot. Co-expression of catalytically inactive Cot inhibits Tax stimulation of NF- κ B.

1.13 Inhibitors of Cot

As Cot has an important role in the LPS-induced production of TNF- α (section 1.11), an inhibitor of Cot would block both TNF- α production and the TNF- α signaling pathway. Furthermore, the different features of Cot (section 1.8) increase the potential for discovering a selective inhibitor. Two classes of inhibitor molecules, 1,7-naphthyridine-3-carbonitriles and quinoline-3-carbonitriles, have been found to be reversible and ATP-competitive inhibitors [82, 83].

Chapter 2

Aims of This Study

The aims of this study were focused on the relationship between the structure and function of Cot protein. To achieve this, the expression of Cot will be performed in *E. coli* and insect cells using different Cot truncations. This will be followed by purification and crystallization screening of the Cot protein. Since the Cot protein overexpressed in a range of human tumor cells, Cot may be a potentially valuable target for chemotherapeutic intervention. From the studies discussed in Introduction, it seems probable that Cot is involved in several cellular pathways and therefore it is likely that Cot has several binding partners and substrates. Functional studies including a 2D (2 dimensional) proteomics study and a substrate screening study will be performed in an attempt to uncover new binding partners and new substrates of Cot.

Chapter 3

Materials and Methods

3.1 Molecular cloning and expression

3.1.1 Molecular cloning and expression of 6×His-tagged Cot in *E. coli*

The nine pET24a constructs with a C-terminal 6×His tag [Cot₁₋₃₈₈, Cot₁₋₄₁₃, Cot₁₋₄₃₈, Cot₁₋₄₅₇, Cot_{full-length}, Cot_{full-length} (with the codon usage of the last 10 amino acids optimized), Cot₁₃₂₋₃₈₈, Cot₁₃₂₋₄₆₇] and two pET16 constructs with an N-terminal 6×His tag (Cot₁₋₃₈₈, Cot_{full-length}) were generated by PCR using Cot cDNA (Genecopiea) as the template. The sequences of the primers used were as follows: forward, 5'TTAGGATCCATGGAGTACATGAGCACTGG3', reverse, 5'AATCTCGAGGCCA TATTCAAGCGTTGGTG3' for pET24-Cot_{full-length} (inserted between *Bam*HI and *Xho*I); forward, 5'CGCGGATCCATGTGGAAGCTGACTTACAGG3', reverse, 5'GC GCTCGAGCAGGGCCTCATGTTTTAG3' for pET24-Cot₁₋₃₈₈ (inserted between *Bam*HI and *Xho*I); forward, 5'ATACATATGGAGTACATGAGCACT3', reverse, 5'AATCTCGAGCCTGTAAGTCAGCTTCCA3' for pET24-Cot₁₋₁₃₇ (inserted between *Nde*I and *Xho*I); forward, 5'ATACATATGGAGTACATGAGCACTGG3', reverse, 5'AATCTCGAGTCAGCCATATTCAAGCGTTG3' for pET16-Cot_{full-length} (inserted between *Nde*I and *Xho*I); forward, 5'ATACATATGGAGTACATGAGCACTGG3', reverse, 5'GCGCTCGAGTCACAGGGCCTCATGTTTTAG3' for pET16-Cot₁₋₃₈₈ (inserted between *Nde*I and *Xho*I). forward, 5'TTAGGATCCATGGAGTAC

ATGAGCACTGG3', reverse, 5'AATCTCGAGACTCAGCAGCCTCTTGCG3' for pET24-Cot₁₋₄₁₃ (inserted between *EcoRI* and *XhoI*); forward, 5'TTAGGATCCATG GAGTACATGAGCACTGG3', reverse, 5'AATCTCGAGGAGCATCTCAGATTCC TC3' for pET24-Cot₁₋₄₃₈ (inserted between *EcoRI* and *XhoI*); forward, 5' TTAGGATCCATGGAGTACATGAGCACTGG3', reverse, 5'AATCTCGAGAAG ATTGAAGTAGCCAGC3' for pET24-Cot₁₋₄₅₇ (inserted between *EcoRI* and *XhoI*); forward, 5'TCCGAATTCATGGAGTACATGAGCACT3', reverse, 5'AATCTCGAG TCAGCCATATTCcagCGTTGGTGGgccgcgAAC3' for pET24-Cot_{full-length} mutation (inserted between *EcoRI* and *XhoI*, lowercase indicates the base pairs changed in order to optimize the codon usage for *E. coli*); forward, 5'TTAGGAT CCTGGAAGCTGACTTACAGG3', reverse, 5'AATCTCGAGCAGGGCCTCA TGTTTTAG3' for pET24-Cot₁₃₂₋₃₈₈ (inserted between *EcoRI* and *XhoI*); forward, 5'TTAGGATCCTGGAAGCTGACTTACAGG3', reverse, 5'AATCTCGAGGCC ATATTCAAGCGTTGGTG3' for pET24-Cot₁₃₂₋₄₆₇ (inserted between *EcoRI* and *XhoI*).

PCR reactions were performed with the Faststart high fidelity PCR system (Roche) according to the manufacturer's protocol. The PCR was performed for 30 reaction cycles [incubated at 95 °C for 10 min, then 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1-2 min (depending on the length of PCR fragment, 1 kb min⁻¹)]. The reactions were refined by another 10 min incubation at 72 °C. Ligation of the double-enzyme digested PCR fragment and the vector was performed with T4 ligase (NEB) at 18 °C overnight. The ligation product was transformed into *E. coli* DH5α competent cells which were prepared using the conventional calcium chloride method [140]. The

competent cells were mixed with the plasmid and incubated on ice for 30 min. The cells were heat shocked at 42 °C for 60 sec. The cells were chilled on ice for 2 min. LB (Luria-Bertani) medium (0.5 mL) was added to the cells and incubated at 37 °C with shaking for 1 hr. The cells were spread to LB plates (10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl, 20 g L⁻¹ agar) with the correct antibiotic (100 µg mL⁻¹ ampicillin for pET16 constructs, 50 µg mL⁻¹ kanamycin for pET24 constructs). Colonies from the selective LB plates were picked for plasmid extraction (Qiagen) and further verification. All the insertions were verified by double restriction enzyme digestion and DNA sequencing.

The constructs were transformed into *E. coli* BL21 (DE3) cells for protein expression as described above. A single colony was inoculated into 5 mL of culture medium containing the correct antibiotic and grown overnight. LB medium (1L, 10 g L⁻¹ tryptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl) was inoculated with 5 mL overnight culture and grown at 37 °C. After the OD₆₀₀ (optical density) reached 0.6-0.8, IPTG (isopropyl-β-D-1-thiogalactopyranoside) at a final concentration of 0.5 or 1 mM was added. After a 6 hr induction at low temperature (16, 18, 20 or 25 °C), cells were harvested by centrifuging at 5000×g for 10 min. The cell pellet was resuspended in the lysis buffer [25 mM Tris-Cl pH 8, 10% (v/v) glycerol, 2.5 mM DTT (1,4-dithiothreitol), 1 mM PMSF (phenylmethylsulfonyl fluoride), 1 µM pepstatin A] and disrupted by sonication (100 Watts, Sonic). The expression was checked using a 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gel. For

protein purification, the lysate was clarified by centrifuging at 25000×g for 20 min.

3.1.2 Expression of Cot in BL21-CodonPlus (DE3)-RIPL

The expression construct was transformed into BL21-CodonPlus (DE3)-RIPL (Stratagene) using the conventional calcium chloride method as described previously. A single colony was inoculated into 5 mL of culture medium containing the proper antibiotic (100 µg mL⁻¹ ampicillin and 75 µg mL⁻¹ streptomycin for pET16 constructs, 50 µg mL⁻¹ kanamycin and 75 µg mL⁻¹ streptomycin for pET24 constructs) and grown overnight. LB medium (1 L) was inoculated with 5 mL overnight culture and grown at 37 °C. After the OD₆₀₀ reached 0.6-0.8, IPTG at a final concentration of 0.5 or 1 mM was added. After a 6 hr induction at low temperature (16, 18, 20 or 25 °C), cells were harvested by centrifuging at 5000×g for 10 min. The cell pellet was resuspended in the lysis buffer and disrupted by sonication (100 Watts, Sonic) as described previously. The expression was checked using a 10% SDS-PAGE gel. For protein purification, the lysate was clarified by centrifugation at 25000×g for 20 min.

3.1.3 Expression of GST-tagged Cot in E. coli BL21(DE3)

The constructs of pGEX-4T-1-Cot₁₋₃₈₈, pGEX-6P-1-Cot₁₋₃₈₈, pGEX-6P-1-Cot₁₋₄₅₇, pGEX-6P-1-Cot_{full-length}, pGEX-6P-1-Cot₁₃₀₋₃₉₉, pGEX-6P-1-Cot₁₋₁₃₇ were generated by PCR using Cot cDNA as the template. pGEX-4T-1 (GE Healthcare) has a thrombin cleavage site between the GST (glutathione-S-transferase) and the recombinant protein. pGEX-6P-1 was purchased from GE Healthcare and has a Prescission

protease (GE Healthcare) cleavage site between the GST and the recombinant protein.

The sequences of the primers used were as follows: forward, 5'TCCGAATTCATGGAGTACATGAG CACT3', reverse, 5'GCGCTCGAGCAGG GCCTCATGTTTTAG3' for pGEX-4T-1-Cot₁₋₃₈₈ and pGEX-6P-1-Cot₁₋₃₈₈ (inserted between *EcoRI* and *XhoI*); forward, 5'TCCGAATTCATGGAGTACATGAGCACT3', reverse, 5'AATCTCGAGA AGATTGAAGTAGCCAGC3' for pGEX-6P-1-Cot₁₋₄₅₇ (inserted between *EcoRI* and *XhoI*); forward, 5'TCCGAATTCATGGA GTACATGAGCACT3', reverse, 5'AATCTC GAGTCAGCCATATTCcagCGT TGGTGGgccgcgAAC3' for pGEX-6P-1-Cot_{full-length} (inserted between *EcoRI* and *XhoI*, lowercase indicates the base pairs changed in order to optimize the codon usage for *E. coli*); forward, 5'TCCGAATTCT GGAAGCTGACTTTA3' , reverse, 5'AATCTCGAGCTAAATTTTGCCTTCTAG3' for pGEX-6P-1-Cot₁₃₀₋₃₉₉ (inserted between *EcoRI* and *XhoI*); forward, 5'TCCG AATTCATGGAGTACATGAGCACT3', reverse, 5'AATCTCGAGCCTGTAAGTCA GCTTCCA3' for clone pGEX-6P-1-Cot₁₋₁₃₇ (inserted between *EcoRI* and *XhoI*). All the insertions were verified by double restriction enzyme digestion and DNA sequencing. PCR reactions were performed with Faststart high fidelity PCR system (Roche) as previously described. The plasmids were transformed into *E. coli* BL21 (DE3) cells. LB medium (500 mL) was inoculated with 5 mL overnight culture and grown at 37 °C. After the OD₆₀₀ reached 0.6-0.8, IPTG at a final concentration of 0.5 mM was added. After overnight induction at 16 °C, cells were harvested by centrifuging at 5000×g for 10 min. The pellets were resuspended in 20 mL cold lysis buffer (PBS, 1 mM PMSF, 1 μM pepstatin A) and

disrupted by sonication as described previously. Expression was checked using a 10% SDS-PAGE gel. For protein purification, the lysate was clarified by centrifuging at 25000×g for 20 min.

3.1.4 Expression of selenomethionine-substituted protein

The plasmid (pGEX-4T-1-Cot₁₋₃₈₈) was transformed into *E. coli* BL21 (DE3) cells. A single colony was inoculated into 5 mL of LB medium containing 100 µg mL⁻¹ ampicillin at 37 °C with shaking overnight. The starter culture was transferred to 50 mL M9 medium (0.6% Na₂HPO₄, 0.3% KH₂PO₄, 0.05% NaCl, 0.1% NH₄Cl, 1 mM MgSO₄, 0.4% glucose, 0.1 mM CaCl₂, 0.00005% thiamine) with 100 µg mL⁻¹ ampicillin for another 5 hr incubation with shaking. The culture was transferred to 1 L M9 medium for further incubation. After the OD₆₀₀ reached 0.6-0.8, amino acid supplements (50 mg L-isoleucine, 50 mg L-leucine, 50 mg L-lysine, 100 mg L-phenylalanine, 100 mg L-threonine, 50 mg L-valine, 50 mg L-selenomethionine) were added and incubated at 37 °C for 20 min with shaking. IPTG at a final concentration of 1 mM was added. The incubation was continued at 25 °C overnight with shaking. Cells were harvested by centrifuging at 5000×g for 10 min. The pellets were resuspended in 20 mL cold lysis buffer (PBS, 1 mM DTT, 0.1% Triton X-100, 0.5 M NaCl, 1 mM PMSF, 1 µM pepstatin A) and disrupted by sonication as described previously. The lysate was clarified by centrifuging at 25000×g for 20 min.

3.1.5 Molecular cloning of *Cot* for expression in insect cells

In order to express the Cot_{1-388} and $Cot_{full-length}$ in insect cells, following primers were used: forward, 5'GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGAGTACATGAGCCAC3'; reverse, 5'GGGGACCACTTTGTACAAGAAAGCTGGGTCCTAACAGCGTGGCTGATCCTC3' (for pENTRY- Cot_{1-388}). Since the *Cot* full-length was already in the plasmid pENTRY- $Cot_{full-length}$, the BP (*attB* and *attP*) reaction step was not performed. For pENTRY- Cot_{1-388} , PCR reactions were performed with the Faststart high fidelity PCR system (Roche) as previously described. The PCR product was separated by agarose gel electrophoresis and purified using a gel extraction kit (Qiagen). A BP reaction was performed by mixing the purified PCR product (2 μ g) with pDONR (300 ng) (Invitrogen), 5 $\times$ BP reaction buffer (4 μ L), and BP enzyme mix (4 μ L) (Invitrogen), followed by incubation at 25 °C for 1 hr. The enzyme was removed by adding protease K (2 μ L) (Invitrogen) and incubating at 37 °C for 10 min. The reaction solution (1 μ L) was incubated with DH5 α competent cells (50 μ L) (Novagen) on ice for 30 min and then heat-shocked at 42 °C for 30 sec. SOC medium (450 μ L) (Novagen) was added to the cell mixture and incubated at 37 °C for another 1 hr. The cell culture (100 μ L) was spread onto a LB plate containing kanamycin (50 μ g mL⁻¹). The plasmids from the positive colony was extracted using the plasmid extraction kit (Qiagen) and verified by sequencing. This plasmid was named the Entry clone. The LR (*attL* and *attR*) reaction was performed by mixing the Entry clone (2 μ g) with pDEST 10 (300 ng) (Invitrogen), 5 $\times$ LR reaction buffer (4 μ L), and LR enzyme mix (4 μ L) (Invitrogen), followed by incubation at 25 °C for 1 hr. The

enzyme was removed by adding protease K (2 μ L) (Invitrogen) and incubating at 37 °C for 10 min. Transformation was performed as previously described. However, different competent cells were used. For the transformation of the LR reaction, DB3.1 competent cells (Invitrogen) were used. The positive colony was selected by the LB plate containing ampicillin (100 μ g mL⁻¹). The plasmids from the positive colony was extracted using the plasmid extraction kit (Qiagen) and verified by DNA sequencing. In order to form the recombinant baculovirus, the constructs of pDEST-Cot₁₋₃₈₈ and pDEST-Cot_{full-length} were transformed into competent cells DH10Bac (Invitrogen). The positive colony was selected by an LB plate (20 mL) containing 20% IPTG (7 μ L), 2% X-gal (40 μ L), 50 mg mL⁻¹ kanamycin (20 μ L), 50 mg mL⁻¹ gentamicin (5 μ L), 10 mg mL⁻¹ tetracyclin (20 μ L) and plasmids were extracted using the large construct plasmid extraction kit (Qiagen) and verified by DNA sequencing.

3.1.6 Culture of insect cells *Spodoptera frugiperda* sf9 and transfection

Spodoptera frugiperda sf9 cells were cultured at 27 °C without additional CO₂ and passaged every 3 days. The 1×10⁷ sf9 cells were seeded per 60-mm cell culture dish and transfected with 5 μ g of recombinant baculovirus DNA, using the lipofectamine transfection kit (Invitrogen). Transfected cells were incubated at 27 °C for 4 hr, then the transfection medium was removed and replaced with fresh sf-900 II SFM medium (Invitrogen). After 72 hr incubation at 27 °C, the virus-containing supernatant was harvested. A total of 7×10⁶ sf9 cells was seeded in a 75-cm² cell culture flask and infected with the recombinant viral stock. A hemocytometer was used for measuring

cell density and trypan blue exclusion was used to check the cell viability. Cells were harvested after 24 hr transfection by centrifuging at 500×g for 5 min, washed with phosphate-buffered saline (PBS) buffer, and resuspended in 500 µL lysis buffer [25 mM Tris pH 8, 10% (v/v) glycerol, 2.5 mM DTT, 1 mM PMSF, 1 µM pepstatin A]. Whole cell lysate was mixed with the SDS loading buffer, and a western blot performed with anti-Cot (N17).

3.1.7 Molecular cloning of plasmids for expression in mammalian cells

N-terminal 6×His tagged Cot₁₃₂₋₃₉₉, Cot₁₋₃₈₈, Cot₁₋₄₁₃, Cot₁₋₄₃₈, Cot₁₋₄₅₇, Cot_{full-length} and Plk1 were constructed by subcloning *EcoRI* and *XhoI* digested PCR fragments into pcDNA4 HisMax vector (Invitrogen). The Cot kinase-dead mutant (167K to R, AAA to AGA) was generated by the fusion PCR method [141]. The *EcoRI* and *XhoI* digested PCR fragments (Cot_{full-length} kinase-dead mutant and Plk1) were inserted into pcDNA4 HisMax (Invitrogen). The PCR primers used were as follows: forward, 5'TCCGAATTCCCCTGGAAGCTGACTTAC3' and reverse, 5'AATCTCGAGAC TCTGACAGCGTGGCTG3' (pcDNA-Cot₁₃₂₋₃₉₉); forward, 5'TCCGAATTTCGAGTACATGAGCACT3', reverse, 5'AATCTCGAGCAGGGCCTCATGTTTTAG3' (pcDNA-Cot₁₋₃₈₈), 5'AATCTCGAGACTCAGCAGCCTCTTGCG3' (pcDNA-Cot₁₋₄₁₃), 5'AATCTCGAGGAGCATCTCAGATTCCTC3' (pcDNA-Cot₁₋₄₃₈), 5'AATCTCGAGAGATTGAAGTAGCCAGC3' (pcDNA-Cot₁₋₄₅₇), 5'AATCTCGAGGCCATATTCAAGCGTTGG3' (pcDNA-Cot_{full-length}); forward, 5'TCCGAATTCATGAGTGCTGCAGTGACT3'; reverse, 5'AATCTCGAGTTAGGAGGCCTTGAGACG3' for Plk1

(inserted between *EcoRI* and *XhoI*); forward 1, 5'TCCGAATTCCCCTGGAAGCTGACTTAC3'; forward 2, 5'AAAAGAATGGCGTGTagaCTGATCCCAGTAGAT3' and reverse 1, 5'AATCTCGAGGCCATATTCAAGCGTTGG3'; reverse 2, 5'ATCTACTGGGATCAGtctACACGCCATTCTTTT3' for the fusion PCR of pcDNA-Cot_{full-length} kinase-dead mutant [inserted between *EcoRI* and *XhoI*, lowercase indicates the base pairs changed in order to generate the mutation from Lys(AAA) to Arg(AGA)]. All insertions were verified by double restriction enzyme digestion and DNA sequencing.

3.1.8 Mammalian cell culture

HEK293, MCF7, A549 and Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 4 mM L-glutamine, 1 mM sodium pyruvate, 100 µg mL⁻¹ ampicillin, 100 µg mL⁻¹ streptomycin at 37 °C in a humidified atmosphere of 5% CO₂. The cells were grown in 100 mm dishes or 96-well plate and were transfected at 60-70% confluency using the standard calcium phosphate method with 10 µg constructs. Cells were harvested at 48 hr post-transfection for the expression test and 2D gel analysis, or changed to the medium without FCS (fetal calf serum) for overnight starvation. Cells were left untreated or treated with 200 ng mL⁻¹ LPS or 20 ng mL⁻¹ TNF-α for another 6 hr for the kinase activity analysis.

3.1.9 Transfection and expression of Cot in HEK293 cells

Calcium phosphate transfection [142]: the cells (60-70% confluency) were plated to a 100 mm dish one night before transfection. The medium was changed to fresh complete medium with 25 μ M chloroquine to enhance the transfection efficiency. One hour later, endotoxin-free plasmid (5 μ g) was mixed with 548 μ L of ddH₂O. The 2 M CaCl₂ (78 μ L) was mixed with the plasmid solution drop-wise. Finally, 2 $\times$ HBS (HEPES-buffered saline, 625 μ L) was mixed with the plasmid solution drop-wise. This solution was added to a 100 mm cell dish drop-wise. The dish was incubated for 7-11 hr, then washed with fresh medium followed by further incubation in fresh medium for another 48 hr. Cells were harvested if they were used for the binding partner experiment (2D gel electrophoresis). To activate the expressed Cot, 48 hr post-transfection, the cells were changed to the medium without FCS for overnight starvation. Cells were treated with 200 ng mL⁻¹ LPS for another 6 hr before harvest. During the cell lysis, an extra step, 6 s ultrasonication (7 watt), was introduced to remove the possible binding partners of Cot.

3.1.10 Co-transfection and expression of Cot with Plk1

Plasmids (5 μ g each) of Cot_{full-length} and Plk1 or Cot_{full-length} kinase-dead mutant and Plk1 were co-transfected into HEK293 cells by the standard calcium phosphate transfection method as described previously. After 48 hr post-transfection, the cells were starved overnight and treated with 200 ng mL⁻¹ LPS for another 6 hr before harvesting.

3.2 Purification

3.2.1 Purification of 6×His-tagged protein

Clarified cell lysate was loaded onto Ni-NTA (nickel nitrilotriacetic acid) Superflow resin (Qiagen) (5 mL) in a glass econo-column (15×10, Bio-Rad). The column was washed with 2 column volumes of buffer A (20 mM Tris-Cl pH 8.0, 500 mM NaCl), and then the protein was eluted with buffer B (20 mM Tris-Cl pH 8.0, 500 mM NaCl, 500 mM imidazole). The buffer of the eluant was changed to 50 mM Tris-Cl pH 8.0 and concentrated with a centrifugal filter unit (Amicon, Millipore) for further purification steps.

3.2.2 Purification of 6×His-tagged protein under denaturing conditions

The cell debris was washed with 20 mM Tris-Cl buffer pH 8.0 (20 mL) 10 times, resuspended in 20 mL buffer C (100 mM Na₂HPO₄, 10 mM Tris-Cl, 8 M urea, pH 8.0), and loaded onto Ni-NTA superflow resin (Qiagen) (10 mL) in a XK16/20 column (GE Healthcare). The column was washed with buffer D (100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea, pH 6.3) for 2 column volumes, and then the protein was eluted with buffer E (100 mM NaH₂PO₄, 10 mM Tris-Cl, 500 mM imidazole, 8 M urea, pH 5.9). Refolding by dialysis bag (10000 Da cut-off, Millipore) against a urea buffer of lower concentration was performed in the following steps: 4 M urea, 2 M urea, 0.5 M urea, 0.1 M urea and refolding buffer (20 mM Tris-Cl, 1 mM EDTA, pH 8.0). The final solution was then concentrated with a centrifugal filter unit and loaded onto a Superdex 75 column (24 mL) (GE Healthcare) and eluted with the elution

buffer (50 mM Tris-Cl, 100 mM NaCl). The target protein was collected and then desalted with a BioSpin-6 column (Bio-Rad). Protein concentration was measured using the Bradford method. The protein (20 to 200 μ g) was mixed with Bradford reagent (5 mL) (Sigma-Aldrich) and incubated at room temperature for 5 min. The absorbance at 590 nm was measured. BSA (bovine serum albumin) standard (Sigma-Aldrich) was used for the standard curve.

3.2.3 Purification of GST (glutathione S-transferase)-tagged protein

Clarified cell lysate was incubated with 2 mL glutathione sepharose 4B slurry (GE Healthcare) at room temperature for 2 hr. The slurry was washed with 5 volumes of PBS. The GST-tagged protein was eluted with 2 volumes of elution buffer (10 mM reduced glutathione, pH 8.0). The eluant buffer was changed to 50 mM Tris-Cl pH 8.0 and concentrated with a centrifugal filter unit for further purification steps.

3.2.4 Cleavage of GST tag

For on-column cleavage, GST-tagged protein (0.4 mg) was bound to the GST affinity column. The column was washed with PBS twice to remove unbound proteins, and then washed with cleavage buffer (50 mM Tris-Cl pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT) twice to exchange the buffer. The 40 μ L of GST-tagged thrombin (2000 units mL⁻¹, GE Healthcare) or GST-tagged Prescission protease (2000 units mL⁻¹, GE Healthcare) was added to the column. The column was incubated at 37 °C for thrombin or 4 °C for Prescission protease for 1-12 hours. For off-column cleavage,

The 40 μL of purified GST fusion protein (10 mg mL^{-1}) was mixed with 40 μL of the protease. The cleavage reaction was achieved by incubation at 37 °C for thrombin or 4 °C for Prescission protease overnight. The flow-through was collected as the cleaved protein.

3.2.5 Gel filtration chromatography

Superdex 75 10/300 GL (24 mL), Superdex 75 16/60 Hiloal (120 mL), Superdex 200 10/300 GL (24 mL) and Superdex 200 16/60 Hiloal (120 mL) were purchased from GE Healthcare. Superdex 75 is suitable for purifying proteins from 3000 to 70000 Da (Dalton). Superdex 200 is suitable for purifying proteins from 10000 to 600000 Da. The column was connected to an AKTA FPLC (fast performance liquid chromatography, GE Healthcare). The buffers were degassed and filtered (0.2 μm) prior to use. The column was equilibrated with 2 column volumes of buffer (50 mM Tris-Cl, 100 mM NaCl). The sample was filtered using a 0.2 μm syringe filter (Millipore) before loading. The protein fractions were collected according to the UV_{280} spectrum peaks. The protein was detected with an SDS-PAGE gel.

3.2.6 Anion exchange chromatography

Q-sepharose and Mono-Q HR 5/5 (1 mL) column were purchased from GE Healthcare. The column was washed with 10 column volumes of buffer (20 mM Tris-Cl pH 8.0, 1 M NaCl) to remove impurities and then equilibrated with 10 column volumes of buffer (20 mM Tris-Cl pH 8.0). Sample was desalted using a BioSpin-6

column and the buffer was changed to 20 mM Tris-Cl pH 8.0 using a centrifugal filter unit and filtered using a 0.2 µm syringe filter (Millipore) before loading. After sample loading, the column was washed with two column volumes of buffer (20 mM Tris-Cl pH 8.0). The protein was then eluted with a NaCl gradient (0-1 M, 20 column volumes) in 20 mM pH 8.0 Tris-Cl. The protein fractions were collected according to the UV₂₈₀ spectrum peaks. The protein was detected with an SDS-PAGE gel.

3.2.7 Removal of contaminating E. coli chaperone

The cell culture was carried out as previously described. The pellet was resuspended with 20 mL of 20 mM Tris-Cl pH 7.0 buffer containing 5 mM ATP and 5 mM MgCl₂. The cells were disrupted by sonication. Clarified cell lysate was incubated with 2 mL glutathione sepharose 4B slurry at room temperature for 2 hr. The column was washed with 5 column volumes of PBS containing 4 M, 5 M or 6 M urea. The bound proteins were eluted with elution buffer (10 mM reduced glutathione pH 8.0) and resolved on a 10% SDS-PAGE gel.

3.2.8 Alternative method for the purification of GST-tagged protein

The cell culture was prepared as previously described. The pellet was resuspended with 20 mL PBS and disrupted by sonication. Clarified cell lysate was passed through the GST affinity column twice to remove impurities. The buffer of the eluant from the GST column was changed to 50 mM pH 8.0 Tris-Cl and concentrated. The sample was loaded onto a Q-sepharose column XK16/20 (GE Healthcare) and eluted with a

NaCl gradient (0-1 M) in 50 mM pH 8.0 Tris-Cl. The samples were collected when the NaCl concentration was between 0.23 to 0.28 M. The fractions were combined and the buffer was changed to 50 mM pH 8.0 Tris-Cl together with 100 mM NaCl followed by concentration. The concentrated sample was loaded onto a Superdex 200 (24 mL) column. The fractions were collected every 0.5 mL. The fractions that came out from 11.5 mL to 15.5 mL were combined and the buffer was changed to 50 mM pH 8.0 Tris-Cl. The concentrated protein solution was loaded onto a Mono-Q column. The target protein was eluted with a NaCl gradient (0-1 M) in 50 mM pH 8.0 Tris-Cl. The fractions which came out from the NaCl concentration 0.23 to 0.28 M were collected. The fractions were analyzed by a 10% SDS-PAGE gel.

3.2.9 Protein secondary structure measurement

The protein secondary structure was analyzed by circular dichroism (CD, Chirascan, Applied Photophysics). The buffer of purified protein sample was changed to 20 mM pH 7.0 phosphate buffer and concentrated to 1 mg mL⁻¹ with a centrifugal filter unit. The sample (60 µL) was loaded into a micro-cuvette and scanned from 260 nm to 180 nm (1 second for 1 nm). 20 mM pH 7.0 phosphate buffer was used as the baseline. After all measurements were finished, the data was exported and converted to an ASCII two column text file by the software APLDataConverter, and the data was subtracted from the baseline and analyzed using the program CDNN.

3.3 Crystallization

3.3.1 Crystallization screen

The purified protein was concentrated with a centrifugal filter unit to a final concentration of 5-30 mg mL⁻¹ and desalted. Initial crystallization trials were set up using the hanging drop vapor diffusion method with the Crystal Screen I and II crystallization kits (Hampton Research). Subsequently, different combinations of precipitant, buffers, and salt solutions were used to screen the optimal conditions for crystallization. The crystallization solution (500 µL) was placed in each of the reservoirs of a 24-well tissue culture plate. The protein solution (1 µL), mixed with an equal volume of crystallization solution from each reservoir, was placed on a plastic cover slip. Plates were incubated at 18 °C and checked for crystals at periodic intervals.

In addition, many different commercial solutions (such as the pre-crystallization test kit, PEG/Ion screen kit, crystal screen kit, additive screen kit from Hampton research) and home-made screens (over 200 different unique solutions). Different temperatures (4 °C, 16 °C and 25 °C), different protein concentrations (10 mg/mL⁻¹, 20 mg/mL⁻¹, 30 mg/mL⁻¹) and different methods (sitting drop and hanging drop) were tried for growing crystals.

3.3.2 Optimization of the crystallization screen

Purified protein was concentrated to 10 mg mL⁻¹. Crystallization screens were set up by the same method previously described. Further screen conditions were extended from the conditions from which crystals had appeared. This included different precipitate concentrations, pH buffers (4-10), protein concentrations (5-30 mg mL⁻¹), incubation temperatures (4, 10, 18 °C), crystallization methods (hanging drop or sitting drop), and additives (based on the Hampton additive screen).

3.3.3 X-ray diffraction

Crystals were picked up with a nylon loop (Hampton Research) and placed into a cryoprotection solution (30% glycerol with 70% well solution). The crystals were then mounted on the X-ray diffractometer (Rigaku MicroMax-007 X-ray generator and R-axis IV++ image plate detector) at a low temperature (-170 °C) using cold dry nitrogen gas. The distance from crystal to detector was set between 100 to 200 mm. CrystalClear was used to control the data collection. The oscillation angle was usually 1° and the exposure time for each image was 10-40 min. Images were analyzed by the program MOSFLM (CCP4 suite [143]).

3.4 2D proteomics

3.4.1 2D electrophoresis

The first dimension IEFs (isoelectric focusing) were performed on the 18 cm IEF gel strips (3-11NL, GE Healthcare) following the manufacturer's instruction. The dry

strips were rehydrated at room temperature overnight with 375 μ L rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate], 50 mM DTT, 3% IPG (immobilized pH gradient) buffer, 0.02% bromophenol blue). The protein (50 μ g per strip) was loaded by cup-loading. IEF was performed by Ettan IPGphor II (GE Healthcare) at 20 °C using the following parameters: 500 V for 500 Vhr; 1000 V for 500 Vhr; 8000 V gradient for 4500 Vhr; 8000 V for 64000 Vhr. Strips were soaked in 10 mL of equilibration solution (6 M urea, 0.05 M Tris-Cl pH 6.8, 30% glycerol, 2% SDS) supplemented with 100 mg DTT for 15 min. The strips were soaked in 10 mL equilibration solution supplemented with 250 mg iodoacetamide and 0.02% bromophenol blue for 15 min. Strips were placed on the top of a 10% SDS-PAGE gel (Protean II Xi, Bio-Rad). The second dimension electrophoresis was performed at 5 °C. The current was kept constant at 7 mA per gel for 1 hr then increased to 25 mA per gel for another 6 hr.

3.4.2 Silver staining

Silver staining was performed following the manufacturer's instruction. The gel was fixed with 40% (v/v) ethanol and 10% (v/v) acetic acid for 30 min. The gel was sensitized with 30% (v/v) ethanol, 0.125% (w/v) glutardialdehyde, 0.5% (w/v) sodium thiosulfate, 6.8% (w/v) sodium acetate for 30 min, followed with 3 times washing with Milli-Q water. The gel was soaked in silver reaction solution [0.25% (w/v) silver nitrate, 0.4% formaldehyde] for 30 min. After 2 times washing with Milli-Q water, the gel was developed with 2.5% (w/v) sodium carbonate and 0.2% (v/v) formaldehyde.

The reaction was stopped by 2.5% (w/v) EDTA- Na_2 solution. The gel was scanned with ImageScanner II (GE Healthcare) and analyzed with the program ImageMaster (GE Healthcare) and DYMENSION (Syngene).

3.4.3 Protein identification by mass spectrometry

To destain the silver-stained gel slices: the gel slices were soaked in freshly prepared 200 μL of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate for 15 min. The chemicals were washed away by 3 $\times$ 5 min Milli-Q water. The gel slices were soaked in 100% acetonitrile (85 μL) for 5 min followed by 5 min concentration using a Speedvac concentrator (Eppendorf).

To destain the CBB (Coomassie brilliant blue)-stained gel slices: the gel slices were soaked in 200 μL of 50% (v/v) acetonitrile with 50 mM ammonium bicarbonate and incubated at 37 °C for 30 min. The destained gel slices were dried by 5 min concentration using a Speedvac concentrator.

Each gel slice was soaked in 20 μL sequencing grade trypsin (3.33 ng μL^{-1} , diluted with 50 mM ammonium bicarbonate) (Promega) at 30 °C overnight or 37 °C for 4 hr. The peptides were extracted with 200 μL of 50% (v/v) acetonitrile and 0.5% (v/v) TFA (trifluoroacetic acid) followed by 10 min ultrasonic water bath (20 Watts, 37 °C). The supernatant was collected and dried using a Speedvac concentrator for 4 hr. The peptides were dissolved in 0.5% (v/v) TFA (1.5 μL). The peptides were desalted with

a ZipTip (Millipore) following the manufacturer's instruction. The tip was equilibrated with 100% acetonitrile, followed by 0.5% (v/v) TFA. The ZipTip was used to aspirate the peptide sample for 10 times for maximum binding. The ZipTip was washed with 0.5% (v/v) TFA twice. The peptides were eluted with 1 μ L of a solution of 50% acetonitrile containing 0.5% (v/v) TFA. The peptides were mixed with an equal volume of freshly prepared CHCA matrix solution [α -cyano-4-hydroxycinnamic acid (5 mg) dissolved in 50% (v/v) acetonitrile with 0.5% (v/v) TFA (1 mL)]. The mixture (1 μ L) was spotted onto a MALDI plate [384 opti-TOF (time-of-flight), 123 $\times$ 81 mm RecA, ABI] and air dried. A microscope was used for checking the matrix following crystallization. The spots were identified by a pre-programmed method (combined MS and MS/MS) with the ABI MALDI TOF/TOF 4800 analyzer following the manufacturer's instruction. Trypsin-derived peptides were used as an internal standard. The MS/MS results were analyzed by searching the Mascott V1.9 database. Parameters used for searching were: one missed cleavage, oxidation on methionine, and 0.3 Da mass tolerance. The results of the MS and MS/MS were matched to the NCBI protein database. Any MOWSE score higher than 75 was regarded as significant.

3.5 Functional experiments

3.5.1 Immunoprecipitation

Clarified lysate (500 μ g) was incubated with 20 μ L primary antibody for 2 hr at 4 $^{\circ}$ C, followed by incubation with 20 μ L protein G-agarose slurry (Invitrogen) for another

1 hr at 4 °C. The agarose was harvested by centrifugation (14000×g for 1 min), washed with the buffer (20 mM pH7.5 HEPES, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 mM Na₃VO₄) twice and resuspended in 50 µL of 2× kinase buffer (40 mM pH 7.2 MOPS, 100 mM β-glycerophosphate, 6 mM EDTA, 2 mM EGTA, 20 mM NaF, 2 mM Na₃VO₄, 2 mM DTT, 40 mM MgCl₂). The immunoprecipitate (5 µL) was resolved on a 10% SDS-PAGE gel and probed with the appropriate antibody to verify the precipitated protein.

3.5.2 Western blotting

The anti-6×His (H20) was from Delta Biolabs. The anti-Cot (H212), anti-Cot (M20), anti-Cot (N17), anti-PDI (protein disulphide isomerase), anti-p105, anti-p-MEK1/2 (Ser 217/221), anti-Plk1 (F8), anti-Hsp90, anti-mouse-HRP (horseradish peroxidase), anti-rabbit-HRP and anti-goat-HRP were from Santa Cruz. The anti-MEK1/2 and anti-p-Plk1 (Ser137) were from Cell Signaling Technology. The anti-p-Plk1(Thr210) was purchased from BD Pharmingen.

Cells were lysed in the lysis buffer (0.1% Triton X-100, 25 mM pH 7.5 Tris-Cl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 20 mM β-glycerophosphate, 1 mM Na₃VO₄, Roche complete protease inhibitor cocktail). The proteins in the soluble fraction were isolated by centrifugation (14500×g, 10 min) at 4 °C. The supernatant was kept for later experiments. The total protein concentration was determined by the Bradford assay (Sigma-Aldrich).

For western blotting, the protein solution (50 μ g for total cell lysates, 5 μ g for immunoprecipitants and 1 μ g for purified proteins) was mixed with an equal volume of SDS loading buffer (100 mM Tris-Cl pH 6.8, 1% SDS, 20% glycerol, 2% mercaptoethanol, 0.2% bromophenol blue) and separated by an SDS-PAGE gel. The proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare) by the semi-dry blotting (Bio-Rad) following the manufacturer's instruction. The PVDF membrane was blocked for 1 hr at room temperature with 2% BSA in TBST (Tris-Buffered Saline Tween-20), and incubated for 1 hr with the appropriate diluted primary antibody. After washing 3 times with TBST, the PVDF membrane was incubated for another 1 hr with the appropriate diluted secondary antibody conjugated to horseradish peroxidase (HRP) at room temperature. After 3 times washing with TBST, the membrane was stained by enhanced chemiluminescence (ECL plus, GE Healthcare) and visualized using an X-ray film (Kodak).

3.5.3 Kinase assay in vitro

The [γ - 32 P]ATP-labeled: kinase assay was modified from previous papers [144]. The reaction mixture (20 μ L) containing 70 mM Tris-Cl (pH 8.0), 10 mM MgCl_2 , 5 mM DTT, 0.05 $\mu\text{g } \mu\text{L}^{-1}$ upstream activator (Cot protein), 0.05 $\mu\text{g } \mu\text{L}^{-1}$ substrate [MEK1 (Cell Signaling Technology)], 100 μM ATP, 1.66 μCi [γ - 32 P]ATP (GE Healthcare) was incubated at 30 $^{\circ}\text{C}$ for 30 min. The reaction was stopped by the addition of 20 μL SDS loading buffer. The proteins were separated by an SDS-PAGE

gel. The gel was dried and then exposed to the X-ray film.

The chemiluminescence assay: the precipitated Cot protein (5 µg) was incubated with 1 µg substrate (GST-Plk1, MEK1, or designed peptide), 2.5 mM ATP at 30 °C for 30 min. The reaction was mixed with the same volume of SDS-PAGE Laemmli buffer (160 mM Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 0.1% β-mercaptoethanol, 0.04% bromophenol blue) and resolved by an SDS-PAGE gel. The gel was CBB stained for the mass spectrometry or transferred to a PVDF membrane for the western blotting analysis probed with phospho-specific primary antibody. The PVDF membrane was incubated with HRP-conjugated secondary antibody and stained by enhanced chemiluminescence.

3.5.4 Kinase assay in vivo

Co-expression of two genes: co-transfected and expressed pcDNA4 HisMax constructs (Cot_{full-length} with Plk1 or Cot_{full-length} kinase-dead mutant with Plk1) were pulled down using anti-6×His and resolved on a 10% SDS-PAGE gel. The proteins were transferred to a PVDF membrane and probed with anti-Cot (Santa Cruz), anti-Plk1 (Santa Cruz), anti-p-Plk1 (Ser137), anti-p-Plk1 (Thr210) for western blots analysis.

Fast activated cell-based ELISA (FACE): FACE (Active Motif) was performed following the manufacturer's instruction. Untreated or treated cells were grown on a 96-well plate, fixed by 4% formaldehyde in PBS for 20 min. After washing 3 times

with wash buffer (0.1% Triton X-100 in PBS), cells were treated with quenching buffer (1% H₂O₂ and 0.1% azide in wash buffer) for 20 min at room temperature. Following with washing twice, the cells were blocked in 2% BSA for 1 hr at room temperature. After washing twice, cells were incubated with 40 µL of properly diluted primary antibody (anti-MEK1/2 and anti-p-MEK1/2 accordingly) overnight at 4 °C. After washing 3 times, cells were incubated with 100 µL of properly diluted secondary antibody for 1 hr at room temperature. After washing 3 times with PBS, cells were incubated with 100 µL of developing solution for about 20 min at room temperature. The reaction was stopped by stop solution. The absorbance was measured at 450 nm with a reference wavelength of 655 nm using the microplate reader (Benchmark, Bio-Rad). After the reading at 450 nm, the plate was washed with the wash buffer and PBS for two times each, and air dried for 5 min. The cells were incubated with 100 µL of crystal violet for 30 min at room temperature. After washing with PBS for 3 times, cells were incubated with 1% SDS solution for 1 hr with shaking. The absorbance at 595 nm was measured. The OD₄₅₀ readings were corrected for the cell number by dividing the OD₄₅₀ reading for a given well by the OD₅₉₅ reading for this well.

3.5.5 Sub-cellular localization of Cot in mammalian cells

Cells were plated onto a 6-well plate. A sterilized cover slip was placed onto this plate. After the cells had grown to 80% confluency, formaldehyde was added to the plate at a final concentration of 3.7% (v/v). The cells were incubated for 30 min to fix. The

cover slip was washed twice with PBS and soaked in 0.5% Triton X-100 in PBS for 5 min to permeabilize the cells. The cover slip was washed twice with PBS again and probed with 50 μ L of primary antibody (100 times diluted with 3% BSA in PBS) for 2 hr at room temperature, or 4 °C for overnight. The cover slip was washed twice with PBS then probed with 50 μ L of fluorescence-conjugated secondary antibody (100 times diluted with 3% BSA in PBS) for 1 hr at room temperature. The cover slip was washed twice with PBS and once with Milli-Q water. Extra solution was removed by tapping the cover slip on tissue paper. For DAPI (4',6-diamidino-2-phenylindole) staining, the cover slip was soaked in 300 nM DAPI solution for 5 min followed by two times rinsing with PBS. Two drops of mounting solution (13.3% Mowiol 4-88, 33.3% glycerol, 0.13 M Tris-Cl pH 8.5) was added to the slide. The cover slip was put on one drop of mounting solution with the cells facing down. The cover slip was dried overnight before observation using a confocal microscope (LSM 510, Zeiss).

3.5.6 Peptide library screen for Cot substrates

Peptide library screening was performed following the manufacturer's instruction. The 25 μ L of kinase reaction cocktails [1 μ g Cot protein, 0.2 mM ATP, 10 μ M biotinylated peptide substrate (Cell Signaling technology)] were incubated at 30 °C for 30 min. The reactions were stopped with 100 μ L of TBST supplemented with 10% BSA and 100 mM EDTA. 100 μ L of each reaction was transferred to a 96-well streptavidin-coated plate (PerkinElmer) and incubated at room temperature for 30 min for the capture. After three times washing with TBST to remove the unreacted kinase,

100 μ L of phospho-antibody (Cell Signaling technology) was transferred from the antibody plate to the matched well of the streptavidin-coated plate. The solutions were mixed well and incubated at room temperature for 60 min followed by washing three times with TBST. Europium-labeled secondary antibody (100 μ L, 500 ng ml⁻¹, DELFIA, PerkinElmer) was added to each well. The plates were incubated at room temperature for 30 min followed by washing five times with TBST. The 100 μ L of enhancement solution (DELFIA, PerkinElmer) was added to each well and the plate was incubated at room temperature for 5 min. The fluorescence signal emission at 615 nm (excitation 340 nm, gate and lag 400 μ s) was measured using a time-resolved fluorescence (TRF) plate reader (Safire², TECAN).

3.5.7 Phospho-peptide isolation and analysis by mass spectrometry

The 5 μ g of precipitated protein Cot₁₋₄₁₃ and Cot_{full-length} was incubated with 1 μ g of GST-Plk1 (Cell Signaling Technology) and 2.5 mM ATP at 30 °C for 30 min. Cot₁₋₄₁₃ was incubated with GST-Plk1 but without ATP as a negative control. The reactions were mixed with an equal volume of SDS-PAGE Laemmli buffer (160 mM Tris-Cl pH 6.8, 4% SDS, 20% glycerol, 0.1% β -mercaptoethanol, 0.04% bromophenol blue) and resolved on a 10% SDS-PAGE gel. GST-Plk1 bands (99 kD) were cut out for the mass spectrometry analysis. Duplicate lanes were left for the western blot analysis. The GST-Plk1 bands were destained and digested with the trypsin as previously described (section 2.4.3). The peptides were dissolved in 1.5 μ L 0.5% TFA. The peptide solution was mixed with the binding solution (5% acetic acid, pH lower than

3.5) then transferred to a IMAC (Immobilized metal ion affinity chromatography) column (Pierce), and mixed with the resin gently. Unbound peptides were removed by centrifugation (1000×g, 1 min). The column was washed with 50 µL of wash solution A (0.1% acetic acid) twice followed by another two times washing with 50 µL of wash solution B (10% acetonitrile with 0.1% acetic acid). Water (75 µL) was used to change the pH value of the resin. The flow-through was removed by centrifugation (1000×g, 1 min). The phospho-peptides were eluted with elution buffer (0.1 M ammonium bicarbonate, pH 9.0). Centrifugation (1000×g, 1 min) was used to collect the eluant. The eluant was desalted with a ZipTip (Millipore) and mixed with an equal volume of matrix solution. The molecular weight and peptide sequence were obtained using the ABI MALDI-TOF/TOF 4800 analyzer as described previously.

Peptides RRRSLLELHKR (Mw: 1463, amino acid 134 to 144 of Plk1) and RKKTLCGTPNY (Mw: 1280, amino acid 207 to 217) were synthesized by Research Biolabs. The rest peptides in Table 8-2 were synthesized by GL Biochem. The peptide (1 µg) was incubated with precipitated Cot_{full-length} kinase (5 µg) and with or without ATP (2.5 mM) at 30 °C for 30 min. The kinase reaction solution was desalted by a ZipTip and mixed with an equal volume of matrix solution followed by spotting onto a MALDI plate (ABI). The molecular weight of the peptide was obtained using the ABI MALDI-TOF/TOF 4800 analyzer as described previously.

3.5.8 RNA interference

The 1×10^6 Hela cells were plated onto a 6-well tissue culture plate (about 60%-80% confluency). The 0.8 μg siRNA duplexes (UUACUGUCUUGACACAUGG and CCAUGUGUCAAGACAGUAA, AUCAGAACCAAUAUCCUG and CAGGAAUAUUGGUUCUGAU, UGUAAAUCUCUGUCCUCG and CGAGGAACAGAGAUUUACA, Santa Cruz) were delivered into the cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Solution A (0.8 μg siRNA in 750 μL serum-free DMEM) was mixed gently with solution B (10 μL Lipofectamine 2000 in 750 μL serum-free DMEM) and incubated at room temperature for 45 min. Serum-free DMEM (1500 μL) was added to the combined solution and overlaid onto the serum-free DMEM washed cells. After 5 hr incubation, the DMEM supplemented with 20% FBS (3 mL) was added to the cells. The medium was replaced with normal DMEM 24 hr later. Forty eight hours after transfection, the cells were harvested and lysed for the western blot analysis with anti-Cot(M20), anti-Plk1(F8), anti-p-Plk1(Ser137), anti-p-Plk1(Thr210), and anti-PDI. The non-targeting siRNA duplex (AAGUCUCCAAGCGGAUCUCGU and antisense, Dharmacon) was used as the negative control. The cell lysate was analyzed by a western blot. Anti-PDI was used as a loading control.

3.6 Computational analysis

3.6.1 Cot kinase domain structure predication

The Cot kinase domain 3D structure was modeled using the program MODELLER

[145]. Protein sequence of Cot kinase domain was converted into PIR format which was readable by MODELLER.

```
>P1;cot
sequence:cot:.....0.00: 0.00
IPRGAFGKVYLAQDIKTKKRMACKLIPVDQFKPSDVEIQACFRHENIAELYGAVLWG
ETVHLFMEAG
EGGSVLEKLESCGPMREFEIIWVTKHVLKGLFLHSSKVIHHDIKPSNIVFMSTKAVLV
DFGLSVQMTEDVYFPKDLRGT
EIYMSPEVILCRGHSTKADIYSLGATLIHMQTGTPPWVKRYPRSA YPSYLYIIHKQAPP
LEDIADDCSPGMRELIEASL
ERNPNHRPRAADLLKH*
```

A search for potentially related sequences of known structure was performed by the command `profile.build()` of MODELLER. The most appropriate template for Cot kinase domain sequence was the Tao2 sequence. The Cot kinase domain sequence was aligned with Tao2 sequence by the command `align2d()` in MODELLER.

```
>P1;cot
sequence:cot:.....0.00: 0.00
IPRGAFGKVYLAQDIKTKKRMACKLIPVDQFKPSDVEIQACFRHENIAELYGAVLWG
ETVHLFMEAG
EGGSVLEKLESCGPMREFEIIWVTKHVLKGLFLHSSKVIHHDIKPSNIVFMSTKAVLV
DFGLSVQMTEDVYFPKDLRGT
EIYMSPEVILCRGHSTKADIYSLGATLIHMQTGTPPWVKRYPRSA YPSYLYIIHKQAPP
LEDIADDCSPGMRELIEASL
ERNPNHRPRAADLLKH*

>P1;tao
structureX:tao:34 : :278 : :tao2:HS: 2.10: 0.24
*
```

Once a target-template alignment was constructed, MODELLER calculated a 3D model of the target using its automodel class. The script is shown below. A PDB file was generated and was viewed by program PyMOL.

```
# Initial test of Modeller
from modeller import *
from modeller.automodel import *
log.verbose()
env = environ()
a = automodel(env,
               alnfile = 'alignment.seg',
               knowns   = 'tao',
               sequence = 'cot')
a.starting_model = 1
a.ending_model = 5
a.auto_align()
a.make()
```

3.6.2 Protein interaction modeling

Interaction modeling of Cot and Plk1 was performed by the online program ZDOCK [146]. ZDOCK used a fast Fourier transform to search all possible binding modes for the proteins, with evaluation based on shape complementarity, desolvation energy, and electrostatics. The top 2000 predictions from ZDOCK were then given to RDOCK where they were minimized by CHARMM to improve the energies and eliminate clashes, and then the electrostatic and desolvation energies were recomputed by RDOCK. A PDB file was generated and viewed by program PyMOL.

3.6.3 Peptide 3D structure prediction and solvent accessible surface calculation (performed by Dr Y. Mu)

Two peptides with sequences RRRSLLELHKR and RRRTLLLELHKR were solvated

in a cubic box of size of 4.5nm separately with an explicit SPC water model. Four chloride ions were added and a total of 2880 and 2878 water molecules in the S and T systems respectively. The initial structures were extended with backbone dihedral angles, ϕ and ψ set to -150 and 150 degrees. Replica exchange simulations with the Poisson Boltzmann hybrid Hamiltonian method [147] were applied to sample the configurational space of the two peptides. Eight replicas were used to cover a range of temperatures from 300.0 K to 517.7 K. The exchange rates were maintained at 30%. Each simulation ran for 52 ns and every 1 ps structure snapshots were output. Finally, two ensembles of 52,000 structures each at 300.0 K were obtained for analysis. A modified Gromacs simulation package [148] with an OPLSAAI all-atomic force field [149] was used.

Chapter 4

Expression of Cot in *E. coli* and Insect Cells

4.1 Expression of Cot in *E. coli*

The four constructs of C-terminally and N-terminally 6×His-tagged Cot₁₋₃₈₈ (encompassing the N-terminal and kinase domains) and Cot_{full-length} were generated by PCR using *cot* cDNA (Genecopiea) as the template. The positive recombinant plasmids were verified by double digestion and DNA sequencing. Cot₁₋₃₈₈ is the same as Cot_{full-length} except for the truncation of 79 amino acids at the C-terminus. However, different expression levels were observed on an SDS-PAGE gel (Figure 4-1). Cot₁₋₃₈₈ has a high level of expression, whereas Cot_{full-length} has no obvious expression. This difference appeared to be independent of the vector used (pET16, pRSET, pETM-30) and the type of fusion protein [N-terminal 6×His, C-terminal 6×His, N-terminal GST, N-terminal MBP (maltose binding protein)].

Figure 4-1 Different expression levels of Cot_{full-length} and Cot₁₋₃₈₈ in *E. coli*. The total cell extract from *E. coli* was separated on a 10% SDS-PAGE gel. **A**, pET16-Cot₁₋₃₈₈ construct, with a 6×His tag at the N-terminus. **B**, pET24-Cot₁₋₃₈₈ construct, with a 6×His tag at the C-terminus. **C**, pET16-Cot_{full-length} construct, with a 6×His tag at the N-terminus. **D**, pET24-Cot_{full-length} construct, with a 6×His tag at the C-terminus. **1**, without induction; **2**, 1 hr induction; **3**, 2 hr induction. Cot₁₋₃₈₈ is identical to Cot_{full-length} except for the truncation of 79 amino acids from the C-terminus. The molecular weight of Cot_{full-length} and Cot₁₋₃₈₈ is 52 kD and 45 kD, respectively. Dashed arrow represents no expression.

4.2 Expression in *E. coli* of different Cot constructs

To investigate which part of the Cot protein may be affecting the protein expression level, seven constructs of C-terminally 6×His-tagged Cot₁₋₃₈₈, Cot₁₋₄₁₃, Cot₁₋₄₃₈, Cot₁₋₄₅₇, Cot_{full-length}, Cot₁₃₂₋₃₈₈, Cot₁₃₂₋₄₆₇ were generated by PCR using Cot cDNA as the template (Figure 4-2). The positive recombinant plasmids were verified by double

digestion and DNA sequencing. The constructs were expressed in *E. coli* BL21(DE3). After growing the cells to an OD₆₀₀ of 0.8, the recombinant protein expression was induced with 1 mM IPTG and the cells were incubated at 18 °C for 4 hr. The total cell lysate was separated on a 10% SDS-PAGE gel. One was stained with Coomassie blue. The duplicate was transferred to a PVDF membrane and probed with anti-Cot (M20). From the result (Figure 4-3), different protein expression levels can be detected among the seven constructs. The results are similar to those previously obtained (Figure 4-1). Only two constructs (Cot_{full-length} and Cot₁₃₂₋₄₆₇), both of which included the last 10 amino acids, had no obvious expression whilst the rest gave good expression in *E. coli*. Subsequent western blotting with anti-Cot confirmed this result.

Figure 4-2 The C-terminus of Cot showing the PEST, degron and kinase repression domain (**A**) and the constructs investigated (**B**).

Figure 4-3 The expression of seven different constructs in *E. coli*. After cells had grown to 0.8 (OD_{600}), the recombinant proteins were induced with 1 mM IPTG and incubated at 18 °C for 4 hr. The total cell lysates were loaded onto 10% SDS-PAGE gels. One was stained with Coomassie blue. The duplicate was transferred to a PVDF membrane and probed with anti-Cot (M20). Dashed arrow represents no expression.

4.3 Correction of *E. coli* codon bias

Due to the low expression of Cot_{full-length} in *E. coli* BL21(DE3) cells, it was thought initially that the codon bias in *E. coli* might have affected the expression. In order to avoid this problem, BL21-CodonPlus (DE3)-RIPL cells, which contain extra copies of rare *E. coli* Arg, Ile, Leu, Pro tRNA genes, were used. Furthermore, the DNA sequence for the last 10 amino acids of Cot was mutated to the codon codes most preferred by *E. coli*. However, expression of Cot_{full-length} was still not observed on an SDS-PAGE gel stained with Coomassie brilliant blue (Figure 4-4). Cot_{full-length} mutation (with the codon usage of last 10 amino acids optimized for *E. coli*) still did

not enhance the expression. These results indicate that the low expression level of Cot may be due to the presence of the last 10 amino acids and not a result of the codon bias of *E. coli*.

Figure 4-4 Expression of Cot_{full-length} with the correction for the codon bias of *E. coli*. The total cell extract from *E. coli* after induction was separated on a 10% SDS-PAGE gel. Lane **1**, before induction; Lane **2**, 2 hr induction in BL21-CodonPlus (DE3)-RIPL cells which contain extra copies of rare *E. coli* Arg, Ile, Leu, Pro tRNA genes; Lane **3**, before induction; Lane **4**, 2 hr induction of Cot with the codon usage of last 10 amino acids optimized for *E. coli*.

4.4 Expression of Cot in insect cells

The baculovirus system has many advantages for foreign gene expression, such as correct folding, and post-translational modifications (proper proteolysis, N- and O-glycosylation, acylation, amidation, carboxymethylation, phosphorylation, prenylation) that are often identical to those occurred in mammalian cells [150]. Expression of foreign proteins is under the control of the strong polyhedrin promoter, allowing levels of expression up to 30% of the total cell proteins. The correct recombinant genomic virus was constructed by the site-specific transposition (Figure 4-5), and then

the insect cells were infected with the recombinant virus genomic DNA. However, the expression level of Cot_{full-length} was still low, and could only be detected by a western blot (Figure 4-6). Subsequently, Cot₁₋₃₈₈ was cloned into baculovirus and expressed in sf9 cells. Although the C-terminal 69 amino acids had been deleted, the expression of Cot₁₋₃₈₈ in insect cells was at the same level as full-length Cot, unlike in *E. coli*, where Cot₁₋₃₈₈ expressed much better than the full-length Cot protein. The expression level of both constructs remained unchanged even after purification of the recombinant virus and scaling up of the sf9 culture.

Figure 4-5 Isolation and verification of recombinant bacmid DNA. **A**, recombinant bacmid DNA (approximately 130 kb) was isolated; and **B**, insertion was verified by PCR. Lane 1, PCR reaction with two primers, forward is M13 (on the bacmid DNA), reverse is *cot* gene specific primer; Lane 2, PCR reaction with two gene specific primers, as a positive control; Lane 3, using two gene specific primers but without template (*cot* gene), as a negative control.

Figure 4-6 Expression of Cot_{full-length} in insect cells by the Bac-to-Bac baculovirus expression system. The membrane was probed with anti-Cot (N17) antibody. A total of 1×10^6 sf9 cells were harvested after 24-hour transfection, washed with PBS buffer, resuspended in lysis buffer, mixed with SDS-PAGE loading buffer, run on a 10% SDS-PAGE gel, and then a western blot performed. Although there was some expression of Cot_{full-length} in insect cells, the amount was still very low. The Cot_{full-length} protein band could only be detected by western blotting.

4.5 Co-expression of NF- κ B and Cot in insect cells

From previous results (section 4.4), the expression of Cot in insect cells is challenging, probably due to the cell toxicity of expressed Cot protein. NF- κ B is a binding partner and activity inhibitor of Cot. Co-expression of Cot with NF- κ B may improve the expression level of Cot. An expression vector pFastBac-Dual (Invitrogen) was used for co-expression. NF- κ B (residues from 367 to 967) and Cot (residues from 31 to

397) were inserted into vector by *XbaI-NheI* and *EcoRI-XhoI*. This construct was cloned into baculovirus and expressed in sf9 cells. After selection by antibiotics for 3 days, the total cell lysates were analyzed by SDS-PAGE and western blotting with anti-NF- κ B or anti-Cot (Figure 4-7). There is an extra band at the position of 67.2 kD (solid arrow) which is same as the molecular weight of NF- κ B (residues from 367 to 967) comparing with control lane (empty vector). There is no obvious band at the position of 41.2 kD (dashed arrow) which is the molecular weight of Cot (residues from 31 to 397). Western blotting result verified the expression of NF- κ B and Cot. However, the expression levels of NF- κ B and Cot are different. NF- κ B has higher expression than Cot. Even when co-expressed with its binding partner and inhibitor, Cot still appears to have a low expression that is almost the same as when expressed alone.

Figure 4-7 Co-expression of NF- κ B and Cot in insect cells. NF- κ B (residues from 367 to 967) and Cot (residues from 31 to 397) were inserted into pFastBac-Dual by *Xba*I-*Nhe*I and *Eco*RI-*Xho*I. This construct was cloned into baculovirus and expressed in sf9 cells. After 3-day selection by antibiotics, the total cell lysates were analyzed by SDS-PAGE and western blotting with anti-NF- κ B or anti-Cot. Dashed arrow represents no expression or very low expression.

4.6 Discussion

Normally, there are three ways to produce an oncogene from a proto-oncogene. first, a deletion or a point mutation in the coding sequence [151]; secondly, abnormal gene amplification [152]; thirdly, the chromosome re-arrangement which including the fusion of the two genes and bringing the growth regulatory gene under the control of stronger promoter to overproduce protein [153]. The *cot* oncogene, due to a deletion in the genomic DNA sequence, encodes a C-terminally truncated and modified protein

(section 1.6). From these results, different expression levels between Cot and C-terminally truncated Cot were observed in *E. coli*. Gandara *et al.* observed that the mRNA levels of Cot_{full-length} and truncated Cot were the same [99]. When the cells were treated with a proteasome inhibitor, Cot_{full-length} and truncated Cot expressed at the same level. This implied that perhaps the C-terminal region of Cot_{full-length} contained some mechanism to degrade Cot_{full-length} itself. The authors believed that a sequence at the C-terminal end contained a target signal for proteasome degradation (Figure 4-2), which they termed a degron. Degron is a target signal peptide for ubiquitin conjugation and proteolysis [154, 155]. Another member of the MAPKKK family, c-Mos, also has a degron at the N-terminus [156]. Interestingly, no ubiquitin was found to conjugate with Cot [99]. Furthermore, a PEST motif was predicted in the C-terminal domain of Cot by the PEST find tool (<http://www.at.emblnet.org/emblnet/tools/bio/PESTfind/>). The PEST motif is a region rich in proline (P), glutamic acid (E), serine (S), and threonine (T). Proteins which have half-lives shorter than 10 hr always contain one or two PEST motifs. However, proteins which have half-lives from 20 to 220 hr only one tenth contain the PEST motif [157]. This indicates that there exist other mechanism for protein digestion except PEST motif. For example, the degron motif maybe involved in the digestion of Cot protein. The exact role of the last 10 amino acids in the Cot degradation or expression still remains unknown.

These results reveal that the last 10 amino acids of Cot can affect the expression of

Cot. The two constructs including Cot₁₃₂₋₄₆₇ and Cot_{full-length} (residues 1-467) gave a low expression. The other constructs which did not contain residues 457-467, gave a high expression level. When the DNA sequence for last 10 amino acids of Cot was mutated to the codon codes most preferred by *E. coli* or when *E. coli* BL21-CodonPlus (DE3)-RIPL cells were used, no improvement in the expression was noted. Interestingly, in the insect cell expression system, there was no apparent difference between Cot_{full-length} and Cot₁₋₃₈₈, both of them gave a low expression level. It seems that the last 10 amino acids or the last 69 amino acids did not influence the expression. The low level of expression for both constructs in sf9 cells may be due to the recombinant protein's toxicity. The different expression in *E. coli* may be due to the last 10 amino acids of Cot targeting the protein for degradation by ClpA protease (analogous to the degron mentioned previously) [158, 159].

Other possible reasons for the lack of expression or minimal expression are: (1) pET vectors are usually stable. However, when a toxic gene is inserted, the plasmid may become unstable; (2) proteolytic degradation can be affected by the N- and C-terminal sequences of a recombinant protein. The presence of Arg, Leu, Lys, Phe, Trp or Tyr at the N-terminus targets the protein for rapid degradation (N-end rule). Furthermore, non-polar amino acids at the C-terminus of the protein can also cause rapid degradation. However, *E. coli* which lacks the proteasome system [160]; (3) the presence of a sequence similar to the ribosome binding site (RBS) appropriately spaced (5-13 nt) upstream of an AUG can result in the formation of truncated protein;

(4) translation efficiency can be modulated by changing the distance between the Shine-Delgarno (SD) sequence and the start codon; (5) the secondary structure which is present at or around the translation initiation site can affect the expression level; (6) the PCR process may introduce mutations which can result in the creation of a new stop codon; (7) the mRNA of a recombinant gene can accumulate in *E. coli* cells and become unstable. This is another plausible explanation for the poor expression of Cot_{full-length}; (8) Most genes which encode hydrophobic, high positive charge and high isoelectric point proteins may be difficult to express in *E. coli* [161]. The Cot protein sequence was analyzed with program Vector NTI. However, the percentage of hydrophobic amino acids is only 32.3%. The charge of Cot at pH 7 is -13.01. The isoelectric point of Cot is 5.54. Furthermore, genes which encode proteins that have a high complexity score, low flexibility and high β -sheet content may be easy to form inclusion bodies [162-164]. In general, expression of full-length human protein kinases in *E. coli* can be challenging and therefore insect cell or mammalian cell expression is often used. However, in my case, insect cell expression was also poor, leading me to concentrate on the expression and purification of truncated Cot constructs.

Chapter 5

Purification and Crystallization of Cot

5.1 Purification and crystallization of 6×His-tagged Cot₁₋₃₈₈

5.1.1 Purification of Cot₁₋₃₈₈ under denaturing condition

From the results of the previous experiments (section 4.1), the Cot₁₋₃₈₈ protein expressed to a much higher degree than Cot_{full-length}. Therefore, the Cot₁₋₃₈₈ construct was used for expression and purification. When the Cot₁₋₃₈₈ protein was expressed in *E. coli*, it was apparent in both the supernatant and inclusion bodies. Initially, Cot₁₋₃₈₈ protein from the supernatant was loaded onto a Ni-NTA column, but no binding was observed (Figure 5-1abc), irrespective of the tag position used (N-terminal or C-terminal 6×His tag). Furthermore, when a cobalt affinity column (cobalt can be used as an alternative to nickel) was used instead of Ni-NTA column, the protein still did not bind. It seemed that both the N-terminal and the C-terminal 6×His tags were inaccessible. A short linker of eight amino acids (SSGHIEGH) between the 6×His tag and the protein was added, but this did not increase the binding to the Ni-NTA column. Thus, purification of the protein was accomplished under denaturing conditions. The protein was denatured by 8 M urea, and loaded onto the Ni-NTA column again. As expected, the 6×His-tagged Cot₁₋₃₈₈ protein bound to the Ni-NTA resin well (Figure 5-2a).

Denatured Cot₁₋₃₈₈ protein was refolded by dialysis against the buffers containing urea of progressively lower concentrations. After refolding, the protein was loaded onto a Superdex 75 gel filtration column for further purification (Figure 5-2b). The Cot protein (about 95% pure) (Figure 5-2c) was concentrated, desalted and used in the kinase assay, protein secondary structure detection and the crystallization screen.

Figure 5-1 Native Cot with 6×His tag can not be purified by Ni-NTA affinity column. **A**, Cot₁₋₃₈₈ was expressed in *E. coli*. The band of Cot₁₋₃₈₈ can be seen either in the pellet or the supernatant. **B** and **C** are western blots of the same samples in the same order except they were probed with different antibodies (anti-6×His for B; anti-Cot for C).

Figure 5-2 Purification of Cot₁₋₃₈₈ under denaturing conditions. Two liters of supernatant was loaded onto a Ni-NTA column, but no binding was detected. **A**, Cot₁₋₃₈₈ was denatured by 8 M urea (Lane 1) and then loaded onto a Ni-NTA column; Lane 2 was flow through; Lane 3 was eluant. **B**, Concentrated eluant of Ni-NTA column (Lane 1) was loaded onto a Superdex 75 column; Lanes 2-9 were the collected fractions. The first two fractions were combined as the purified Cot₁₋₃₈₈ (**C**). The molecular weight of Cot₁₋₃₈₈ is 45.3 kD.

5.1.2 Secondary structure of refolded protein Cot₁₋₃₈₈

The secondary structure of the Cot₁₋₃₈₈ protein was analyzed with circular dichroism (CD) (Figure 5-3). The data was deconvoluted with the program CDNN [165]. The result was α -helical 31.1%, antiparallel β -sheet 7.6%, parallel β -sheet 9.3%, β -turn 15.9%, and random coil 36.1%. The secondary structure analysis varies according to which program is used. The prediction of the secondary structure from the website www.predictprotein.org, is α -helical 27.0%, strand 12.0%, and loop 61.0%. Thus, it appears that the proportion of the secondary structure elements in the refolded proteins is partially correct. However, CD is unable to determine whether all the

protein molecules in the solution are correctly folded. Any heterogeneity in the protein folding would affect crystallization. Several different methods were tried to improve the refolding process efficiency, including: 1) rapid dilution with the optimization of solution pH value and the concentration of arginine; 2) dialysis with the denaturant Gn-HCl (guanidine hydrochloride); 3) on-column refolding. All gave similar results in respect to the CD spectrum.

Figure 5-3 The purified protein solution (60 μL , 1 mg mL^{-1} in 20 mM pH 7.0 phosphate buffer) was loaded into a microcuvette. 20 mM pH 7.0 phosphate buffer alone was used as baseline. The data after subtraction from the baseline was analyzed by the software CDNN.

5.1.3 Kinase assay of *Cot*₁₋₃₈₈

In order to check the activity of the refolded *Cot*₁₋₃₈₈, two negative control reactions were performed, one with substrate (MEK1) but without kinase (*Cot*₁₋₃₈₈), and using

the Cot kinase-dead which was obtained by immunoprecipitation from transfected HEK293 cells. Two negative reactions were performed to correct for ^{32}P incorporation into Cot₁₋₃₈₈ and MEK1 by autophosphorylation (Figure 5-4). The gel was visualized by autoradiography. By comparing the two parts of the gel, a phosphorylated band at approximately 47 kD (p-MEK1) was observed which indicated that MEK1 could be phosphorylated by Cot₁₋₃₈₈. At the same time, it can also be phosphorylated at other residues by itself. Therefore, it was important to do a reaction in the absence of Cot₁₋₃₈₈ to measure the autophosphorylation. However, there was no obvious band for autophosphorylation of MEK1 and 10 times MEK1. Compared with the two controls, it appeared that the recombinant protein Cot₁₋₃₈₈ had some activity after refolding. Combined with the CD result, it appears that the recombinant kinase purified from *E. coli* is at least partially correctly folded and active [166].

Figure 5-4 Kinase assay of Cot₁₋₃₈₈. Enzyme (Cot₁₋₃₈₈) and substrate (MEK1) were mixed with kinase reaction solution, incubated at 30 °C for 30 min, and then resolved on a 10% SDS-PAGE gel and visualized by autoradiography. Two controls, one without enzyme, and one using kinase-dead mutant Cot were performed.

5.1.4 Screening for crystals of Cot₁₋₃₈₈

Crystal screens were set up using 5 mg mL⁻¹, 20 mg mL⁻¹ and 30 mg mL⁻¹ Cot₁₋₃₈₈ protein with 98 different solutions, including different salts, precipitants, buffers and pH values (Hampton basic and extension crystal screen kits, Table A-1, appendices) by the hanging drop vapor diffusion method [167]. After one week, potential protein crystals were observed (Figure 5-5). Refolded protein tends to aggregate when additional precipitant is added. In the crystal screening of Cot₁₋₃₈₈, there was slight

aggregation when the protein concentration was 5 mg mL⁻¹. When the concentration was higher (30 mg mL⁻¹), precipitation occurred in two-thirds of drops, and the rest had phase separation. Several possible crystals were seen with 30 mg mL⁻¹ Cot₁₋₃₈₈ (initial concentration), 1.0 M lithium sulfate (initial concentration) and 2% (w/v) polyethylene glycol (PEG) 8000 (initial concentration). Subsequent screening was performed using different lithium compounds (lithium acetate, lithium chloride, lithium fluoride, and lithium nitrate) at different concentrations, and with different pH value buffers. However, no improvement in the quality or size of the crystals was apparent. Furthermore, this crystal was mounted for the X-ray diffraction, and unfortunately, no diffraction spots were detected. The probable cause was that the crystal quality or size was too poor to diffract. As mentioned previously, different refolding procedures were performed to improve the efficiency of the refolding procedure. All the refolded proteins were screened for the crystallization. However, no improvement in the quality of crystals was observed.

Figure 5-5 Screening for Cot₁₋₃₈₈ crystals. The hanging drop vapor diffusion method with the Hampton basic and extension crystal screen kits was used with 5-30 mg mL⁻¹ Cot₁₋₃₈₈. After incubation at 18 °C for a week, potential crystals (arrows pointed) were seen in the drop which contained 30 mg mL⁻¹ Cot₁₋₃₈₈ (initial concentration), 1.0 M lithium sulfate (initial concentration), 2% (w/v) PEG 8000 (initial concentration).

5.2 Purification and crystallization of untagged Cot₁₋₃₈₈

Since refolded protein is non-optimal for crystallization, Cot₁₋₃₈₈ was purified without the use of a Ni-NTA affinity column. Using the program Vector NTI (Invitrogen), the predicted pI value of Cot₁₋₃₈₈ is 5.54. So in a pH 8.0 buffer, the target protein should be negatively charged. The pET24-Cot₁₋₃₈₈ construct (with a stop codon before the 6×His tag) was transformed into *E. coli* BL21(DE3) and expressed at 16 °C using 0.5 mM IPTG for the induction. The cell pellet was lysed with sonication. The clarified and desalted cell lysate was loaded onto the Q-Sepharose column (20 mL). Flow rate was 2 mL min⁻¹. The target protein was eluted with a salt gradient (0-50%

1M NaCl, 50 mM Tris-Cl pH 8.0 in 40 min). The fractions were collected every 2 mL and visualized on a 10% SDS-PAGE gel (Figure 5-6). Compared with the Cot band that could be observed in the cell pellet, the fractions A10-12 and B1-2 appeared to be enriched with Cot₁₋₃₈₈.

The fractions A10-12 and B1-2 from the Q-Sepharose column were combined, desalted, concentrated and then loaded onto a Superdex 75 column (24 mL, 10/300 GL). Flow rate was 0.5 mL min⁻¹. Fractions were collected 0.5 mL each and visualized on a 10% SDS-PAGE gel (Figure 5-7). Fractions A3-6 contained the enriched band at the right molecular weight position of Cot₁₋₃₈₈ (44.1 kD).

Fractions A3-6 from the Superdex 75 column was desalted, concentrated and loaded onto a Mono-Q column (HR 5/5). Flow rate was 1 mL min⁻¹. The target protein was eluted by a salt gradient (0-100% 1 M NaCl, 50 mM Tris-Cl pH 8.0 in 40 min). Fractions were collected every 1 mL and visualized on a 10% SDS-PAGE gel (Figure 5-8). Fractions A9 contained the enriched Cot₁₋₃₈₈ at the right molecular weight position. Furthermore the band of Cot₁₋₃₈₈ was also confirmed by a western blot probed with anti-Cot (N17) (Figure 5-8).

Figure 5-6 The purification of untagged Cot₁₋₃₈₈. The clarified and desalted cell lysate (20 mL) was loaded onto a Q-Sepharose column (20 mL). Flow rate was 2 mL min⁻¹. After the UV curve had returned to the zero and stayed flat, the target protein was eluted by a salt gradient (0-50% 1M NaCl, 50 mM Tris-Cl pH 8.0 in 40 min). The fractions were collected every 2 mL and visualized on a 10% SDS-PAGE gel. The molecular weight of untagged Cot₁₋₃₈₈ is 44.1 kD. Fractions A10-12 and B1-2 were enriched with target protein.

Figure 5-7 The purification of untagged Cot₁₋₃₈₈. The fractions A10-12 and B1-2 from the Q-Sepharose column were combined, desalted, concentrated and then loaded onto a Superdex 75 column (24 mL, 10/300 GL) for further purification. Fractions were collected 0.5 mL each and visualized on a 10% SDS-PAGE gel. The molecular weight of untagged Cot₁₋₃₈₈ is 44.1 kD. Fractions A3-6 were enriched with target protein.

Figure 5-8 The purification of untagged Cot₁₋₃₈₈. Fractions A3-6 (from Superdex 75) was desalted, concentrated and loaded on to Mono-Q (HR 5/5). Flow rate was 1 mL min⁻¹. The target protein was eluted with a salt gradient (0-100% 1 M NaCl, 50 mM Tris-Cl pH 8.0 in 40 min). The band of Cot₁₋₃₈₈ (arrow) was confirmed by a western blot with anti-Cot (N17). The molecular weight of untagged Cot₁₋₃₈₈ is 44.1 kD.

After three columns (Q-Sepharose, Superdex 75, Mono-Q), the untagged Cot₁₋₃₈₈ was still not pure (around 50% purity). The difficulty with this purification process was that the Cot protein was mixed with other impurities. It was very hard to separate them using the gel filtration chromatography or anion exchange chromatography. As the 6×His tag did not appear to be beneficial for purification, the next strategy was to use glutathione affinity chromatography with GST-tagged protein.

5.3 Purification and crystallization of GST-tagged GST-Cot₁₋₃₈₈

5.3.1 Purification and crystallization of GST-tagged Cot₁₋₃₈₈

The vectors pGEX-4T-1 and pGEX-6P-1 were used as the expression vector. Between the GST and insertion, there was a thrombin (pGEX-4T-1) or Prescission protease (pGEX-6P-1) cleavage site. The protein was expressed at 18 °C and 0.5 mM IPTG. The clarified and desalted cell lysate (20 mL) was mixed with 5 mL GST affinity resin (Glutathione Sepharose 4B), and the target protein was eluted, concentrated, desalted and loaded onto a Superdex 75 column (24 mL, 10/300 GL) for further separation. Fractions were collected every 1 mL and visualized with a 10% SDS-PAGE gel. Fraction A3 was collected as the purified protein (Figure 5-9).

Figure 5-9 GST-tagged Cot₁₋₃₈₈ purification. The clarified and desalted cell lysate (20 mL) was mixed with 5 mL of GST affinity resin (Glutathione sepharose 4B), and the target protein was eluted, concentrated and loaded onto a Superdex 75 column (24 mL, 10/300 GL). The fractions were collected every 1 mL and observed on a 10% SDS-PAGE gel. Fraction A3 was collected as the purified protein. The molecular weight of GST-Cot₁₋₃₈₈ is 69.1 kD.

Thrombin cleavage was attempted for this protein sample (Figure 5-10). However, the cleavage did not appear to be successful. The band of GST could be observed after adding the protease. However, the band of Cot₁₋₃₈₈ was too weak. Only a very faint band at the right position (45 kD) could be detected. The possible reason is that most of GST-Cot₁₋₃₈₈ remained uncleaved or that the Cot protein is unstable or has low solubility after GST tag removal. After increasing the quantity of the protease or the incubation time, the band of Cot₁₋₃₈₈ was still very weak. Thus, it appears to be difficult to remove the GST tag from Cot₁₋₃₈₈.

Figure 5-10 The cleavage of GST-Cot₁₋₃₈₈ by thrombin. The purified GST-Cot₁₋₃₈₈ (fraction A3 from the Superdex 75) was mixed with different thrombin concentrations and incubated at 37 °C for 2 to 16 hr. The protein was separated and observed on a 10% SDS-PAGE gel. The original thrombin came from GE Healthcare at 2000 unit mL⁻¹. The molecular weight of Cot₁₋₃₈₈ is 45 kD.

After many repeated experiments, the same negative results were obtained. Therefore, the crystallization trays were set up with the 14.8 mg mL⁻¹ uncleaved GST-Cot₁₋₃₈₈. The solution used was Hampton basic screen kit. After 7 days, some small crystals

were seen in condition 12 (0.2 M MgCl_2 , 0.1 M HEPES pH 7.5, 30% propanol). Further optimization were performed under the two conditions (0.2 M MgCl_2 , 0.1 M HEPES-Na pH 7.5, 30% isopropanol; 0.2 M MgCl_2 , 0.1 M Tris-Cl pH 8.5, 3.4 M 1,6-hexanediol). Three buffers (0.1 M HEPES-Na pH 7.5, 0.1 M Tris-Cl pH 8.0, 0.1 M Tris-Cl pH 8.5) and four precipitant conditions (0.2 M MgCl_2 with either 2.6 M, 3.0 M, 3.4 M or 3.8 M hexandiol) were used for the crystal screen. Some larger plate shape crystals grew in the condition of 0.2 M MgCl_2 , 3.8 M hexandiol, 0.1 M HEPES pH 8.5 (Figure 5-11). These plate shape crystals were mounted on the X-ray diffractometer. However, no protein diffraction was detected. This crystal was also sent to a synchrotron (Swiss Light Source; the X-rays produced by a synchrotron source are several magnitudes more intense than those from an in-house X-ray generator). X-ray diffraction was successfully obtained from the protein crystal at the synchrotron (Figure 5-12), but the resolution (2.8 Å) was fairly low and it was difficult to solve the structure.

Figure 5-11 Crystallization of GST-Cot₁₋₃₈₈. The crystallization trays were set up with 14.8 mg mL⁻¹ GST-Cot₁₋₃₈₈ using the hanging drop vapor diffusion method. The solution used was the Hampton basic screen kit. After incubation for 7 days, some small crystals were observed in condition 12 (0.2 M MgCl₂, 0.1 M HEPES pH 7.5, 30 % propanol) (**A**). After optimization, some larger plate shape crystals grew in the condition of 0.2 M MgCl₂, 3.8 M hexandiol, 0.1 M HEPES pH 8.5 (**B**).

Figure 5-12 An X-ray diffraction image (1° oscillation angle) recorded from GST-Cot₁₋₃₈₈ crystal at the Swiss Light Source at a wavelength of 0.9801 Å and a temperature of 100 K using a marCCD detector. Diffraction spots out to a limit of 2.8 Å can be observed.

5.3.2 Purification of selenomethionine-substituted GST-Cot₁₋₃₈₈

Once well-diffracting protein crystals have been obtained, one of the quickest methods to solve the crystal structure is known as multiwavelength anomalous diffraction (MAD). To be successful, well-defined heavy atoms need to be present in the protein crystal. One of the best ways of accomplishing this is to substitute the

methionine in the protein with selenomethionine, *i.e.* sulfur (atomic weight 32.0) is replaced with the heavier selenium (atomic weight 79.0). As the selenomethionine-substituted protein sometimes crystallizes in different conditions from the native protein, expression, purification and crystallization screening of the modified protein is worthwhile even in the absence of good quality native crystals. Expression of selenomethionine-substituted protein can be accomplished either by using a methionine auxotroph *E. coli* strain or by metabolic inhibition. The latter method, which involves suppression of the endogenous methionine biosynthetic pathway by a defined mixture of amino acids, was used in this study.

The culture of *E. coli* cells was conducted following the procedure described in the Materials and Methods. The purification procedure of the GST-tagged selenomethionine-substituted protein was similar to the normal GST-tagged protein except that all buffers used were supplemented with 1 mM DTT and 1 mM EGTA to remove traces of metals that could catalyze oxidation [168]. After elution from the GST affinity column, the protein was loaded onto a Superdex 75 column (24 mL, 10/300 GL). Fractions were collected every 0.5 mL and visualized with a 10% SDS-PAGE gel (Figure 5-13ab). The target protein was enriched in the fractions A4-6. Fractions A4-6 were combined, desalted and loaded onto a Mono-Q column (HR 5/5) for further purification. The target protein was eluted with a salt gradient (0-50% 1 M NaCl, 50 mM Tris-Cl pH 8.0 in 20 min). Fractions were collected every 0.5 mL and visualized with a 10% SDS-PAGE gel (Figure 5-14ab). Fractions A2 and A3 from the Mono-Q column were combined as the purified selenomethionine-substituted protein

GST-Cot₁₋₃₈₈ (around 90% purity). However, the Cot protein was co-purified with the *E. coli* chaperone DnaK (about 70 kD) (Figure 5-14b). DnaK is an acidic *E. coli* heat shock protein which belongs to the molecular chaperone class of proteins. The molecular weight is 70 kD, and the sequence of the DnaK protein is highly homologous with eukaryotic Hsp70 proteins. The binding of Hsp70 to unfolded nascent polypeptides, partially denatured proteins and some native proteins leads to the stabilization of polypeptide chains during folding process, protection of other proteins from aggregation, reactivation of heat inactivated enzymes, promotion of peptide translocation through membranes, and proteolysis of partially denatured proteins [169].

Figure 5-13 GST-tagged selenomethionine-substituted protein GST-Cot₁₋₃₈₈ purification I. After elution from the GST affinity column, the protein was loaded onto a Superdex 75 column (24 mL, 10/300 GL) (A). The fractions were analyzed by SDS-PAGE (B). The molecular weight of selenomethionine-substituted GST-Cot₁₋₃₈₈ is 69.1 kD.

Figure 5-14 GST-tagged selenomethionine-substituted protein GST-Cot₁₋₃₈₈ purification II. The fractions A4-6 from the Superdex 75 column were combined, desalted and loaded onto a Mono-Q column (HR 5/5). The target protein was eluted with a salt gradient (0-50% 1 M NaCl, 50 mM Tris-Cl pH 8.0 in 20 min) (**A**). Fractions A2 and A3 were combined as the purified protein. The target protein co-purified with DnaK and the purity was about 90% as measured by densitometry (**B**). The molecular weight of selenomethionine-substituted GST-Cot₁₋₃₈₈ is 69.1 kD.

5.3.3 Removal of chaperone DnaK protein

Since GST-Cot₁₋₃₈₈ co-purified with the chaperone DnaK, an attempt was made to remove the chaperone DnaK. The cell pellet was resuspended with 20 mL of 20 mM pH 7.0 Tris-Cl buffer supplemented with 5 mM ATP and 5 mM MgCl₂. The cells were lysed with sonication. The clarified cell lysate was incubated with 2 mL GST affinity resin slurry at room temperature for 2 hr and washed with 5 volumes PBS together with 4 M, 5 M or 6 M urea accordingly. The bound proteins were eluted with elution buffer (10 mM reduced glutathione pH 8.0) and resolved on a 10% SDS-PAGE gel. The eluant from the columns washed with 5 M or 6 M urea was free of the chaperone (Figure 5-15). This eluant was loaded onto a Superdex 200 followed by a Mono-Q column for further purification.

Figure 5-15 The cell pellet was resuspended in Tris-Cl buffer supplemented with ATP and MgCl₂. Clarified cell lysate was incubated with GST affinity resin slurry and washed with five volumes PBS together with 4 M (lane 1), 5 M (lane 2) or 6 M (lane 3) urea accordingly. The bound proteins were eluted and resolved on a 10% SDS-PAGE gel. The molecular weight of selenomethionine-substituted GST-Cot₁₋₃₈₈ is 69.1 kD.

5.3.4 Crystallization of selenomethionine-substituted GST-Cot₁₋₃₈₈

The crystallization screen was set up with Hampton basic screen kit (Table A-1, appendices) using 12 mg mL⁻¹ purified selenomethionine-substituted GST-Cot₁₋₃₈₈ from which the DnaK had been removed. After four days, small crystals were observed in solution 21 (0.2 M MgAc, 0.1 M Na-cacodylate pH 6.5, 30% 2-methyl-2,4-pentanediol). After optimization, the crystals grew larger, but their subjective quality was not improved (Figure 5-16). The purified selenomethionine-substituted GST-Cot₁₋₃₈₈ was used for CD analysis (Figure 5-17). However, the CD result (α -helical 99.1%, antiparallel β -sheet 0%, parallel β -sheet 0.2%, β -turn 4.9%, and random coil 0.3%) indicated that the secondary structure may not have folded correctly.

Figure 5-16 Crystallization screen was set up with Hampton basic screen kit using 12 mg mL⁻¹ purified selenomethionine-substituted GST-Cot₁₋₃₈₈ from which the DnaK had been removed using the hanging drop vapor diffusion method. After four days, crystals were observed in solution 21 (0.2 M MgAc, 0.1 M Na-cacodylate pH 6.5, 30 % 2-methyl-2,4-pentanediol).

Figure 5-17 The purified protein solution (60 μL , 1 mg mL^{-1} in 20 mM pH 7.0 phosphate buffer) was loaded into a microcuvette. 20 mM pH 7.0 phosphate buffer alone was used as baseline. The data after subtraction from the baseline was analyzed by the software CDNN.

5.3.5 Alternative method to remove the chaperone during purification

From the above results, some of the Cot₁₋₃₈₈ protein appears to form a tight complex with the chaperone DnaK, while some of it may not have folded correctly. Thus, limited volume of GST resin was used to remove the majority of the mis-folding proteins from the clarified cell lysate of GST-Cot₁₋₃₈₈, and some of the correctly folded proteins may flow through the resin because of the resin's binding capacity limitation. The flow through was collected and loaded onto a Superdex 200 column (24 mL, 10/300 GL) (Figure 5-18). Fractions were collected every 0.5 mL and visualized with a 10% SDS-PAGE gel. Then, fractions A10-12 were combined and the proteins were separated by a Mono-Q column (HR 5/5) with a salt gradient (0-50%

1M NaCl, 50 mM Tris-Cl pH 8.0 in 20 min). Fractions were collected every 0.5 mL and visualized with a 10% SDS-PAGE gel. The fractions A5 and A6 from the Mono-Q column contained the purified protein, and most of the chaperone was removed (Figure 5-19). However, this method has a low recovery (about 5% after 3 columns), and the final purity was not ideal, only around 60%. Thus, it was not considered suitable for large scale purification and crystallization.

Figure 5-18 The GST-Cot₁₋₃₈₈ was expressed in *E. coli*. The cell pellet was lysed and clarified as described previously. The cell lysate was loaded onto 5 mL of GST affinity resin to remove the GST-Cot₁₋₃₈₈ protein which was bound to the DnaK. The flow-through was collected, desalted, concentrated and loaded onto a Superdex 200 column. Fractions A10-12 were combined, desalted and concentrated for further purification. The molecular weight of GST-Cot₁₋₃₈₈ is 69.1 kD.

Figure 5-19 Fractions A10-12 from the Superdex 200 column were combined and loaded onto a Mono-Q column (HR 5/5). The proteins were separated using a salt gradient (0-50% 1 M NaCl, 50 mM Tris-Cl pH 8.0 in 20 min) and visualized with a 10% SDS-PAGE gel. The molecular weight of GST-Cot₁₋₃₈₈ is 69.1 kD.

5.4 Purification of the Cot kinase domain

Since no good crystals from Cot₁₋₃₈₈ were obtained, and the similarity of the Cot kinase domain or the full length kinase to other human kinases is low, the three-dimensional structure of the Cot kinase domain would be of significant interest, and valuable for inhibitor design.

The kinase domain of Cot (residues 132-388) was cloned into pET24a with a C-terminal 6×His tag. The construct was transformed into *E. coli* BL21(DE3) and expressed at 18 °C and induced with 0.5 mM IPTG (Figure 5-20).

Figure 5-20 Cot₁₃₂₋₃₈₈ was expressed in *E. coli*. 0 = before induction; 1 = 1-hr induction; 2 = 2-hr induction. The molecular weight of Cot₁₃₂₋₃₈₈ (kinase domain) is 29.0 kD.

The cell pellet was lysed with sonication. The clarified and desalted cell lysate was loaded onto a Ni-NTA affinity column and the eluant was desalted, concentrated and

then loaded onto a Superdex 75 column (24 mL, 10/300 GL). The fractions were collected every 0.5 mL and observed on a 10% SDS-PAGE gel. Fraction A7 contained the purified protein (Figure 5-21). The Cot₁₃₂₋₃₈₈ was concentrated and desalted for the crystallization screen. The crystal screen was set up with Hampton basic and extension screen kits with 10.5 mg mL⁻¹ Cot kinase domain. However, after 2 years, there was still no evidence of crystal formation. Most of the drops formed heavy precipitate once mixed with the screen solution.

Furthermore, the secondary structure of Cot₁₃₂₋₃₈₈ was analyzed with CD spectrometry. However, Cot₁₃₂₋₃₈₈ (α -helical 55.2%, antiparallel β -sheet 1.4%, parallel β -sheet 4.4%, β -turn 13.0%, and random coil 16.8%) seems not to have folded correctly since the percentage of random coil is high (Figure 5-22).

Figure 5-21 Purification of Cot₁₃₂₋₃₈₈. The cell lysate was loaded onto a Ni-NTA column. The eluant from Ni-NTA column was loaded onto a Superdex 75 column (24 mL) (**A**). The fractions were collected and observed on a 10% SDS-PAGE gel. Fraction A7 contained the purified Cot kinase domain (**B**). The molecular weight of Cot₁₃₂₋₃₈₈ (kinase domain) is 29.0 kD.

Figure 5-22 The purified protein solution (60 μL , 1 mg mL^{-1} in 20 mM pH 7.0 phosphate buffer) was loaded into a microcuvette. 20 mM pH 7.0 phosphate buffer alone was used as baseline. The data after subtraction from the baseline was analyzed by the software CDNN.

5.5 Purification of Cot₁₋₁₃₇

Although the crystal structure of N-terminal domain would not be useful for inhibitor studies, it would still be useful for studying the interaction with other ligands and proteins because the N-terminal domain may act as the binding site or a recognition site for another kinase [170]. The N-terminal domain of Cot (residues 1-137) was cloned into pET24a with a 6 $\times$ His tag at the C-terminus. The expressed recombinant protein Cot₁₋₁₃₇ was solely located in inclusion bodies. In order to increase the solubility of Cot₁₋₁₃₇, pGEX-6p-1 was used to place a GST tag at the N-terminus. The clarified and desalted cell lysate was loaded onto a GST affinity column. The GST-Cot₁₋₁₃₇ was eluted and the eluant (loading sample) was loaded onto a Superdex 200

column (120 mL) for further purification (Figure 5-23a). The fractions were collected every 0.8 mL and observed on a 10% SDS-PAGE gel (Figure 5-23b). Fractions A9-10 were collected as the purified protein. For the on-column cleavage, purified GST-Cot₁₋₁₃₇ was loaded onto the GST affinity column again and then mixed with Prescission protease (GST-tagged) for cleavage (Figure 5-24). The flow-through was collected as the cleaved Cot₁₋₁₃₇. The uncleaved GST-Cot₁₋₁₃₇ and protease (GST-Prescission) were bound to the resin and eluted with 10 mM reduced glutathione. The cleaved Cot₁₋₁₃₇ was present in the flow-through of the GST affinity column. Though Cot₁₋₁₃₇ was purified (about 75%), the recovery efficiency during the cleavage was low. The level of purity was not high enough for crystallization screening.

Western blotting was performed to verify that it was the right protein (Figure 5-24). From the western blot result, it can be seen that the protein (before cleavage and after cleavage) tended to aggregate. Even though the GST has been removed, Cot₁₋₁₃₇ still can bind to the GST or the GST resin. This reduced the yield. Many methods, such as adding a small amount of non-ionic detergent, for reducing the interaction between Cot₁₋₁₃₇ and GST were tried, but no improvement was observed. N-terminal domains of protein may be disordered when expressed alone [171], thus it is possible that the folding of the N-terminal domain of Cot alone may not be correct.

Figure 5-23 Purification of Cot₁₋₁₃₇. The construct pGEX-6p-1-Cot₁₋₁₃₇ was expressed in *E. coli*.

The clarified and desalted cell lysate was loaded onto a GST affinity column. The eluant was loaded onto a Superdex 200 column (120 mL, Hiload) (A). The fractions collected were observed on a 10% SDS-PAGE gel (B). The molecular weight of Cot₁₋₁₃₇ is 15.6 kD.

Figure 5-24 Cleavage of GST-Cot₁₋₁₃₇. Fractions A9-10 from the Superdex 200 column were collected as the purified protein. Purified GST-Cot₁₋₁₃₇ was loaded onto a GST affinity column again and mixed with the protease for different incubation times at 4 °C. The 10 μ L of GST resin was mixed with the same volume SDS-PAGE loading buffer and visualized with a 15% SDS-PAGE gel. The flow-through was collected for the purified Cot₁₋₁₃₇. The remainder of the uncleaved GST-Cot₁₋₁₃₇ and the protease (GST-Prescission) were bound to the resin and eluted with 10 mM reduced glutathione. Western blotting was performed with anti-Cot (N17) to verify protein identity. The molecular weight of GST-Cot₁₋₁₃₇ is 40.6 kD. The molecular weight of Cot₁₋₁₃₇ is 15.6 kD.

5.6 Discussion

From these results, the 6 $\times$ His tag, regardless of which terminus it is fused to, cannot bind to Ni-NTA resin. However, after the protein was denatured by 8 M urea, the 6 $\times$ His tag bound to Ni-NTA. This may imply, that the 6 $\times$ His tag was hidden from the surface of the Cot protein molecule, or that the surface of Cot was binding the 6 $\times$ His

tag thus preventing its exposure.

The expression and purification of a number of Cot constructs including Cot₁₋₁₃₇, Cot₁₃₂₋₃₈₈, Cot₁₋₃₈₈ (6×His-tagged, GST-tagged and untagged), Cot₁₋₄₁₃, Cot₁₋₄₃₈, Cot₁₋₄₅₇, Cot₁₋₄₆₇, Cot₁₃₂₋₄₆₇ were attempted (Table 5-1). Different fusion tags including N-terminal 6×His, C-terminal 6×His, GST, MBP, Flag, and ubiquitin were tried. Different expression host including *E.coli* (DH5α, BL21, BL21 RIPL), yeast (*Pichia pastoris*), insect cell (sf9, Hi5), and mammalian cell (HEK293) were tried. For purification, ion-exchange columns (Q-sepharose, Mono-Q, Mono-S), gel filtration columns (Superdex 75, Superdex 200), HIC and all fusion tag affinity columns were tried to obtain purified protein. For fusion tag cleavage, factor Xa, thrombin, Prescission protease and TEV protease were tried. For crystallization screening, many different commercial solutions (such as the pre-crystallization test kit, PEG/Ion screen kit, crystal screen kit, additive screen kit from Hampton research) and home-made screens (over 200 different unique solutions). In addition, different temperatures (4 °C, 16 °C and 25 °C), different protein concentrations (10 mg mL⁻¹, 20 mg mL⁻¹, 30 mg mL⁻¹) and different methods (sitting drop and hanging drop) were tried for growing crystals.

After extensive screening, only GST-Cot₁₋₃₈₈ which was purified via its GST fusion tag, and Cot₁₋₃₈₈ which was purified via its 6×His fusion tag in the denatured condition, formed crystals. Diffraction from either of these crystals could not be obtained using

the in-house X-ray diffractometer, probably due to their small size or thinness. Larger crystals for GST-Cot₁₋₃₈₈ could be grown and low resolution diffraction was obtained using a synchrotron X-ray source. Another 200 different solutions including the PEG/ion screen were used for screening. However, no improvement was noted in the quality of crystals. The trays were incubated longer but there was no evidence for showing that thicker crystals had grown. The possible reason for the poor quality of the GST-Cot₁₋₃₈₈ crystals is that the connection between the GST tag and the protein is flexible, and thus makes it difficult to form a tight or rigid structure. Therefore the crystals stopped growing larger. Within the RCSB PDB database, few GST-fusion protein crystal structures are found. Most proteins that had been expressed with a GST tag had the GST tag removed before the crystallization trays were set up. One crystal structure was solved with a GST tag [172]. The possible reason for this success is that the GST was fused with a very short peptide (a six-amino acid conserved neutralizing epitope of gp41 from HIV).

Researchers have reported that Cot overexpression and purification are challenging [99, 173]. Researchers have tried multiple constructs with different amino- and carboxy-terminal boundaries starting at either amino acid 1 or 30 and ending at amino acid 397, corresponding to the oncogenic form of Cot, or the Cot full-length protein. A series of N-terminal truncations based on secondary structure analysis were also examined, for instance the residues 51-397, 68-397, 81-397, 99-397, 121-397, and 134-397. Furthermore, many affinity tags were tried, including FLAG, 6×His, GST,

calmodulin-binding protein, and maltose-binding protein in combination with different Cot truncations for the purification. In addition, different expression hosts such as *E. coli*, yeast, insect cell, and HEK293 cells were used. Eventually, it was found that FLAG-tagged protein expressed in insect cells was satisfactory levels in terms of both expression and purification. Insect cells and HEK293 cells were better than the other hosts. However, the insect cell expressed protein in insect cells remained bound to the chaperone Hsp70 and after a three-column purification procedure, the Cot purity was still around 40% due to the presence of the chaperone. Furthermore, all attempts to remove the chaperone failed. This result was similar to the work described in this thesis. To increase the stability of Cot, they used co-expression of p105 and Cot in the insect cells to make the Cot protein more stable. However, this did not improve the purification greatly and the final purity of this complex was around 50% [173].

TAK, transforming growth factor-beta (TGF- β)-activated kinase, is another MAPKKK. Solving the structure of TAK was difficult due to the poor expression and misfolding problem. However, when the authors generated TAK1 chimeric protein with TAB (TAK1-binding protein), the protein complex seemed more stable and easier to express, purify and crystallize [174]. There are some reports that the p105 is the binding partner of Cot [133, 136]. Researchers have performed the co-expression of p105 and Cot protein in insect cells. The result showed that p105 can enhance the stability of Cot, and protect Cot from degradation. However, the purity of Cot and

p105 complex is still not ideal for crystallization. Furthermore, crystallization of protein-protein complexes is challenging. A co-expression construct of Cot₃₀₋₃₉₇ with p105 (369-969) was generated with the insect cell dual expression vector pFastBac Dual (Invitrogen). However, expression and purification of the complex was not satisfactory. Furthermore, obtaining the crystal structure of a complex of two large proteins is challenging. Therefore, most MAPKs are crystallized in complex with docking peptides [175, 176]. The next stratagem is to find suitable Cot binding partners and derive the docking peptide sequences. However, the sequence and the length of the docking peptide will affect the crystallization, diffraction limit and electron density. Currently, commonly used methods of finding the putative docking peptide from a sequence are scanning the sequence by eye or using a simple partner matching algorithm. The program Scansite can help to identify the docking site [177]. Good results are likely to be obtained by screening many peptides in the co-crystallization experiments.

Table 5-1 Expression, purification and crystallization of a number of Cot constructs

Expression vector	Fragment inserted	Fusion tag				<i>E. coli</i>			Insect cell		Crystallization
		N-6×His	N-9aa-6×His	C-6×His	N-GST	solubility	purification	tag cleavage	expression	purification	
pET24a	Cot ₁₋₁₃₇			•		×	-				Poor crystals
	Cot ₁₋₃₈₈			•		√	×				
	Cot ₁₋₃₈₈			•		√	√				
	Cot ₁₋₄₁₃			•		√	×				
	Cot ₁₋₄₃₈			•		√	×				
	Cot ₁₋₄₅₇			•		√	×				
	Cot _{full-length}			•		-	-				
	Cot _{fl-mutant}			•		-	-				
	Cot ₁₃₂₋₃₈₈			•		√	√				
	Cot ₁₃₂₋₄₆₇			•		-	-				
pET16b	Cot ₁₋₃₈₈		•			√	×				
	Cot _{full-length}		•			-	-				
pGEX-4T-1	Cot ₁₋₃₈₈				•	√	√	×			Poor crystals
pGEX-6P-1	Cot ₁₋₁₃₇				•	√	√	×			×
pDEST	Cot ₁₋₃₈₈	•				√	-		low	×	
	Cot _{full-length}	•				√	-		low	×	

Chapter 6

Expression of Cot in Mammalian Cells

6.1 Expression of Cot in mammalian cells

To investigate the overexpression of Cot protein in tumor cells, 29 tumor cell lines and one cow kidney cell line (MDBK, used for comparison) were used for western blotting probed with anti-Cot (M20) (Figure 6-1). Whole blots were shown in appendices (Figure A-1). The tumor cell lines used included skin cancers (A375, A431, SK-MEL-28, C32), lung cancer (A549), soft tissue cancer (rhabdomyosarcoma) (A673), colorectal adenocarcinoma (Colo 320DM, WiDr, Caco-2, HCT-8), prostate cancer (DU145), ovarian cancer (ES-2), uterine cancer (AN3 CA), cervical cancer (Hela), leukemia (HL-60, HEL 92.1.7, K-562, MEG-01, TF-1), breast cancer (MCF7, MDA-MB-231, ZR-75-1), T cell leukemia (Jurkat), lymphoma (Daudi, EB1, HuT 78), kidney carcinoma (Caki-1), neuroglioma (H4), and hepatocellular carcinoma (Hep G2). It appeared that most of the tumor cells overexpressed the Cot protein. Only A431, WiDr, AN3 CA, Caco-2 and H4 demonstrated no obvious band for Cot. This is in line with previous studies, examining the activation of its substrate MEK1/2, which showed that Cot was found highly active in a variety of primary human tumors including cancers of breast, lung, colon, pancreas, prostate (section 1.7). This may indicate that, since Cot can activate the MEK1/2-ERK1/2 pathway parallel to Raf, the activity of MEK1/2 may be partially contributed by the overexpressed Cot protein [178]. Furthermore, in HEK293 cells, no obvious expression of Cot was detected with

the western blot.

Figure 6-1 Overexpression of Cot protein in tumor cell lines. Total cell lysate (50 μ g) was mixed with the same volume of SDS-PAGE loading buffer. The proteins were separated with 10% SDS-PAGE gels. Western blots were performed with anti-Cot (M20) antibody. PDI was loading control.

6.2 Sub-cellular localization of Cot in the mammalian cells

MCF7, A549, and HeLa cells, which express Cot endogenously, were used to investigate the sub-cellular localization of Cot. The cells were grown on a coverslip, probed with anti-Cot (N17), and mounted with fluorescence-conjugated secondary antibody (anti-rabbit-FITC, green color). The figure showed that the Cot protein was localized in the cytosol for all three cell lines (Figure 6-2). There is no evidence for Cot being a membrane-associated or nuclear-localized protein.

Figure 6-2 MCF7, A549, and HeLa cells, all of which have been shown to express Cot, were used for investigation of the sub-cellular localization of Cot. Cells were grown on a coverslip and probed with anti-Cot (N17), mounted with fluorescence conjugated secondary antibody (anti-rabbit-FITC, green color). Cells were probed with fluorescence conjugated secondary antibody alone as a negative control. Cell nuclei were stained with DAPI. The coverslip was mounted with Mowiol and dried before observation with confocal microscopy.

6.3 Expression of Cot constructs

To determine the binding site of potential binding partners, a series of different truncations of Cot were constructed and expressed in HEK293 cells (which express very low level of endogenous Cot). Due to the toxicity of the expressed protein for the host cells (Figure 6-3), in the third day after transfection of Cot_{full-length}, most of the transfected cells died. In the control experiment, cells were still viable after 3-day transfection with empty vector. Therefore, the cells were harvested two days post-transfection for the maximum yield of the Cot protein. Only for LPS- or TNF- α -stimulation, the cells were harvested on the third day.

As mentioned in chapter 4, the six constructs were designed according to the domain structure of Cot. Some PEST sequences appear to be proteolytic signals for degradation by the 26S proteasome [179]. Cot₁₃₀₋₃₉₉ represents the kinase domain; Cot₁₋₃₈₈ represents the N-terminal domain plus the kinase domain; Cot₁₋₄₁₃ is almost same as Cot₁₋₃₈₈ but longer at the C-terminus; Cot₁₋₄₃₈ contains the putative PEST domain; Cot₁₋₄₅₇ contains the PEST domain and the degron but only part of the kinase repression domain; Cot_{full-length} and empty vector (pcDNA4) were used as positive control and negative control, respectively.

Figure 6-3 The HEK293 cells were transfected with Cot_{full-length} construct by the standard calcium phosphate transfection method. Empty vector was used as a negative control. The images were taken at one day intervals.

The six constructs were transfected into HEK293 cells using the calcium phosphate transfection method. In order to determine the steady-state expression level of all constructs, the total cell lysate was probed with anti-6×His 48 hr after transfection. From the western blots, it can be seen that Cot₁₋₄₁₃ was expressed as a higher level than the other constructs (Figure 6-4). However, it has been reported that the mRNA

level, determined by Northern blotting, of C-terminally truncated Cot kinase is similar to that of the full-length protein [99]. When the cells were treated with a proteasome inhibitor such as 10 μ M lactacystin, all the Cot constructs expressed protein at the same level [99]. It is therefore possible that the rate of degradation of Cot₁₋₄₁₃ may be less than the other constructs. This implied that the PEST and the degron sequence or the N-terminal region of Cot may have different binding partners to keep their stability.

Figure 6-4 Western blot analysis of the expression of Cot constructs. HEK293 cells were transfected with 10 μ g of empty vector or Cot constructs. After 48-hr posttransfection, cells were harvested and lysed in lysis buffer. Expression of all constructs was determined by western blotting using anti-6xHis antibody. Anti-PDI was used as the control for protein loading.

6.4 Immunoprecipitation

The pull-down efficiency for the expressed full-length Cot by three antibodies [anti-6×His, anti-Cot (N17), anti-Cot (H212)] was compared (Figure 6-5a) and it was found that anti-6×His was optimal. Therefore, the expressed Cot proteins from HEK293 cells were precipitated using anti-6×His antibody and detected by western blot using anti-Cot (M20) (Figure 6-5b). As p105 has been reported to be a physiological binding partner and an inhibitor of inactivated Cot, it was used as a control. The PVDF membrane was stripped and then probed with anti-NF-κB1 (p105). Only the lane of Cot₁₃₀₋₃₉₉ did not have a p105 band (Figure 6-5b). It seems that the N-terminus which has unknown function may be involved in the binding of NF-κB1 (p105).

The C-terminal half of NF-κB1 (p105) was shown to bind to Cot at two sites [17]: the C-terminal domain of Cot bound to the p105 ankyrin repeat region (497-534), and the Cot kinase domain bound to the p105 death domain (808-892) region. Both p105 binding sites appeared to be required for optimal Cot interaction, and it was shown that this interaction regulated the metabolic stability of Cot. The binding of the p105 death domain to Cot inhibits its MEK kinase activity; this inhibitory effect was reduced with C-terminally truncated Cot. It was therefore suggested that p105 negatively regulates Cot MEK kinase activity by preventing access to its substrate. Although Cot phosphorylated the PEST domain of p105, it did not phosphorylate the death domain despite its interaction with the kinase domain. The membrane was

stripped and re-probed with anti-NF- κ B1 (p105). In this work, only Cot₁₃₀₋₃₉₉ did not display a distinct p105 band (Figure 6-5b) which may indicate that the N-terminus of Cot is also involved in the binding of NF- κ B1 (p105).

Figure 6-5 Western blot analysis of the immunoprecipitated Cot proteins. **(A)** Immunoprecipitation of HEK293 lysate was performed using anti-6×His, anti-Cot (N17) or anti-Cot (H212) followed by incubation with protein G-agarose. **(B)** HEK293 lysates transfected with Cot were performed immunoprecipitation by anti-6×His. Western blotting was performed using anti-Cot (M20) and anti-NF- κ B1 (p105). Random IgG chain C was used as negative control.

6.5 Kinase assays *in vitro* and *in vivo*

When the constructs were expressed in *E. coli*, the kinase domain was found to be completely insoluble and the full-length protein did not express, whilst the rest were partially soluble. When expressed and purified with a GST-tag, the Cot protein always co-purified with chaperones such as Hsp60, DnaK and GroEL. This means the protein expressed in *E. coli* may not have folded correctly. In order to make sure the protein expressed in HEK293 cells was active, *in vitro* kinase assays were performed with the immunoprecipitated Cot proteins from HEK293 cells. The precipitated proteins were incubated with the reported substrate MEK1 in presence of ATP, and following western blotting, were probed with anti-phospho-MEK1/2 (Figure 6-6). All the Cot proteins appeared to be active. An endotoxin-free plasmid maxi-prep kit (Qiagen) was used in an attempt to minimize the possibility of contamination with the *E. coli*-derived plasmids with LPS.

Figure 6-6 Kinase assay *in vitro*. Immunoprecipitates (1 μ g) from HEK293 cells transfected with Cot constructs were incubated with 200 μ M ATP and MEK1 (1 μ g) in the kinase buffer for 1 hr at 30 °C. Phosphorylated-MEK1 was visualized by western blotting probed with anti-p-MEK1/2 antibody. The first lane (MOCK) used the kinase buffer instead of the immunoprecipitate as the negative control. Cot kinase-dead protein was used as an additional negative control.

Because the pull-down process may interrupt the kinase complex, an *in vivo* kinase assay which can reduce the effects of degradation during cell lysis and interruption during the process of pull-down, was performed. The ratio between p-MEK1/2 and total MEK1/2 for the untreated transfected cells, LPS-treated transfected cells, TNF- α -treated transfected cells was measured (Figure 6-7). The ratios of p-MEK1/2 to total MEK1/2 for untreated and LPS-treated were the highest for the Cot_{full-length} protein. The ratio of Cot₁₋₄₁₃ with TNF- α -treatment was the highest. However, after TNF- α and LPS-treated ratios of p-MEK1/2 to total MEK1/2 were substrated with Non-treated, Cot₁₋₃₈₈ showed the highest ratio for TNF- α -treatment and LPS-treatment. The

possible answer for this may due to the highest stability of Cot₁₋₃₈₈. Overall, all Cot proteins showed similar activity *in vivo* assay which was constant with *in vitro* assay result before. This result also is in line with previous experiments [82, 99, 112] on wild-type Cot, all constructs demonstrated higher activity following LPS and TNF- α stimulation. However, these experiments additionally demonstrated that C-terminally truncated Cot behaves in a similar manner to the Cot full-length protein.

Figure 6-7 Fast activated cell-based ELISA (FACE). Untreated, LPS-treated, TNF- α -treated cells were fixed, quenched, and incubated with primary antibody (anti-MEK1/2 and anti-p-MEK1/2 accordingly). The absorbance was read at 450 nm with an optional reference wavelength of 655 nm. The absorbance was read at 595 nm for the staining of crystal violet. OD₄₅₀ were corrected for the cell number by dividing the OD₄₅₀ for a given well by the OD₅₉₅. The data were the average of three individual experiments and the error bars represent one standard deviation.

6.6 Discussion

From the Cot expression screen in 29 tumor cell lines, a band for Cot was found in most of the cell lines. The cow kidney cell line MDBK, which is a non-human, non-tumor cell line, was studied as a comparison. In the 29 tumor cell lines studied, only five cell lines (A431, WiDr, AN3 CA, Caco-2 and H4) did not show a band of Cot. This implies that the expression of Cot in tumor cells was a common phenomenon. Of the four skin cancer cell lines (A375, A431, SK-MEL-28, and C32) studied, three of them demonstrated expression of Cot. Only A431 did not show a band for Cot. Interestingly, A375, SK-MEL-28, and C32 are malignant melanoma; but A431 is epidermal squamous cell carcinoma. Furthermore, in three breast cancer cell lines studied, both ER⁺ (estrogen receptor⁺) cell lines (MCF7 and ZR-75-1) show heavier Cot bands than ER⁻ cell line (MDA-MB-231). However, the expression of Cot in cancer-derived cell lines may not reflect the status of tumor tissues from patients.

A series of Cot constructs with an N-terminal 6×His tag were transiently expressed in HEK293 cells: Cot₁₃₀₋₃₉₉ (kinase domain), Cot₁₋₃₈₈ (N-terminal and kinase domains), Cot₁₋₄₁₃, Cot₁₋₄₃₈ (containing a putative PEST sequence), Cot₁₋₄₅₇ (containing both PEST and degron sequences) and Cot₁₋₄₆₇ (full-length protein). From the *in vivo* and *in vitro* Cot kinase assays, all the expressed Cot proteins demonstrated activation. The Cot protein expressed in HEK293 cells was thus suitable for the functional studies.

Overexpression of Cot in some cancer cells implied that Cot may play a role in maintaining the survival of cancer cells [180]. This would suggest an anti-apoptotic function for Cot, in contrast to the pro-apoptotic function mentioned previously. As discussed in the chapter 1, Cot mRNA expression level is enhanced in breast cancer cells and large granular leukemia cells (section 1.7). The deregulation of Cot in cancer cells may also occur posttranslationally. Cot is constitutively activated in human T cells transformed by the oncogenic retrovirus HTLV-1 by the mechanism involving the oncogenic action of Tax protein [139]. Tax activates some transcription factors such NF- κ B, leading to deregulated expression of various genes which are involved in the control of cell growth and survival. Furthermore, overexpression of Cot did not lead to the activation of p38. In PC12 cells, Cot induced the stimulation of ERK and JNK activation. Recent studies revealed that the function of Cot is tightly regulated by p105 [131-133, 138]. In macrophages and various other cell types, Cot forms a stable complex with p105, IKK, TRAF6 [125], hKSR (kinase suppressor of Ras) [107], Syk [103], ABIN-2 [136] and AKT [97]. The binding with other proteins may contribute to the Cot stability and its activation in the signaling pathway.

It was shown that the C-terminally truncated Cot was approximately five times more active in autophosphorylation and five to seven times more active in the phosphorylation of histone substrates H3 and H2A, respectively [181]. The co-expression of GST-C-terminal tail of Cot with Cot (wild-type or truncated) has been shown to down-regulate the Cot kinase activity. This is possibly the result of an

inhibitory interaction between the Cot kinase domain and the C-terminal tail. However, my results indicated that the C-terminal tail of Cot has little effect on Cot activity, which may be due to the different substrate used. The activation loop of Cot for different substrates is different. Furthermore, Cot transgenic mouse expressed the wild-type Cot protein without biological function, whereas those expressing the C-terminally truncated Cot protein developed large-cell lymphoblastic lymphomas of T-cell origin. When the expression level of Cot was examined in greater detail, it was found that the C-terminally truncated protein is only oncogenic when expressed at high levels. Therefore, the oncogenic potential of Cot depends on the deregulation of its kinase activity combined with high levels of expression (overexpression).

C-terminally truncated Cot has a longer half-life (95 min) than the wild-type protein (35 min) [182]. Addition of a proteasome inhibitor to total cell extracts reduced degradation. After 30 min incubation with the 20 S proteasome, 55% of full-length Cot was degraded whilst only 20% of C-terminally truncated Cot was degraded. Degron at the C-terminal domain of Cot may target the protein for proteasome-mediated degradation. The phosphorylation of the C-terminal domain of Cot by AKT did not affect the degradation. The C-terminally truncated Cot appeared to have higher specific kinase activity than the full-length Cot. Therefore, in the next chapter, the binding partners of Cot and their binding sites were investigated.

Chapter 7

Proteomics Analysis of Proteins Associated with Cot

7.1 2D gel electrophoresis

As discussed in Chapter 4, the expression of Cot in *E. coli* and insect cells was not satisfactory. The co-expression of Cot with p105 obtained by other researchers is reasonable, but the level of purity that can be obtained is not high [173]. One possible reason is that the binding partner is helpful for the stability of Cot, but the interaction between these two proteins needs to be strong. The object of this proteomics study was try to find new binding partners of Cot protein and the binding site for these binding partners.

The six different Cot constructs (Cot₁₃₀₋₃₉₉, Cot₁₋₃₈₈, Cot₁₋₄₁₃, Cot₁₋₄₃₈, Cot₁₋₄₅₇, Cot_{full-length}) were expressed in HEK293 cells as mentioned previously. After 48 hr post-transfection, the total cell lysate was mixed with anti-6×His antibody and followed by incubation with protein-G-agarose. The proteins that co-immunoprecipitated with Cot kinase were separated by 2D electrophoresis (pH 3-10 non-linear IEF as the first dimension; 10% SDS-PAGE as the second dimension) in an attempt to identify potential binding partners in the absence of extracellular stimulation (Figure 7-1). 2D electrophoresis is a powerful and widely used method for the analysis of complex protein mixtures extracted from cells and tissues [183-185]. Thousands of different

proteins can thus be separated and information such as protein pI value, molecular weight, and quantity is obtained. Each spot can then be excised from the gel and the protein contained within extracted. The identity of the protein can be ascertained by trypsin digestion followed by mass spectrometric analysis of the resultant peptides. This procedure of separating and analyzing a complex mixture of proteins is generally known as proteomics.

This experiment was repeated for each of the Cot constructs to allow the binding region to be determined. As the anti-6×His antibody is not completely specific to the expressed Cot proteins, HEK293 cells transfected with an empty vector (pcDNA4 HisMax) were used as a negative control.

Thirteen regions (A-M, corresponding to those shown in Figure 6-1) were enlarged in Figure 7-2. Twenty-one protein spots were found in the Cot-transfected cell lysates that did not appear or had substantially reduced intensity, in the control sample (Figure 7-2, 7-3 and Table 7-1). Three spots (No. 1, 2, 20) appeared to bind to the C-terminal domain of Cot protein. Four spots (No. 6, 7, 9, 13) bound to the Cot protein in the absence of the N- and C-terminal domains. The interesting spots were picked for the protein identification by MALDI-MS/MS mass spectrometry. Spot No. 4 was identified to be chaperone Hsp90 (MOWSE score 101). Spot No. 5 was identified to be chaperone Hsp70 (MOWSE score 277). Spot No. 6 was identified to be chaperone Grp78 (MOWSE score 80). Spot No. 12 was identified to be MAPK-like protein

(NCBI NP057597, score 37). Spot No 19 was identified to be Mu-adaptin 2 (adaptor-related protein complex1, mu 2 subunit, NCBI Q9Y6Q5, score 34) or complement C1q tumor necrosis factor-related protein 7 precursor (NCBI Q9BXJ2, score 23). Since the MOWSE scores of the last two spots are lower than 75, the identification can only be regarded as tentative. Three of these proteins were identified successfully with the peptides sequenced. They are heat-shock protein Hsp70 and Hsp90 and glucose-regulated protein Grp78. The other spots have not yet been successfully identified, probably due to the quantity of protein being too low. From the pattern of association with the different Cot constructs (Table 7-1), it can also be determined that Hsp90 appears to bind to the kinase domain.

Figure 7-1 (previous page) 2D analysis of the proteins obtained from immunoprecipitation (anti-6×His) of the cell lysate from HEK293 cells that had been transfected with the six different constructs and the empty vector as a control. Thirteen regions (A-M) have been highlighted which contain 21 mismatched spots. These gels represent examples from at least two repeats. The molecular weight positions were determined using markers (not shown), and the pI positions were determined from information supplemented with the IPG gel strips (GE Healthcare).

7.2 Interaction between Cot and Hsp90

Hsp90 appeared to bind to all six constructs. Although there is a small spot for Hsp90 in the empty vector control, the ratio of upper spot to lower spot increased with the expression of Cot constructs (Figure 7-3b). Therefore, the increase of Hsp90 was due to the expression of Cot. However, the results shown here cannot address whether the binding of Hsp90 was an artefact as a consequence of the overexpression of the Cot protein or a real interaction with the Cot protein that is functionally specific. First, co-immunoprecipitations were performed with HEK293 cell lysate after the transfection of pcDNA4 HisMax six different Cot constructs (Cot₁₃₀₋₃₉₉, Cot₁₋₃₈₈, Cot₁₋₄₁₃, Cot₁₋₄₃₈, Cot₁₋₄₅₇, Cot_{full-length}). The expressed Cot was precipitated by its 6×His tag with anti-6×His antibody. The precipitated protein was probed with anti-Hsp90, anti-Hsp70, and anti-Grp78 antibodies. The bands of Hsp90, Hsp70 and Grp78 were detected at the right position (Figure 7-4). Furthermore, Hsp90 was also precipitated with anti-Hsp90 from Cot-transfected HEK293 cell lysate; Hsp70 was also precipitated with anti-Hsp70 from Cot-transfected HEK293 cell lysate; Grp78 was also precipitated

with anti-Grp78 from Cot-transfected HEK293 cell lysate. These three immunoprecipitates (anti-Hsp90, anti-Hsp70 and anti-Grp78) were probed with anti-Cot (M20). The bands corresponding to the Cot protein were detected at the right position (Figure 7-4). These immunoprecipitation results match the 2D electrophoresis results (Figure 7-2 and Table 7-1).

Figure 7-2 Thirteen enlarged regions (A-M, corresponding to those shown in Figure 7-1) from seven 2D gels (six different Cot constructs and the empty vector) showing the 21 mismatched spots.

Figure 7-3 2D analysis of empty vector and Cot₁₋₄₁₃. **(A)** 2D gels of empty vector (negative control) and Cot₁₋₄₁₃ were analyzed with program DYMENSION. **(B)** Circled regions were viewed in 3D. Each peak was marked with normalized volume. The regions were rotated 90° (anti-clockwise) for a better view. All Cot constructs gave similar results.

Table 7-1 Isoelectric points (pI) and molecular weight (Mw) values of cellular proteins associated with Cot

<i>Spot number</i>	<i>Approximate Mw (kD)</i>	<i>Approximate pI value</i>	<i>Empty vector</i>	<i>Cot₁₃₀₋₃₉₉</i>	<i>Cot₁₋₃₈₈</i>	<i>Cot₁₋₄₁₃</i>	<i>Cot₁₋₄₃₈</i>	<i>Cot₁₋₄₅₇</i>	<i>Cot₁₋₄₆₇</i>	<i>Protein identification</i>	<i>SwissProt ID</i>	<i>MOWSE score</i>	<i>Tryptic peptides sequenced by MS/MS</i>
1	200	5.75					+	+	+				
2	120	6.05					+	+	+				
3	150	6.35							+				
4	85	5.15		+	+	+	+	+	+	Hsp90	P07900	101	HFSVEGQLEFR GVVDSIDLPLNISR NPDDITNEEYGEFYK
5	69	5.15			+			+		Hsp70	P08107	277	DAGVIAGLNVLR TTPSYVAFTDTER IINEPTAAAIAYGLDR NQVALNPQNTVFDAKR
6	58	5.05		+						Grp78	P11021	80	IEIESFYEGEDFSETLTR ITPSYVAFTPEGER
7	52	5.35		+									
8	44	5.15			+								
9	62	5.85		+									
10	65	5.95				+	+						
11	63	5.95				+							
12	63	6.05					+	+					
13	45	5.80		+									
14	46	6.00				+							
15	49	6.75							+				
16	34	6.35			+			+					
17	44	7.35			+	+	+	+					
18	37	5.45					+						
19	31	5.85					+	+	+				
20	26	5.25				+	+	+	+				
21	24	5.20					+

Figure 7-4 The co-immunoprecipitations were performed with HEK293 cell lysate after the transfection of pcDNA4 HisMax-Cot_{full-length}. The expressed Cot was precipitated by its 6×His tag with anti-6×His antibody. Hsp90 was precipitated with the anti-Hsp90. Hsp70 was precipitated with the anti-Hsp70. Grp78 was precipitated with the anti-Grp78. The precipitated protein was performed the western blots using anti-Hsp90, anti-Hsp70, anti-Grp78 and anti-Cot.

Secondly, the co-immunoprecipitation was performed with the Hela cell lysates using the anti-Cot (H212) antibody. Hela cells express active Cot endogenously as mentioned previously (section 6.1). The immunoprecipitate was probed with antibodies anti-Cot (M20), anti-Hsp90, anti-p105, anti-ABIN-2, and anti-AKT (Figure 7-5). For full blots please refer to appendices (Figure A-2). The anti-Cot (H212) precipitated Cot protein was confirmed using anti-Cot (M20) antibody. Hsp90, ABIN-

2, and AKT were detected at the right position.

Figure 7-5 Co-immunoprecipitation analysis of endogenous Cot. The endogenous Cot was precipitated with anti-Cot (H212) antibody. The immunoprecipitate was performed the western blots with antibodies anti-Cot (M20), anti-Hsp90, anti-p105, anti-ABIN-2, and anti-AKT. Non-specific IgG (IgG chain C) was used as the negative control.

7.3 Discussion

Hsp90, Hsp70 and Grp78 were found to bind with recombinant Cot in HEK293 cells by 2D gel electrophoresis. The results were confirmed later by the western blot using anti-Cot (N17) with the immunoprecipitate of anti-Hsp90, anti-Hsp70 and anti-Grp78.

In order to verify that the binding of Hsp90 to Cot is functionally specific, endogenous Cot from Hela cells was precipitated using anti-Cot (H212), and the immunoprecipitate was probed with anti-Cot (M20), anti-Hsp90, anti-p105, anti-ABIN-2, and anti-AKT. Anti-Cot (M20) was used to verify that the right protein was precipitated. Anti-p105, anti-ABIN-2, and anti-AKT were used to test the cellular roles of expressed Cot protein. When probed with anti-p105, there was no obvious band at the right position. That means the expressed Cot in Hela cells was released from p105. Without the inhibitor (p105), the Cot protein may be active. Furthermore, when probed with anti-ABIN-2 and anti-AKT, there are bands for each of them at the right position. Considering the relationship between Cot and ABIN-2 or AKT, the expressed endogenous Cot in Hela cells may be active. Therefore, the binding of Hsp90 to Cot protein is not the result of overexpression of Cot. Hsp90 may possibly be involved in the signaling pathway of Cot.

Hsp70 and Hsp90 represent two different classes of chaperone [186]. Hsp70 holds the unfolded substrates to prevent irreversible aggregation by refolding denatured proteins. As a chaperone, the role of Hsp90 does not depend on the presence of nucleoside triphosphates, Hsp90 suppresses the formation of protein aggregates by binding to the target proteins at a stoichiometry of one Hsp90 dimer to one or two substrate molecule(s) [187]. AKT is critical for Cot function in the induction of NF- κ B-dependent transcription [97]. Ser400 of Cot will be phosphorylated by AKT. The phosphorylation of Ser400 may lead Cot to release from IKK complex. However, Cot

was not phosphorylated by AKT *in vitro* [97]. This indicates that a protein is needed to bring these two proteins together. ABIN-2 was a negative regulator of NF- κ B activation and required for the activation of ERK induced by the receptor TLR4 via Cot [188]. Furthermore, ABIN-2 was also involved in the maintenance of Cot stability [189]. The p105 protein was not detected in my experiments. p105 is an inhibitor of Cot and is released from Cot after its activation. Since endogenous Cot was co-immunoprecipitated with AKT, ABIN-2 but not p105, overexpressed Cot in HeLa cells appears to be active. As Hsp90 was co-immunoprecipitated with Cot, this implies that Hsp90 is being used to prevent the aggregation of Cot rather than to correct the folding in cells. Hsp90 is an essential and highly abundant molecular chaperone of eukaryotic cells. It is needed for the final activation step of several key signaling and regulatory proteins rather than acting at an early stage of protein folding. Hsp90 is associated with these proteins when they are already substantially folded, inducing the structural changes and protein associations whereby they attain full activity.

Hsp90 has been shown to interact with around 100 proteins including many kinases involved in signaling pathways, including AKT [190], IKK [191], KSR-1 [192], Src [193] and Raf [194]. The role of Hsp90 in the Ras-Raf signal transduction pathway has been well studied [194]. Hsp90 can recruit Raf to the membrane like Ras; Hsp90 can change the conformation of the regulatory loop of Raf for the activation; and most importantly, Hsp90 can stabilize the active conformation of Raf [195].

Several Hsp90 inhibitors have been developed and are currently in clinical trials as anti-cancer therapeutic agents [196]. Hsp70 is essential for the assembly of signaling protein:Hsp90 heterocomplexes, and this relates to the finding of this study that Hsp70 binds to Cot in the presence of Hsp90. The Hsp90/Hsp70 chaperone system is an important regulator of signal transduction pathways for steroid hormones receptors, kinase signaling, and transcription factors [197]. Hsp90 has been demonstrated to bind directly to the kinase catalytic domain [198] and can directly affect kinase activity by blocking the access of the phosphatase and hence modulating phosphorylation [190]. Grp78 (also known as BiP) is a chaperone that is abundant in the endoplasmic reticulum and is a member of Hsp70 family [199]. It is therefore possible that Cot, ABIN-2, AKT and the Hsp90/Hsp70/Grp78 chaperone proteins are a part of a signaling complex (Figure 7-6).

Figure 7-6 A schematic diagram of the Cot and Hsp90 signaling complex. Hsp90 can alter the conformation of Cot thus helping it to bind with other proteins, and keeping it both stable and active.

Chapter 8

Substrate Specificity of Cot Protein

8.1 Peptide library screening

The most well-established substrates of Cot are MEK1/2 [200] and MKK6 [201], but given the involvement of Cot in a diverse range of cellular pathways (sections 1.10 and 1.11), it is possible that not all the target proteins of Cot have yet been identified. Therefore, identification of putative substrates of Cot through a peptide-based screening assay was performed.

Cot₁₋₄₁₃ and Cot_{full-length} were transiently expressed in HEK293 cells, immunoprecipitated using anti-6×His antibody and verified by *in vitro* and *in vivo* kinase assays, using MEK1/2 as the substrate, as described previously (Section 6.5). As full-length Cot (residues 1-467) may be less stable than C-terminally truncated Cot [182], Cot₁₋₄₁₃ was used for the substrate screening assay (Figure 8-1). Later work, including the *in vitro* and *in vivo* kinase assays, was performed with Cot₁₋₄₁₃ or the full-length protein. The serine/threonine kinase substrate assay (Cell Signaling Technology) allowed the potential phosphorylation of different proteins by Cot to be screened. The assay contained 87 peptides which encompass the phosphorylation site of various potential protein substrates (Table A-2, appendices). Phosphorylation of these peptides by Cot was detected by a phospho-specific primary antibody. The

primary antibodies were detected using europium-labeled secondary antibody (DELFIA, PerkinElmer). The fluorescence emission was recorded at 615 nm (excitation 340 nm, gate and lag 400 μ s) using a time-resolved fluorescence (TRF) Safire² plate reader (Tecan). The results are shown in Table A-3 (appendices). The values in the table correspond to the average intensity readings for two separate experiments. The readings between the two experiments were within 7.5% of each other. The SNR (signal:noise ratio) was calculated using the following formula: $(\text{signal} - \text{negative control})/(\text{positive control} - \text{negative control}) \times 100$. Substrates which gave a higher signal:noise ratio (SNR) than 66% are shown in Table 8-1 (the target indicates the protein from which the peptide sequence was derived).

From Table 8-1, several proteins may be the potential substrates of Cot. They are Ezrin, CHK1 (checkpoint kinase), Plk1, p90RSK (p90 ribosomal S6 kinase), eIF4G, 4E-BP1 (eIF-4E-binding protein 1), MKK3/6, Ab1, cdc2, CREB, Cofilin, Histone H2B and, including the well-established substrate of Cot: MEK1/2 [133] and MKK3/6 [100]. SNR for MEK1/2 and MKK3/6 are 76.3 % and 66.7 % accordingly. The SNR values of some proteins such as Ezrin, CHK1, Plk1, p90RSK, 4E-BP1, Ab1, cdc2, CREB, Cofilin, Histone H2B are higher than that of MEK1/2 and MKK3/6. This indicates that some of these proteins may be more suitable as substrates for Cot. Plk1 (probed with anti-phospho-Plk1, highlighted in bold) did not give the highest SNR (93.8%). However, the magnitude of the signal (45,009) was the largest and significantly higher than for the other targets. Furthermore, the substrate screening kit

contained an identical peptide which was detected by anti-phospho-PKC rather than anti-phospho-Plk1; this also gave a significant SNR (Table 8-1). Plk1 was of especial interest given the current ambiguity surrounding the identity of the upstream kinase that phosphorylates Plk at Ser137 [202]. Further experiments were performed to verify the phosphorylation of Plk1 by Cot.

Figure 8-1 Cot₁₋₄₁₃ was transiently expressed in HEK293 cells. The expressed Cot₁₋₄₁₃ was immunoprecipitated using anti-6×His antibody. Western blot was performed with anti-Cot (N17) to verify the right protein.

Table 8-1 Potential substrates of Cot identified by the peptide-based screening assay

Target	Positive control	Negative control	Signal	SNR (%)
Histone H2B (Ser32) (detected by anti-phospho-PKC)	255	10	254	99.6
Plk1 (Ser137) (detected by anti-phospho-PKC)	3702	76	3680	99.4
p90RSK (Ser380) (detected by anti-phospho-Phe)	3994	150	3936	98.5
Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558)	636	112	627	98.3
cdc2 (Thr161)	119	22	117	97.9
Chk1 (Ser345)	421	87	402	94.3
Plk1 (Ser137)	47954	462	45009	93.8
Abl (Thr735)	1203	83	1089	89.8
CREB (Ser133) (detected by anti-phospho-PKA)	10404	12	9311	89.5
Cofilin (Ser3)	180	35	163	88.3
4E-BP1 (Thr70)	88	9	74	82.3
eIF4G (Ser1108)	912	43	731	79.2
MEK1/2 (Ser217/221)	2827	39	2165	76.3
MKK3/MKK6 (Ser189/207)	642	21	435	66.7

8.2 Kinase assay (protein-protein) *in vitro* of Plk1

To provide an initial verification of the substrate screening assay result indicating phosphorylation of Plk1 at Ser137, immunoprecipitated Cot_{full-length} and Cot₁₋₄₁₃ was incubated in the presence and absence of ATP with GST-tagged Plk1 for 30 min at 30 °C. Cot_{full-length} kinase-dead was used as a negative control. Kinase assay reactions (Cot proteins with GST-Plk1) were separated on a 10% SDS-PAGE gel stained with Coomassie blue dye (Figure 8-2a). A band of about 99 kD was observed which matched the molecular weight of GST-Plk1 and one sample of this band was analyzed by the MALDI MS/MS and verified the right protein. The duplicate was transferred to

PVDF membrane for western blotting probed with anti-Cot (N17), anti-Plk1, anti-phospho-Plk1 (Ser137) and anti-phospho-Plk1 (Thr210). The anti-Cot (N17) and anti-Plk1 were used as the kinase reaction controls. Compared with the negative control lanes (Cot₁₋₄₁₃ with GST-Plk1 without ATP and Cot kinase-dead), the rest two lanes present the positive reactions of phosphorylation at Ser137. However, very faint bands from anti-phospho-Plk1 (Thr210) can also be detected (if increasing exposure time), which may be due to the phosphospecific antibody's lack of specificity rather than real phosphorylation, and there was no obvious difference in their intensity between the samples in the lanes of presence, absence of ATP and Cot kinase-dead (Figure 8-2b).

Figure 8-2 Kinase assay (protein-protein) *in vitro* I. Cot₁₋₄₁₃, Cot_{full-length} and kinase-dead mutant Cot were incubated with GST-Plk1 and 2.5 mM ATP at 30 °C for 30 min for the kinase assay *in vitro*. Cot₁₋₄₁₃ was incubated with GST-Plk1 but without ATP as the negative control. GST-Plk1 was detected with Coomassie blue (**A**) and anti-Plk1 (**B**). Cot proteins were detected with anti-Cot. Phospho-Plk1 at Ser137 and Thr210 were detected by probing with anti-p-Plk1 (Ser137) and anti-p-Plk1 (Thr210).

From the previous Coomassie blue stained gel (Figure 8-2a), bands of GST-Plk1 (99 kD) were cut out and destained. Following trypsin digestion, the phosphopeptides were isolated by an IMAC column (Pierce), desalted with a ZipTip and analyzed by MALDI-MS to obtain the molecular weight of phosphopeptides. It can be seen that the mass spectrum following reaction with Cot₁₋₄₁₃ (Figure 8-3b) contains a significantly higher number of potentially phosphorylated peptides than the control sample (Figure 8-3a). The sequence of GST-Plk1 was analyzed by the program GPMW [203] to give a list of the theoretical tryptic peptides (Figure 8-4). The total number of possible tryptic peptides is large due to the presence of repeated arginine and lysine residues in the Plk1 sequence. Three peaks visible in the Cot but not the control sample with molecular weights of 1073.0, 1516.7 and 1670.7 Da may correspond to the phosphorylated forms (+80 Da) of the following tryptic peptides: RSLLELHK/SLLELHKR (identity of the peptide is uncertain as they have identical mass, the intensity is too low for MS/MS analysis), RSLLELHKRRK and RRSLLELHKRRK which encompass Ser137 (underlined) (the calculated molecular weights of the unphosphorylated peptides are 994.6, 1434.9 and 1591.0 Da respectively). Three peaks of similar mass were also seen in an identical experiment when Cot_{full-length} was replaced with Cot₁₋₄₁₃. No significant peaks could be mapped to phosphorylated tryptic peptides encompassing Thr210, suggesting that Cot does not phosphorylate Plk1 at this site.

A **B**

Figure 8-3 Kinase assay (protein-protein) *in vitro* II. Coomassie blue stained GST-Plk1 bands from Figure 8-2a were picked for trypsin digestion, the phospho-peptides were isolated by the IMAC column (Pierce). After desalting with a ZipTip, the samples were mixed with matrix solution and spotted onto a MALDI plate for the molecular weight analysis. Three peaks at 1073.0, 1516.7 and 1670.7 Da which are present in the positive reaction (**B**) and not the negative control (**A**), may represent the phosphorylated peptides of Ser137 (* indicates phosphorylation). Only the result of the incubation of Cot₁₋₄₁₃ with GST-Plk1 is presented, as the incubation of Cot_{full-length} with GST-Plk1 gave the same result.

Mass	From-To	MonoIso	HPLC	B&B	Ch.	Sequence
97*	87- 97	1347.80	17.92	-920	3	Ser-Leu-Leu-Leu-Lys-Pro-His-Gln-Arg-Glu-Lys-
98*	96-106	1359.66	17.89	-520	1	Glu-Lys-Met-Ser-Met-Glu-Ile-Ser-Ile-His-Arg-
47	325-337	1389.69	18.10	-480	0	Phe-Ser-Ile-Ala-Pro-Ser-Ser-Leu-Asp-Pro-Ser-Asn-Arg-
325*	51- 61	1406.04	10.14	-510	5	Arg-Arg-Tyr-Val-Arg-Gly-Arg-Phe-Leu-Gly-Lys-
86*	39- 50	1408.77	17.26	-1510	-1	Glu-Ile-Pro-Glu-Val-Leu-Val-Asp-Pro-Arg-Ser-Arg-
145*	501-512	1430.75	20.23	-2120	-1	Glu-Gly-Asp-Glu-Leu-Ala-Arg-Leu-Pro-Tyr-Leu-Arg-
339*	136-146	1434.89	17.00	-340	5	Arg-Ser-Leu-Leu-Glu-Leu-His-Lys-Arg-Arg-Lys-
338*	133-145	1462.90	17.38	-110	5	Arg-Arg-Ser-Leu-Leu-Glu-Leu-His-Lys-Arg-Arg-
107*	147-158	1480.77	19.72	-2420	0	Ala-Leu-Thr-Glu-Pro-Glu-Ala-Arg-Tyr-Tyr-Leu-Arg-
310*	590-603	1480.84	16.02	1100	3	Leu-Leu-Ser-Ser-Arg-Ser-Ala-Ser-Asn-Arg-Leu-Lys-Ala-Ser-
32	179-191	1502.80	20.44	-4190	-2	Leu-Gly-Asn-Leu-Phe-Leu-Asn-Glu-Asp-Leu-Glu-Val-Lys-
226*	508-518	1507.84	24.15	-4970	3	Leu-Pro-Tyr-Leu-Arg-Thr-Trp-Phe-Arg-Thr-Arg-
96*	83- 95	1527.96	21.36	-3800	4	Ile-Val-Pro-Lys-Ser-Leu-Leu-Lys-Pro-His-Gln-Arg-
130*	146-158	1533.83	15.86	1240	0	Gly-Leu-Glu-Asn-Pro-Leu-Pro-Glu-Arg-Pro-Arg-Glu-Lys-
237*	502-514	1530.02	10.27	-1950	2	Tyr-Ala-Arg-Thr-Met-Val-Asp-Lys-Leu-Leu-Ser-Ser-Arg-
249*	53- 66	1554.88	21.31	-720	4	Tyr-Val-Arg-Gly-Arg-Phe-Leu-Gly-Lys-Gly-Gly-Phe-Ala-Lys-
166*	39- 51	1564.87	17.43	-820	0	Glu-Ile-Pro-Glu-Val-Leu-Val-Asp-Pro-Arg-Ser-Arg-Arg-
144*	492-507	1568.79	14.99	4370	-1	Ala-Gly-Ala-Asn-Ile-Thr-Pro-Arg-Glu-Gly-Asp-Glu-Leu-Ala-Arg-
4	20- 38	1568.87	15.00	5420	1	Ala-Gly-Val-Pro-Gly-Val-Ala-Ala-Pro-Gly-Ala-Ala-Pro-Pro-Ala-Lys-
411*	42- 76	1566.73	21.48	-3020	-1	Gly-Gly-Phe-Ala-Lys-Cys-Phe-Glu-Ile-Ser-Asp-Ala-Lys-Thr-Lys-
415*	135-146	1590.99	17.19	350	6	Arg-Arg-Ser-Leu-Leu-Glu-Leu-His-Lys-Arg-Arg-Lys-
109*	159-171	1597.04	22.27	-660	3	Gln-Ile-Val-Leu-Gly-Cys-Gln-Tyr-Leu-His-Arg-Asn-Arg-
142*	481-492	1599.79	21.82	-4680	2	Tyr-Phe-Arg-Asn-Tyr-Met-Ser-Glu-His-Leu-Leu-Lys-
186*	146-158	1608.86	19.46	-1960	1	Lys-Ala-Leu-Thr-Glu-Pro-Glu-Ala-Arg-Tyr-Tyr-Leu-Arg-
401*	49- 61	1649.97	18.42	600	6	Ser-Arg-Arg-Tyr-Val-Arg-Gly-Arg-Phe-Leu-Gly-Lys-
230*	505-509	1663.87	15.25	1210	2	Thr-Met-Val-Asp-Lys-Leu-Leu-Ser-Ser-Arg-Ser-Ala-Ser-Asn-Arg-
153*	561-574	1674.77	26.14	-3400	0	Thr-Tyr-Arg-Leu-Ser-Leu-Leu-Glu-Glu-Tyr-Gly-Cys-Cys-Lys-
37	210-225	1703.87	22.29	-2630	0	Thr-Leu-Cys-Gly-Thr-Pro-Asn-Tyr-Ile-Ala-Pro-Glu-Val-Leu-Ser-Lys-
326*	82- 66	1710.98	21.34	-30	8	Arg-Tyr-Val-Arg-Gly-Arg-Phe-Leu-Gly-Lys-Gly-Gly-Phe-Ala-Lys-
245*	39- 52	1720.97	17.62	-130	1	Glu-Ile-Pro-Glu-Val-Leu-Val-Asp-Pro-Arg-Ser-Arg-Arg-Arg-
94*	67- 82	1757.81	21.41	1020	-2	Cys-Phe-Glu-Ile-Ser-Asp-Ala-Asp-Thr-Lys-Glu-Val-Phe-Ala-Gly-Lys-
264*	148-158	1764.96	19.57	-1270	2	Arg-Lys-Ala-Leu-Thr-Glu-Pro-Glu-Ala-Arg-Tyr-Tyr-Leu-Arg-
114*	192-207	1768.86	20.72	-80	-2	Ile-Gly-Asp-Phe-Gly-Leu-Ala-Thr-Lys-Val-Glu-Tyr-Asp-Gly-Glu-Arg-
176*	83- 97	1785.10	21.21	-2830	4	Ile-Val-Pro-Lys-Ser-Leu-Leu-Lys-Pro-His-Gln-Arg-Glu-Lys-
69	541-556	1790.94	27.48	-7410	-1	Leu-Ile-Leu-Cys-Pro-Leu-Met-Ala-Ala-Val-Thr-Tyr-Ile-Asp-Glu-Lys-
214*	575-589	1807.97	10.40	-560	2	Glu-Leu-Ala-Ser-Arg-Leu-Arg-Tyr-Ala-Arg-Thr-Met-Val-Asp-Lys-
315*	580-594	1808.01	20.65	-2910	3	Leu-Arg-Tyr-Ala-Arg-Thr-Met-Val-Asp-Lys-Leu-Leu-Ser-Ser-Arg-
57	442-456	1810.91	24.52	-5200	-2	Leu-Ile-Leu-Tyr-Asn-Asp-Gly-Asp-Ser-Leu-Gln-Tyr-Ile-Glu-Arg-
154*	564-579	1810.86	26.30	-2370	-1	Leu-Ser-Leu-Leu-Glu-Glu-Tyr-Gly-Cys-Cys-Lys-Glu-Leu-Ala-Ser-Arg-
162*	1- 19	1810.97	15.87	4810	2	Met-Ser-Ala-Ile-Val-Thr-Ala-Gly-Lys-Leu-Ala-Arg-Ala-Pro-Ala-Asp-Pro-Gly-Lys-
117*	209-225	1831.96	22.12	-2170	1	Lys-Thr-Leu-Cys-Gly-Thr-Pro-Asn-Tyr-Ile-Ala-Pro-Glu-Val-Leu-Ser-Lys-
110*	210-226	1831.96	22.12	-2170	1	Thr-Leu-Cys-Gly-Thr-Pro-Asn-Tyr-Ile-Ala-Pro-Glu-Val-Leu-Ser-Lys-Lys-
112*	176-191	1859.00	22.15	-4770	-2	Asp-Leu-Lys-Leu-Gly-Asn-Leu-Phe-Leu-Asn-Glu-Asp-Leu-Glu-Val-Lys-
402*	51- 66	1867.08	21.39	660	6	Arg-Arg-Tyr-Val-Arg-Gly-Arg-Phe-Leu-Gly-Lys-Gly-Gly-Phe-Ala-Lys-
280*	258-272	1881.04	20.16	-2890	2	Glu-Thr-Tyr-Leu-Arg-Ile-Lys-Lys-Asn-Glu-Tyr-Ser-Ile-Pro-Lys-
194*	192-200	1896.96	20.50	300	-1	Ile-Gly-Asp-Phe-Gly-Leu-Ala-Thr-Lys-Val-Glu-Tyr-Asp-Gly-Glu-Arg-Lys-
217*	585-601	1905.05	17.63	20	3	Thr-Met-Val-Asp-Lys-Leu-Leu-Ser-Ser-Arg-Ser-Ala-Ser-Asn-Arg-Leu-Lys-
143*	484-500	1913.98	19.34	-140	2	Asn-Tyr-Met-Ser-Glu-His-Leu-Leu-Lys-Ala-Gly-Ala-Asn-Ile-Thr-Pro-Arg-
341*	144-150	1921.06	19.70	-580	3	Arg-Arg-Lys-Ala-Leu-Thr-Glu-Pro-Glu-Ala-Arg-Tyr-Tyr-Leu-Arg-
108*	156-169	1923.11	28.16	-6260	3	Tyr-Tyr-Leu-Arg-Gln-Ile-Val-Leu-Gly-Cys-Gln-Tyr-Leu-His-Arg-
150*	541-557	1947.04	27.35	-6720	0	Leu-Ile-Leu-Cys-Pro-Leu-Met-Ala-Ala-Val-Thr-Tyr-Ile-Asp-Glu-Lys-Arg-
54	397-413	1959.82	25.98	-1140	-3	Gln-Glu-Glu-Ala-Glu-Asp-Pro-Ala-Cys-Ile-Pro-Ile-Phe-Trp-Val-Ser-Lys-
196*	208-225	1960.06	21.89	-1710	2	Lys-Lys-Thr-Leu-Cys-Gly-Thr-Pro-Asn-Tyr-Ile-Ala-Pro-Glu-Val-Leu-Ser-Lys-
197*	209-226	1960.06	21.89	-1710	2	Lys-Thr-Leu-Cys-Gly-Thr-Pro-Asn-Tyr-Ile-Ala-Pro-Glu-Val-Leu-Ser-Lys-Lys-

Figure 8-4 The sequence of GST-Plk1 was analyzed by the program GPMW [203] to give a list of the theoretical tryptic peptides.

8.3 Kinase assay (peptide-protein) *in vitro* of Plk1

To obtain further confirmation of the previous result, the peptides RRRSLLELHKR (M_w : 1463.8 Da) and RKKTLCGTPNY (M_w : 1280.9 Da) were synthesized, encompassing the regions of Plk1 (residues 134 to 144 and 207 to 217 respectively) that include Ser137 and Thr210 (underlined). The peptides were incubated with immunoprecipitated Cot_{full-length} protein with or without ATP, desalted and analyzed by MALDI-MS. In the presence of ATP, a new peak, shifted by 80 Da (to 1543.7 Da), was apparent for the peptide RRRSLLELHKR (Figure 8-5a and b). Consequent

MS/MS analysis for the peak of 1543.7 Da confirmed that phosphorylation occurred uniquely at the residue corresponding to Ser137 (Figure 8-5c). However, no additional peaks were detected for the peptide RKKTLCGTPNY in the presence of ATP (Figure 8-6). These results provide further *in vitro* evidence that Cot phosphorylates Plk1 at Ser137 and not at Thr210.

Figure 8-5 Kinase assay (peptide-protein) *in vitro* I. Synthesized peptide RRRSLLLELHKR (MW: 1463) was incubated with precipitated Cot_{full-length} protein without or with ATP at 30 °C for 30 min. The reactions were desalted with a ZipTip and mixed with the matrix solution. The peptides were analyzed by MALDI-MS/MS. (**A**) represents the control sample (Cot_{full-length} without ATP) and (**B**) the sample following reaction (Cot_{full-length} and ATP). (**C**) The peak at 1543.7 Da (Figure 8-5b) was selected and the peptide sequence identified by MS/MS. *, phosphorylation.

Figure 8-6 Kinase assay (peptide-protein) *in vitro* II. Synthesized peptide RKKTLCGTPNY (M_w : 1280.9 Da) was incubated with precipitated Cot_{full-length} protein without or with ATP at 30 °C for 30 min. The peptides were analyzed by MALDI-MS/MS as previously described. **(A)** the control (Cot_{full-length} without ATP) and **(B)** the sample following reaction (Cot_{full-length} and ATP).

To investigate the phosphorylation site specificity of Plk1 by Cot, another two peptides were synthesized. One was RRRTLLELHKR (M_w : 1477.9 Da) in which Ser137 was substituted by Thr. The other was RKKSLCGTPNY (M_w : 1266.5 Da) in

which Thr210 was substituted by Ser. Cot *in vitro* kinase assays were performed using these two peptides. Peptide molecular weights were detected by MALDI-TOF (Figures 8-7 and 8-8). Neither of them demonstrated a 80 Da shift indicating that these two peptides were not phosphorylated. This suggests that the phosphorylation of Plk1 at Ser137 by Cot is site specific and Ser-specific.

A

B

Figure 8-7 Kinase assay (peptide-protein) *in vitro* III. Synthesized peptide RRRTLLELHKR (M_w : 1477.9 Da) was incubated with precipitated Cot_{full-length} protein without or with ATP at 30 °C for 30 min. The peptides were analyzed by MALDI-MS/MS as previously described. **(A)** the control (Cot_{full-length} without ATP) and **(B)** the sample following reaction (Cot_{full-length} and ATP).

A

B

Figure 8-8 Kinase assay (peptide-protein) *in vitro* IV. Synthesized peptide RKKSLCGTPNY (M_w : 1266.5 Da) was incubated with precipitated Cot_{full-length} protein without or with ATP at 30 °C for 30 min. The peptides were analyzed by MALDI-MS/MS as previously described. **(A)** the control (Cot_{full-length} without ATP) and **(B)** the sample following reaction (Cot_{full-length} and ATP).

To investigate which amino acid residues were critical for the phosphorylation of Ser137, another five peptides related to Ser137 were synthesized. Three were

truncated at the C-terminus (RRRSLLELH, RRRSLLE and RRRSLL); another two were truncated at the N-terminus (RRSLLELHKR and RSLLELHKR). These five peptides were used in an *in vitro* Cot kinase assay. Peptide molecular weights were detected by MALDI-TOF (Figures 8-9, 8-10, 8-11, 8-12 and 8-13). An 80 Da shift for the peptides RRRSLLELH and RRRSLLE could be observed, suggesting the phosphorylation of the serine residue corresponding to Ser137 (Figures 8-9 and 8-10). The phosphorylation of peptide RRRSLLE was less than that of peptide RRRSLLELH by comparing the ratio of phosphorylated peptide and unphosphorylated peptide. When the peptide was shorter at the C-terminus, for example in the case of peptide RRRSLLE, no 80 Da shift could be detected (Figure 8-11) which implies that the serine residue corresponding to Ser137 for this peptide is not phosphorylated. The five residues, LLELH, at the C-terminal side of Ser137 are therefore demonstrated to be important for the phosphorylation of Ser137. Although only a minimum of three residues, LLE, at the C-terminal side was absolutely required for the phosphorylation of Ser137, the phosphorylation efficiency was substantially lower than in the case of five residues. This suggests that the length of the peptide on the C-terminal side of Ser137 is important, with five residues being optimal. When residues were truncated from the N-terminal side of the serine corresponding to Ser137, for example in the case of RRSLLELHKR and RSLLELHKR, no 80 Da shift was observed (Figure 8-12 and Figure 8-13). These results imply that the three sequential arginine residues on the N-terminal side of Ser137 are essential for phosphorylation. Table 8-2 is a summary of the Cot-Plk

peptide kinase assays.

A

B

Figure 8-9 Kinase assay (peptide-protein) *in vitro* V. Synthesized peptide RRRSLLELH (M_w : 1179.7 Da) was incubated with precipitated Cot_{full-length} protein without or with ATP at 30 °C for 30 min. The peptides were analyzed by MALDI-MS/MS as previously described. **(A)** the control (Cot_{full-length} without ATP) and **(B)** the sample following reaction (Cot_{full-length} and ATP). *, phosphorylation.

A

B

Figure 8-10 Kinase assay (peptide-protein) *in vitro* VI. Synthesized peptide RRRSLLE (M_w : 929.5 Da) was incubated with precipitated Cot_{full-length} protein without or with ATP at 30 °C for 30 min. The peptides were analyzed by MALDI-MS/MS as previously described. **(A)** the control (Cot_{full-length} without ATP) and **(B)** the sample following reaction (Cot_{full-length} and ATP). *, phosphorylation.

Figure 8-11 Kinase assay (peptide-protein) *in vitro* VII. Synthesized peptide RRRSLL (M_w : 799.9 Da) was incubated with precipitated Cot_{full-length} protein without or with ATP at 30 °C for 30 min. The peptides were analyzed by MALDI-MS/MS as previously described. **(A)** the control (Cot_{full-length} without ATP) and **(B)** the sample following reaction (Cot_{full-length} and ATP).

Figure 8-12 Kinase assay (peptide-protein) *in vitro* VIII. Synthesized peptide RRSLLLELHKR (M_w : 1307.7 Da) was incubated with precipitated Cot_{full-length} protein without or with ATP at 30 °C for 30 min. The peptides were analyzed by MALDI-MS/MS as previously described. **(A)** the control (Cot_{full-length} without ATP) and **(B)** the sample following reaction (Cot_{full-length} and ATP).

A

B

Figure 8-13 Kinase assay (peptide-protein) *in vitro* IX. Synthesized peptide RSLLEELHKR (M_w : 1151.5 Da) was incubated with precipitated $Cot_{full-length}$ protein without or with ATP at 30 °C for 30 min. The peptides were analyzed by MALDI-MS/MS as previously described. **(A)** the control ($Cot_{full-length}$ without ATP) and **(B)** the sample following reaction ($Cot_{full-length}$ and ATP).

Table 8-2 Summary of Cot-Plk1 peptide kinase assays

Peptides	Phosphorylation by Cot	Peptide description
RKK <u>T</u> LCGTPNY	Negative	Thr210
RRR <u>S</u> LLELHKR	Positive	Ser137
RRR <u>T</u> LLELHKR	Negative	S137T
RKK <u>S</u> LCGTPNY	Negative	T210S
RRR <u>S</u> LLELH	Positive	2 amino-acids shorter at C-terminal
RRR <u>S</u> LLE	Positive, but partial phosphorylation	4 amino-acids shorter at C-terminal
RRR <u>S</u> LL	Negative	5 amino-acids shorter at C-terminal
RR <u>S</u> LLELHKR	Negative	1 amino-acids shorter at N-terminal
R <u>S</u> LLELHKR	Negative	2 amino-acids shorter at N-terminal

(Note: S or T underlined represents the potential phosphorylation site for Cot)

8.4 Peptide structure prediction and analysis

8.4.1 Cluster analysis results

The clear difference observed in the *in vitro* phosphorylation of two almost identical 11-residue peptides (Ser137 and S137T, Table 8-2) was striking. The sole difference between these two peptides is the addition of a methyl ($-\text{CH}_3$) group to Ser137, and the resultant additional chiral centre. In an effort to understand this result, a prediction of the structure of these peptides was determined using quantum mechanical calculations (due to their complexity, these experiments were performed by Dr Y. Mu). Although these two peptides (Ser137, RRRSLLELHKR and S137T, RRRTLLELHKR) did not appear to have a unique structure in solution, cluster analysis based on pair-wise root mean squared deviation (RMSD) does resolve a structural preference for them. The RMSDs were calculated based on backbone atoms and the cutoff for clustering is 0.1 nm. The two representative structures (the center of the cluster) of the highest populated clusters for the two peptides are shown in Figure 8-14. Both peptides are abundant in charged residues. The conformations are

stabilized by hydrogen bonds and salt bridges between the polar groups of side chains as well as backbone atoms. Interestingly, the hydroxyl group of the S137T peptide is observed to face inside and is hydrogen-bonded with an electronegative oxygen on the carboxylate group of the glutamate side chain (Figure 8-14b) and may prevent its interaction with approaching kinase. The hydroxyl group of the Ser137 peptide, by contrast, is exposed to the solvent and may be readily available for attack by the kinase and subsequently phosphorylated.

Figure 8-14 Representative structure of the highest populated clusters of both wild type (Ser137, a) and mutant (S137T, b) peptides. The sidechains of serine or threonine are displayed in stick form. Red dashed lines demonstrate the polar contacts (hydrogen bonds or salt bridges) between residues.

8.4.2 Solvent accessible surface of the hydroxyl group on Ser/Thr

To test the hypothesis that an observable difference of orientation of the hydroxyl group of the Ser/Thr on the two peptides exists, the solvent accessible surface (SAS)

of the hydroxyl group on both the serine and threonine was calculated over 52 ns at 300 K. The distribution of the hydroxyl SAS is plotted in Figure 8-15. The SAS of serine hydroxyl was distributed around one peak (0.3 nm^2). This is consistent with the finding in Figure 8-14a where the hydroxyl group of the serine residue is solvent-exposed. By comparison, there is an additional probability (a second peak) for the SAS of the hydroxyl group of the threonine residue to be small (0.1 nm^2). This indicates that the hydroxyl group on the threonine residue is partially buried and thus phosphorylation by the kinase is hindered. The result of the SAS distribution is consistent with the direct structural interrogation shown in Figure 8-14.

The change from Ser to Thr would normally be considered to be a conservative mutation. However, our modeling shows that the orientation of hydroxyl group on Ser may be different from that on Thr, and thus a possible structural explanation for the inability of Cot to phosphorylate the S137T peptide is demonstrated.

Figure 8-15 Distribution of the solvent accessible surface (SAS) of the hydroxyl group in the serine residue in the Ser137 peptide (black) and the threonine residue in the S137T peptide (red).

8.5 *In vivo* phosphorylation of Plk1

To investigate the *in vivo* phosphorylation of Plk1 Ser137 by Cot, two expression constructs derived from pcDNA4 HisMax [Cot_{full-length} with Plk1 or Cot_{full-length} kinase-dead mutant (K167 R) with Plk1] were co-transfected into HEK293 cells. After 48-hr post transfection, cells were starved for 16 hr and followed by the stimulation with 200 ng mL⁻¹ LPS to activate the Cot protein. Cells were harvested after 6-hr stimulation. The expressed proteins (both Cot proteins and Plk1 with 6×His tag at the N-terminus) were precipitated by anti-6×His antibody and probed with anti-Cot, anti-Plk1, anti-phospho-Plk1 (Ser137) and anti-phospho-Plk1 (Thr210) respectively (Figure 8-16). For whole blots please refer to Figure A-3 (appendices). Empty

pcDNA4 vector was used as a negative control. From Figure 8-16, it can be seen that the expression of Cot_{full-length} and Cot_{full-length} kinase-dead mutant was almost at the same level. Furthermore, the expression of Plk1 for both co-transfections was also similar. However, when the membranes were stripped and reprobed with anti-p-Plk1 (Ser137), the density of the bands was different. The density of the band from Cot_{full-length} with Plk1 was higher than the band from kinase-dead mutant Cot_{full-length} with Plk1. Furthermore, the density of the Plk1 and phospho-Plk1 (Thr210) bands was almost identical. Cot kinase-dead mutant has been reported as being inactive [113, 204]. This was also confirmed by my experimental result. With the activated Cot, Plk1 was phosphorylated on Ser137. With kinase-dead mutant Cot, phosphorylation of Ser137 was inhibited. These results indicate that Plk1 is phosphorylated by Cot at Ser137 and not at Thr210 *in vivo*.

Figure 8-16 Kinase assay *in vitro*. Constructs of Cot_{full-length} with Plk1 or Cot_{full-length} kinase-dead mutant with Plk1 were co-transfected into HEK293 cells. After 48-hr post transfection, Cot was activated with 200 ng mL⁻¹ LPS. The expressed proteins (both Cot kinases and Plk1 had a 6×His tag at N-terminus) were precipitated by anti-6×His antibody and probed with anti-Cot, anti-Plk1, anti-phospho-Plk1 (Ser137) and anti-phospho-Plk1 (Thr210) respectively. Empty vector (pcDNA4) was performed the whole procedure as the negative control. The faint bands for anti-p-Plk1(Thr210) appeared to be the result of non-specific reactions.

8.6 Interaction between Cot and Plk1

To further investigate whether the interaction between Cot and Plk1 is physiologically relevant, Cot protein was precipitated from the cell lysate of Hela cells (endogenous Cot) using anti-Cot (H212) or from HEK293 cells (transfected with Cot_{full-length}) using anti-6×His. The precipitated proteins were analyzed by western blot probed with anti-

Cot (M20) and anti-Plk1 (F8). Anti-Cot (M20) antibody was used as the pull-down control. Both of them demonstrated a band corresponding to Plk1 at the correct position (Figure 8-17), suggesting a physiological interaction between Cot and Plk1 proteins *in vivo*. For whole blots please refer to Figure A-4 (appendices).

Figure 8-17 Interaction between Cot and Plk1. The total cell lysates of Hela cells (endogenous Cot) and HEK293 cells (transfected with Cot_{full-length}) were subjected immunoprecipitation with anti-Cot (M20) and anti-6×His accordingly. The precipitated proteins were analyzed by western blot probed with anti-Cot (M20) and anti-Plk1 (F8).

8.7 RNA interference

Further investigation of the functional relevance of Cot and Plk1 was performed with RNA interference in Hela cells (endogenous Cot). Three Cot-specific siRNAs (Santa Cruz) were delivered into Hela cells to knock down the expression of Cot protein. After 48-hr transfection, the cells were harvested and the lysates were analyzed by western blot with anti-Cot (M20), anti-Plk1 (F8), anti-p-Plk1(Ser137), anti-p-Plk1(Thr210), and anti-PDI (Figure 8-18). For whole blots please refer to Figure A-5 (appendices). Anti-PDI was used as the loading control. The density of the bands was

measured by densitometry (Bio-Rad GS-800). The expression of endogenous Cot was knocked down by a significant amount (24 % of the control), whilst that of Plk1 remained at the same level. The density of the p-Plk1(Ser137) band was noticeably lower compared to the control siRNA sample (36 % of the control). The density of the p-Plk1(Thr210) bands was faint and almost similar in both cases. This result suggests that the phosphorylation of Plk1 at Ser137 is relevant with Cot *in vivo*.

Figure 8-18 RNA interference of Cot. The Cot specific siRNA duplexes were delivered into Hela cells. Forty eight hours after transfection, the cells were harvested for western blot analysis with anti-Cot (M20), anti-Plk1(F8), anti-p-Plk1(Ser137), anti-p-Plk1(Thr210), and anti-PDI. The non-targeting siRNA duplex (AAGUCUCCAAGCGGAUCUCGU and antisense) was used as the negative control. The anti-PDI was used as the loading control. The experiments were repeated twice and similar results were obtained. The faint bands for anti-p-Plk1(Thr210) appeared to be the result of non-specific reactions.

8.8 Cot kinase domain structure predication and interaction modeling with Plk1

Having structural information, for example the X-ray crystallographic or NMR structure of the complex between Cot and Plk1, would be valuable in trying to understand the functional relationship between Cot and Plk1. However, obtaining such protein:protein complex structures is challenging. Although there is partial structural information available on Plk1 (the kinase domain has had its structure determined by X-ray crystallography), there is no structural information available on Cot.

Therefore, a comparative model was constructed for the Cot kinase domain by using the program MODELLER [145]. The first step was to use MODELLER to search for proteins that contained a similar sequence to the Cot kinase domain sequence, and had had their three-dimensional structure solved (preferably by X-ray crystallography). The comparison result showed that Tao2 had highest sequence similarity of those kinases that had their structure solved.. The sequence of the Cot kinase domain was aligned with the template, the Tao2 kinase domain, and a prediction of the Cot kinase domain tertiary structure was performed by MODELLER. The prediction is shown in Figure 8-19. The structure displays a typical kinase fold in which the ATP binding site is located in a cleft formed between the N-terminal lobe composed predominantly of antiparallel β -sheets and the primarily α -helical C-terminal lobe. The two lobes are connected by a hinge region that makes conserved backbone interactions with the bound ligands.

Figure 8-19 Ribbon representations of the predicted tertiary structure of Cot kinase domain. The structure was predicted by program MODELLER based on the known structure of Tao2 kinase domain (PDB code 1u5q).

The tertiary structure of the Plk1 kinase domain was determined recently [205]. The structure is shown in Figure 8-20. Based on the predicted structure of Cot and the known structure of Plk1, an interaction modeling of the Cot and Plk1 kinase domains was performed by the program ZDOCK [146]. The result is shown in Figure 8-21. Cot appears to interact with Plk1 only on the Ser137 side, whilst Thr210 was distant from Cot kinase domain. This modeling result correlates well the peptide-based assays described previously, which indicated that Cot specifically phosphorylates Ser137.

Figure 8-20 Ribbon representations of the tertiary structure of Plk1 kinase domain. Ser137 is located in a cleft at the transition from the hinge region to the alpha helix (red color). Thr210 is located in the activation loop (cyan color).

Figure 8-21 A model of the interaction between the kinase domains of Cot and Plk1 was obtained using the program ZDOCK. The electrostatic surface representation of the interaction of Cot (yellow) and Plk1 (green) (A). The C α atom trace representation of the interaction between Cot (yellow) and Plk1 (green) (B).

8.9 Discussion

The identification of about 500 protein kinases in the human genome indicates the importance of the network of kinases in cell biology [206]. However, many questions still remain to be answered including correctly locating kinases within the network, their mechanism of regulation, identification of their substrates, etc. The difficulty is that protein kinases often have multiple states, such as a phosphorylated active state and an unphosphorylated inactive state. Furthermore one particular kinase often has multiple substrates; binding with scaffold proteins may reduce the unspecificity when the cell encounters extracellular signals [207].

The increase in the Cot mRNA level during the G0 to G1 cell cycle phase transition in T lymphocytes suggested the involvement of Cot in the cell cycle. Other experiments implied that Cot plays a role in G1 to S phase transition [98]. Transfection of Jurkat T cells with C-terminally truncated Cot led to a small increase in COX-2 mRNA transcripts, and the activation of ERK by Cot was shown to lead to the phosphorylation of CREB at Ser133 via the intermediate kinases p90Rsk and MSK1 [208]. Furthermore, Cot protein can form a stable complex with MKK6, MEK, SEK, MEKK5 *in vitro* [100]. This may provide a molecular mechanism to explain how extracellular stimuli can activate more than one MAPK cascade or other cellular functions. The pathways achieve specificity by filtering out spurious cross-talk through mutual inhibition. Furthermore the variability between cells allows for heterogeneity of the decisions [209].

To give an insight into the function of Cot, it was expressed in HEK293 cells and immunoprecipitated. Cot protein was then used for the substrate screening assay. Several putative substrates of Cot that are phosphorylated by Cot *in vitro* were identified. They are Ezrin, CHK1, Plk1, p90RSK, eIF4G, 4E-BP1, MKK3/6, Ab1, cdc2, CREB, Cofilin, Histone H2B and MEK1/2. Interestingly, the well-studied substrates MEK1/2 and MKK3/6 were also on the list. Furthermore, the magnitude of the signals for CREB (10404) and p90Rsk (3994) were higher than that of MEK1/2 (2827) and MKK3/6 (642) which indicates the direct phosphorylation of CREB and p90Rsk by Cot. CREB (cAMP response element binding protein) is a transcription factor, which binds to CRE (cAMP response element) in DNA to control some genes expression [210]. p90RSK (p90 ribosomal S6 kinase), downstream effector of ERK1/2, is phosphorylated upon activation and translocated to the cell nucleus [211].

One of most interesting putative substrates is Polo-like kinase 1 (Plk1) which plays key roles during multiple stages of the cell cycle, including mitosis, G1 to S phase transition, G2 to M transition, bipolar spindle formation, cytokinesis as well as in the response to DNA damage [212]. Plk1 locates mostly in the cytoplasm during interphase and translocates to the nucleus in early mitosis [213]. Polo-box domain (PBD) located at the C-terminus is critical for both Plk location and function and docking site for Ser/Thr phosphorylated protein kinases [214]. The optimal sequence motif recognized by the PBD is Ser-pSer/pThr-Pro/X, suggesting that Cdk (cyclin-dependent kinase), MAP kinases might be its upstream activator [202]. Plk1 was

overexpressed in rapidly proliferating cell and various human tumors [215, 216] and is an important target for anticancer chemotherapeutic treatment. Interestingly, in the cancer cells of breast, colon, ovary, and lung cancer patients, the mRNA level of Cot increases by 40% (section 1.7). The upregulation of two genes (Cot and Plk1) in cancer cells could suggest a functional relationship, although this may be coincidental. Plk1 has several potential phosphorylation sites, of which Ser137 and Thr210 are the best studied. Thr210 has been shown to be involved in the activation of Plk1 [217]. However, it is unclear what the role of phosphorylation at Ser137 *in vivo* is.

Ser137 is preceded by three basic amino acids and followed by a hydrophobic residue (RRRSL), and is very similar to the Thr210 site (RKKTL). However, these results demonstrate, through *in vitro* and *in vivo* experiments, that Cot phosphorylates Plk1 at Ser137 not at Thr210. This may indicate that the conformation of these two phosphorylation sites is different [218]. Ser137 is located in the probable substrate-binding cleft at the start of an α -helix which is hidden from surface, while Thr210 is located in the activation loop which is exposed to the surface [202]. It seems probable that the site of Thr210 is easier for an upstream kinase to access. The Ser137 site, since it is partially hidden from the surface, may be harder to access and hence only structurally specific kinases would be able to phosphorylate this residue. Furthermore, as Ser137 is not located at the activation loop, its phosphorylation may not directly alter the kinase activity of Plk1.

The results described earlier in this chapter appears to indicate that Ser137 can be phosphorylated by Cot. If true, this would suggest that the phosphorylation of Plk1 by Cot might be structurally specific. The kinase assay results indicate that the Cot protein can phosphorylate Plk1 at Ser137, but not at Thr210. Even when threonine substitutes the serine residue corresponding to Ser137 in a short peptide, or when a serine replaces the threonine residue corresponding Thr210, no phosphorylation appears to occur. This suggests that the phosphorylation of Plk1 by Cot may be serine-specific. The peptide structure prediction of two peptides (RRRSLLELHKR and RRRTLLELHKR) also indicated that the hydroxyl group of Ser137 is solvent-exposed. If this serine is changed to a threonine, the hydroxyl group faces inside and the solvent accessible surface is only one-third of that of the serine. A series of peptides related to the sequence surrounding Ser137 were synthesized and assayed for phosphorylation by Cot. These results showed that the sequence RRRSLLE is the minimum requirement for the phosphorylation of Ser137, with a longer sequence RRRSLLELH being optimal.

It was previously indicated that a *Xenopus* kinase, xPlkk1, can phosphorylate Plx1 (*Xenopus* polo-like kinase 1), the *Xenopus* homolog of Plk1, at Thr201 (equivalent to Thr210) [219], but there is some debate whether xPlkk1 is a physiological upstream kinase of Plx1 [220]. It has been shown that xPlkk1 does not phosphorylate Plk1 at Ser137 *in vitro* and that Ser137 is not phosphorylated at M phase [217]. Two research groups found that depletion of Plk1 will cause HeLa cells arrest at mitosis and

apoptosis [221, 222]. Currently, no upstream kinase has been identified for the phosphorylation on Ser137 [202]. S137D and S137D/T210D caused HeLa cells to arrest at the G1/S transition, whereas those expressing wild-type and T210D were arrested or delayed in M phase [217]. These data may provide indirect evidence for the importance of phosphorylation on Ser137. In agreement with earlier work on Plx1 [223], both the T210D and S137D mutations increased the activity of Plk1 [217]. It was suggested, therefore, that phosphorylation at Ser137 prior to M phase might be of physiological significance in the cell cycle.

Although Cot and Plk1 could be precipitated together, when Cot was knocked-down, the phosphorylation of Ser137 at Plk1 was noticeably decreased too. In addition, the docking prediction of Cot and Plk1 also suggested evidence as to why Cot only phosphorylates Ser137 rather than Thr210. All the experimental and computational work presented in this chapter suggests that Cot is an upstream kinase of Plk1 for the phosphorylation of Ser137. However, Cot knock-out mice did not exhibit obvious phenotypic defects [224], suggesting that the cellular pathways in which Cot is involved have a degree of redundancy. This raises the questions as to whether Cot is the sole kinase that is able to phosphorylate Plk1 at Ser137, or if Ser137 phosphorylation is non-critical, what the precise function of the phosphorylation of Ser137 is. Since Ser137 is not located in the activation loop, it is possible that it does not directly affect Plk1's kinase activity. One paper mentioned that the phosphorylation of Ser137 may enhance the kinase activity of Plk1 [217]. Recently,

more papers have been published whose results strengthen the relationship of the MAPK family with cell division [225-227]. Low levels of MAPK activation correlated with low levels of Cdc25c phosphorylation; inhibition of MAPK activation inhibited Cdc25c phosphorylation and activation [225]. One paper found that ERK5, which kinase domain was homologous to ERK1/2, was activated at G2/M and was critical for G2/M transition and timely mitotic entry. In addition, the ERK5-NF- κ B pathway was required for G2/M progression in primary cultured human cells [226]. Based on my findings and others, a plausible hypothesis is that Cot phosphorylates Plk1 at Ser137 at G1/S phase prior to phosphorylation of Plk1 at Thr210, perhaps during G2/M phase, by another upstream kinase. Together with the effects from other MAPK proteins and NF- κ B, Cot regulates Plk1's activity or translocation and hence cell division (Figure 8-22). It is also likely that the translocation of Plk1 from the cytoplasm to the nucleus occurs after phosphorylation by Cot. From this and previous work, it seems clear that the multiple phosphorylations of Plk1, in conjunction with, and perhaps facilitating, other regulatory mechanisms, gives rise to the pleiotropic nature of Plk1 during the cell cycle.

Figure 8-22 The hypothesis of Cot's involvement in the cell cycle via Plk1. Solid arrows, direct relationship; dashed arrows and question marks, undefined relationship.

Appendices

Table A-1 Hampton basic and extension screen
Basic screen

#	Salt	Buffer	Precipitant
1.	0.02 M Calcium chloride dihydrate	0.1 M Sodium acetate trihydrate pH 4.6	30% v/v (+/-)-2-Methyl-2,4-pentanediol
2.	None	None	0.4 M Potassium sodium tartrate tetrahydrate
3.	None	None	0.4 M Ammonium phosphate monobasic
4.	None	0.1 M Tris hydrochloride pH 8.5	2.0 M Ammonium sulfate
5.	0.2 M Sodium citrate tribasic dihydrate	0.1 M HEPES sodium pH 7.5	30% v/v (+/-)-2-Methyl-2,4-pentanediol
6.	0.2 M Magnesium chloride hexahydrate	0.1 M Tris hydrochloride pH 8.5	30% w/v Polyethylene glycol 4,000
7.	None	0.1 M Sodium cacodylate trihydrate pH 6.5	1.4 M Sodium acetate trihydrate
8.	0.2 M Sodium citrate tribasic dihydrate	0.1 M Sodium cacodylate trihydrate pH 6.5	30% v/v 2-Propanol
9.	0.2 M Ammonium acetate	0.1 M Sodium citrate tribasic dihydrate pH 5.6	30% w/v Polyethylene glycol 4,000
10.	0.2 M Ammonium acetate	0.1 M Sodium acetate trihydrate pH 4.6	30% w/v Polyethylene glycol 4,000
11.	None	0.1 M Sodium citrate tribasic dihydrate pH 5.6	1.0 M Ammonium phosphate monobasic
12.	0.2 M Magnesium chloride hexahydrate	0.1 M HEPES sodium pH 7.5	30% v/v 2-Propanol
13.	0.2 M Sodium citrate tribasic dihydrate	0.1 M Tris hydrochloride pH 8.5	30% v/v Polyethylene glycol 400
14.	0.2 M Calcium chloride dihydrate	0.1 M HEPES sodium pH 7.5	28% v/v Polyethylene glycol 400
15.	0.2 M Ammonium sulfate	0.1 M Sodium cacodylate trihydrate pH 6.5	30% w/v Polyethylene glycol 8,000
16.	None	0.1 M HEPES sodium pH 7.5	1.5 M Lithium sulfate monohydrate
17.	0.2 M Lithium sulfate monohydrate	0.1 M Tris hydrochloride pH 8.5	30% w/v Polyethylene glycol 4,000
18.	0.2 M Magnesium acetate tetrahydrate	0.1 M Sodium cacodylate trihydrate pH 6.5	20% w/v Polyethylene glycol 8,000
19.	0.2 M Ammonium acetate	0.1 M Tris hydrochloride pH 8.5	30% v/v 2-Propanol
20.	0.2 M Ammonium sulfate	0.1 M Sodium acetate trihydrate pH 4.6	25% w/v Polyethylene glycol 4,000
21.	0.2 M Magnesium acetate tetrahydrate	0.1 M Sodium cacodylate trihydrate pH 6.5	30% v/v (+/-)-2-Methyl-2,4-pentanediol
22.	0.2 M Sodium acetate trihydrate	0.1 M Tris hydrochloride pH 8.5	30% w/v Polyethylene glycol 4,000
23.	0.2 M Magnesium chloride hexahydrate	0.1 M HEPES sodium pH 7.5	30% v/v Polyethylene glycol 400
24.	0.2 M Calcium chloride dihydrate	0.1 M Sodium acetate trihydrate pH 4.6	20% v/v 2-Propanol
25.	None	0.1 M Imidazole pH 6.5	1.0 M Sodium acetate trihydrate
26.	0.2 M Ammonium acetate	0.1 M Sodium citrate tribasic dihydrate pH 5.6	30% v/v (+/-)-2-Methyl-2,4-pentanediol
27.	0.2 M Sodium citrate tribasic dihydrate	0.1 M HEPES sodium pH 7.5	20% v/v 2-Propanol
28.	0.2 M Sodium acetate trihydrate	0.1 M Sodium cacodylate trihydrate pH 6.5	30% w/v Polyethylene glycol 8,000

29.	None	0.1 M HEPES sodium pH 7.5	0.8 M Potassium sodium tartrate
30.	0.2 M Ammonium sulfate	None	30% w/v Polyethylene glycol 8,000
31.	0.2 M Ammonium sulfate	None	30% w/v Polyethylene glycol 4,000
32.	None	None	2.0 M Ammonium sulfate
33.	None	None	4.0 M Sodium formate
34.	None	0.1 M Sodium acetate trihydrate pH 4.6	2.0 M Sodium formate
35.	None	0.1 M HEPES sodium pH 7.5	0.8 M Sodium phosphate monobasic
			0.8 M Potassium phosphate monobasic
36.	None	0.1 M Tris hydrochloride pH 8.5	8% w/v Polyethylene glycol 8,000
37.	None	0.1 M Sodium acetate trihydrate pH 4.6	8% w/v Polyethylene glycol 4,000
38.	None	0.1 M HEPES sodium pH 7.5	1.4 M Sodium citrate tribasic dihydrate
39.	None	0.1 M HEPES sodium pH 7.5	2% v/v Polyethylene glycol 400
			2.0 M Ammonium sulfate
40.	None	0.1 M Sodium citrate tribasic dihydrate pH 5.6	20% v/v 2-Propanol
			20% w/v Polyethylene glycol 4,000
41.	None	0.1 M HEPES sodium pH 7.5	10% v/v 2-Propanol
			20% w/v Polyethylene glycol 4,000
42.	0.05 M Potassium phosphate monobasic	None	20% w/v Polyethylene glycol 8,000
43.	None	None	30% w/v Polyethylene glycol 1,500
44.	None	None	0.2 M Magnesium formate dihydrate
45.	0.2 M Zinc acetate dihydrate	0.1 M Sodium cacodylate trihydrate pH 6.5	18% w/v Polyethylene glycol 8,000
46.	0.2 M Calcium acetate hydrate	0.1 M Sodium cacodylate trihydrate pH 6.5	18% w/v Polyethylene glycol 8,000
47.	None	0.1 M Sodium acetate trihydrate pH 4.6	2.0 M Ammonium sulfate
48.	None	0.1 M Tris hydrochloride pH 8.5	2.0 M Ammonium phosphate monobasic
49.	1.0 M Lithium sulfate monohydrate	None	2% w/v Polyethylene glycol 8,000
50.	0.5 M Lithium sulfate monohydrate	None	15% w/v Polyethylene glycol 8,000

Extension screen

#	Salt	Buffer	Precipitant
1.	2.0 M Sodium chloride	None	10% w/v Polyethylene glycol 6,000
2.	0.5 M Sodium chloride	None	0.01 M Hexadecyltrimethylammonium bromide
	0.01 M Magnesium chloride		25% v/v Ethylene glycol
3.	None	None	35% v/v 1,4-Dioxane
4.	None	None	5% v/v 2-Propanol
5.	2.0 M Ammonium sulfate	None	1.0 M Imidazole pH 7.0
6.	None	None	10% w/v Polyethylene glycol 1,000
7.	None	None	10% w/v Polyethylene glycol 8,000
			10% v/v Ethanol
8.	1.5 M Sodium chloride	None	2.0 M Sodium chloride
9.	None	0.1 M Sodium acetate trihydrate pH 4.6	30% v/v (+/-)-2-Methyl-2,4-pentanediol
10.	0.2 M Sodium chloride	0.1 M Sodium acetate trihydrate pH 4.6	1.0 M 1,6-Hexanediol
11.	0.01 M Cobalt (II) chloride hexahydrate	0.1 M Sodium acetate trihydrate pH 4.6	30% v/v Polyethylene glycol 400
12.	0.1 M Cadmium chloride hydrate	0.1 M Sodium acetate trihydrate pH 4.6	30% w/v PEG 2,000
13.	0.2 M Ammonium sulfate	0.1 M Sodium acetate trihydrate pH 4.6	2.0 M Ammonium sulfate
14.	0.2 M Potassium sodium tartrate	0.1 M Sodium citrate tribasic dihydrate pH 5.6	1.0 M Lithium sulfate monohydrate
15.	0.5 M Ammonium sulfate	0.1 M Sodium citrate tribasic dihydrate pH 5.6	2% v/v Ethylene imine polymer
16.	0.5 M Sodium chloride	0.1 M Sodium citrate tribasic dihydrate pH 5.6	35% v/v tert-Butanol
17.	None	0.1 M Sodium citrate tribasic dihydrate pH 5.6	10% v/v Jeffamine M-600 ®
18.	0.01 M Iron (III) chloride hexahydrate	0.1 M Sodium citrate tribasic dihydrate pH 5.6	2.5 M 1,6-Hexanediol
19.	None	0.1 M Sodium citrate tribasic dihydrate pH 5.6	1.6 M Magnesium sulfate heptahydrate
20.	None	0.1 M MES monohydrate pH 6.5	2.0 M Sodium chloride
21.	0.1 M Sodium phosphate monobasic	0.1 M MES monohydrate pH 6.5	12% w/v Polyethylene glycol 20,000
	0.1 M Potassium phosphate monobasic		
22.	None	0.1 M MES monohydrate pH 6.5	10% v/v 1,4-Dioxane
23.	1.6 M Ammonium sulfate	0.1 M MES monohydrate pH 6.5	30% v/v Jeffamine M-600
24.	0.05 M Cesium chloride	0.1 M MES monohydrate pH 6.5	1.8 M Ammonium sulfate
25.	0.01 M Cobalt (II) chloride hexahydrate	0.1 M MES monohydrate pH 6.5	30% w/v PEG 5,000
26.	0.2 M Ammonium sulfate	0.1 M MES monohydrate pH 6.5	25% v/v PEG 550
27.	0.01 M Zinc sulfate heptahydrate	0.1 M MES monohydrate pH 6.5	1.6 M Sodium citrate pH 6.5
28.	None	None	30% v/v (+/-)-2-Methyl-2,4-pentanediol
29.	0.5 M Ammonium sulfate	0.1 M HEPES pH 7.5	10% w/v Polyethylene glycol 6,000
30.	None	0.1 M HEPES pH 7.5	5% v/v (+/-)-2-Methyl-2,4-pentanediol

31.	None	0.1 M HEPES pH 7.5	20% v/v Jeffamine M-600
32.	0.1 M Sodium chloride	0.1 M HEPES pH 7.5	1.6 M Ammonium sulfate
33.	None	0.1 M HEPES pH 7.5	2.0 M Ammonium formate
34.	0.05 M Cadmium sulfate hydrate	0.1 M HEPES pH 7.5	1.0 M Sodium acetate trihydrate
35.	None	0.1 M HEPES pH 7.5	70% v/v (+/-)-2-Methyl-2,4-pentanediol
36.	None	0.1 M HEPES pH 7.5	4.3 M Sodium chloride
37.	None	0.1 M HEPES pH 7.5	10% w/v Polyethylene glycol 8,000
			8% v/v Ethylene glycol
			20% w/v Polyethylene glycol 10,000
38.	None	0.1 M HEPES pH 7.5	3.4 M 1,6-Hexanediol
39.	0.2 M Magnesium chloride hexahydrate	0.1 M Tris pH 8.5	25% v/v tert-Butanol
40.	None	0.1 M Tris pH 8.5	1.0 M Lithium sulfate monohydrate
41.	0.01 M Nickel (II) chloride hexahydrate	0.1 M Tris pH 8.5	12% v/v Glycerol
42.	1.5 M Ammonium sulfate	0.1 M Tris pH 8.5	50% v/v (+/-)-2-Methyl-2,4-pentanediol
43.	0.2 M Ammonium phosphate	0.1 M Tris pH 8.5	20% v/v Ethanol
44.	None	0.1 M Tris pH 8.5	20% w/v PEG 2,000
45.	0.01 M Nickel (II) chloride hexahydrate	0.1 M Tris pH 8.5	20% v/v PEG 550
46.	0.1 M Sodium chloride	0.1 M BICINE pH 9.0	2.0 M Magnesium chloride
47.	None	0.1 M BICINE pH 9.0	2% v/v 1,4-Dioxane
48.	None	0.1 M BICINE pH 9.0	10% w/v Polyethylene glycol 20,000

Table A-2 Peptide library screening

Peptide Target	Peptide sequence	Target/CST Antibody Description
Phospho-mTOR (Ser2481)	(biotin)AGAGTVPEsIHsFIGDGLV	Phospho-mTOR (Ser2481) Antibody
Phospho-DARPP-32 (Thr75)	(biotin)AGAGAYtPPSLKAVQRIA	Phospho-DARPP-32 (Thr75) Antibody
Phospho-C/EBPbeta (Ser105) (Rat Specific)	(biotin)AGAGAKPSKKPsDYGYVSL	Phospho-C/EBPbeta (Ser105) Antibody (Rat Specific)
Phospho-Bad (Ser112)	(biotin)AGAGRSRHSsYPAGT	Phospho-Bad (Ser112) Antibody
Phospho-CaMKII (Thr286)	(biotin)AGAGVASMMHRQEtVE	Phospho-CaMKII (Thr286) Antibody
Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558)	(biotin)AGAGRDKYKtLRQIR	Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) Antibody
Phospho-GSK-3beta (Ser9)	(biotin)AGAGMSGRPRTTsFAES	Phospho-GSK-3beta (Ser9) Antibody
Phospho-Chk1 (Ser345)	(biotin)AGAGVQGIsFsQPT	Phospho-Chk1 (Ser345) Antibody
Phospho-MEK1/2 (Ser217/221)	(biotin)AGAGSGQLIDsMANSFVGTR	Phospho-MEK1/2 (Ser217/221) Antibody
Phospho-eNOS (Ser1177)	(biotin)AGAGTSRIRTQsFSLQERQL	Phospho-eNOS (Ser1177) Antibody
Phospho-cdc25 (Ser216)	(biotin)AGAGCGLYRSPsMPENLNRPRL	Phospho-(Ser) 14-3-3 Binding Motif Antibody
Phospho-VASP (Ser239)	(biotin)AGAGKLRKVskQEEA	Phospho-VASP (Ser239) Antibody
Phospho-PKCdelta (Ser643)	(biotin)AGAGLNEKPQLsFSDKN	Phospho-PKCdelta/theta (Ser643/676) Antibody
Phospho-cdc25 (Ser216)	(biotin)AGAGCGLYRSPsMPENLNRPRL	Phospho-cdc25C (Ser216) Antibody
Phospho-PKA (Thr197)	(biotin)AGAGVKGRTWtLCGTPEYL	Phospho-(Ser/Thr) Akt Substrate Antibody
Phospho-FKHR (Ser256)	(biotin)AGAGSPRRRAAsMDNNSKFA	Phospho-FKHR (Ser256) Antibody
Phospho-PLK (Ser137)	(biotin)AGAGRRRsLLELHKR	Phospho-PLK (Ser137) Antibody
Phospho-p90RSK (Ser380)	(biotin)AGAGHQLFRGFsFVATGLM	Phospho-(Ser/Thr) Phe Antibody
Phospho-MSK1 (Ser376)	(biotin)AGAGSEKLFQGYsFVAPS	Phospho-MSK1 (Ser376) Antibody
Phospho-SEK1/MKK4 (Thr261)	(biotin)AGAGSIAKtRDA	Phospho-SEK1/MKK4 (Thr261) Antibody
Phospho-Bad (Ser155)	(biotin)AGAGELRRMsDEFEG	Phospho-Bad (Ser155) Antibody
Phospho-KDR (Tyr996)	(biotin)AGAGEAPEDLyKDFLTLE	Phospho-VEGF Receptor-2 (Tyr996) Antibody
Phospho-p53 (Ser20)	(biotin)AGAGSQETFsDLWKLLPP	Phospho-p53 (Ser20) Antibody
Phospho-STAT3 (Tyr705)	(biotin)AGAGADPGSAAPyLKTKFI	Phospho-Stat3 (Tyr705) Antibody
Phospho-4E-BP1 (Thr70)	(biotin)AGAGRNSPVTKtPPRD	Phospho-4E-BP1 (Thr70) Antibody
Phospho-Elk-1 (Ser383)	(biotin)AGAGWSTLsPIAPRSPAK	Phospho-Elk-1 (Ser383) Antibody
Phospho-eIF4G (Ser1108)	(biotin)AGAGVVQRSSLsRERGE	Phospho-eIF4G (Ser1108) Antibody
Phospho-MKK3/MKK6 (Ser189/207)	(biotin)AGAGYLVDSvAKTM	Phospho-MKK3/MKK6 (Ser189/207) Antibody
Phospho-c-Abl (Thr735)	(biotin)AGAGTEWRSVtLPRDL	Phospho-c-Abl (Thr735) Antibody

Phospho-Rb (Ser780)	(biotin)AGAGTRPPTLsPIPHIPRS	Phospho-Rb (Ser780) Antibody
Phospho-cdc2 (Thr161)	(biotin)AGAGGIPIRVYtHEVVTLWY	Phospho-cdc2 (Thr161) Antibody
Phospho-GSK-3alpha/beta (Ser21/9)	(biotin)AGAGRARTSsFAEPGG	Phospho-GSK-3alpha/beta (Ser21/9) Antibody
Phospho-p70 S6 Kinase (Thr421/Ser424)	(biotin)AGAGSPRtPVsPVKFS	Phospho-p70 S6 Kinase (Thr421/Ser424) Antibody
Phospho-MSK1 (Ser376)	(biotin)AGAGSEKLFQGYsFVAPS	Phospho-(Ser/Thr) Phe Antibody
Phospho-AMPK (Thr172)	(biotin)AGAGSDGEFLRtSCGSPNY	Phospho-AMPK-alpha (Thr172) Antibody
Phospho-Akt (Thr308)	(biotin)AGAGIKDGATMKtFCGTP	Phospho-(Thr) PDK1 Substrate Antibody
Phospho-Aurora 2/AIK (Thr288)	(biotin)AGAGSSRRTtLCGTLDY	Phospho-Aurora 2/AIK (Thr288) Antibody
Phospho-PLK (Ser137)	(biotin)AGAGRRRsLLELHKR	Phospho-(Ser) PKC Substrate Antibody
Phospho-Bad (Ser136)	(biotin)AGAGFRGRSRsAPPNLWA	Phospho-Bad (Ser136) Antibody
Phospho-HSP27 (Ser82)	(biotin)AGAGRQLsSGVSEIRH	Phospho-HSP27 (Ser82) Antibody
Phospho-PKA (Thr197)	(biotin)AGAGVKGRTWtLCGTPEYL	Phospho-(Ser/Thr) PKA Substrate Antibody
Phospho-c-Jun (Ser73)	(biotin)AGAGLKLasPELE	Phospho-c-Jun (Ser73) Antibody
Phospho-p44/42 MAP Kinase (Thr202/Tyr204)	(biotin)AGAGTGFLtEyVATRWYR	Phospho-p44/42 MAP Kinase (Thr202/Tyr204) Antibody
Phospho-Caspase9(ser196)	(biotin)AGAG-LEKLRRRFsSLHFMV	Phospho-(Ser)-Arg-X-Tyr/Phe-X-pSer Motif Antibody
Phospho-CREB (Ser133)	(biotin)AGAGKRREILSRPsYRK	Phospho-(Ser/Thr) PKA Substrate Antibody
Phospho-Cofilin (Ser3)	(biotin)AGAGMAsGVAVSDGVIK	Phospho-Cofilin (Ser3) Antibody
Phospho-PTEN (Ser380)	(biotin)AGAGDHRYRsDTTDSDE	Phospho-PTEN (Ser380) Antibody
Phospho-FKHR (Thr24)/FKHRL1 (Thr32)	(biotin)AGAGSRPRScWPLPRPEI	Phospho-FKHR (Thr24)/FKHRL1 (Thr32) Antibody
Phospho-p90RSK (Ser380)	(biotin)AGAGHQLFRGFsFVATGLM	Phospho-p90RSK (Ser380) Antibody
Phospho-Chk2 (Thr68)	(biotin)AGAGETVStQELYS	Phospho-Chk2 (Thr68) Antibody
Phospho-p38 MAP Kinase (Thr180/Tyr182)	(biotin)AGAGLARHTDDEMtGyVA	Phospho-p38 MAP Kinase (Thr180/Tyr182) Antibody
Phospho-ATF2(Thr71)	(biotin)AGAGVASMMHRQEtVE	Phospho-ATF-2 (Thr71) Antibody
Phospho-Histone H2B (Ser32)	(biotin)AGAGGKKRKRsrKESYSI	Phospho-(Ser) PKC Substrate Antibody
Phospho-c-Jun (Ser63)	(biotin)AGAGRAKNSDLLTsPDVGL	Phospho-c-Jun (Ser63) II Antibody
Phospho-p90RSK (Thr573)	(biotin)AGAGKQLRAENGLLMtP	Phospho-p90RSK (Thr573) Antibody
Phospho-eIF4E (Ser209)	(biotin)AGAGATKSGsTTKNRFVV	Phospho-eIF4E (Ser209) Antibody
Phospho-PKC α /II (Thr633)	(biotin)AGAGAENFDRFFtRHPPV	Phospho-(Ser/Thr) PKA Substrate Antibody
Phospho-SAPK/JNK (Thr183/Tyr185)	(biotin)AGAGSFMMtPyVVTRYR	Phospho-SAPK/JNK (Thr183/Tyr185) Antibody
Phospho-CaMKII (Thr286)	(biotin)AGAGVASMMHRQEtVE	Phospho-(Ser/Thr) PKA Substrate Antibody
Phospho-Histone H2B (Ser32)	(biotin)AGAGGKKRKRsrKESYSI	Phospho-(Ser/Thr) PKA Substrate Antibody
Phospho-Chk2 (Thr26/Ser28)	(biotin)AGAGHGsvtQsQGSS	Phospho-(Ser/Thr) ATM/ATR Substrate

Phospho-PAK1 (Ser144)/PAK2 (Ser141)	(biotin)AGAGTSNSQKYMST	Phospho-(Ser/Thr) Phe Antibody
Phospho-p53 (Ser15)	(biotin)AGAGSVEPPLsQETFSDK	Phospho-(Ser/Thr) ATM/ATR Substrate Antibody
Phospho-Tyrosine Hydroxylase (Ser40)	(biotin)AGAGRFIGRRQsLIEDARK	Phospho-(Ser/Thr) PKA Substrate Antibody
Phospho-VASP (Ser157)	(biotin)AGAGEHIERRVsNAGGPPA	Phospho-(Ser/Thr) PKA Substrate Antibody
Phospho-Rad17 (Ser645)	(biotin)AGAGWSLPLsQNSASEL	Phospho-(Ser/Thr) ATM/ATR Substrate Antibody
Phospho-(Thr) PDK1 Substrate	(biotin)AGAGGLRPGDTSStFCGT	Phospho-(Ser/Thr) Phe Antibody
Phospho-p53 (Ser15)	(biotin)AGAGSVEPPLsQETFSDK	Phospho-p53 (Ser15) (16G8) Monoclonal Antibody
Phospho-SAPK/JNK (Thr183/Tyr185)	(biotin)AGAGSFMMtPyVVTRYR	Phospho-SAPK/JNK (Thr183/Tyr185) (G9) Monoclonal Antibody
Phospho-c-Abl (Thr735)	(biotin)AGAGTEWRSVtLPRDL	Phospho-Threonine (42H4) Monoclonal Antibody
Phospho-4E-BP (Thr45)	(biotin)AGAGGTLFSTtPGGTRII	Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101)
Phospho-IkappaB-alpha (Ser32)	(biotin)AGAGLDDRHDsGLDSMKD	Phospho-IkappaB-alpha (Ser32/36) (5A5) Monoclonal Antibody
Phospho-p38 MAP Kinase (Thr180/Tyr182)	(biotin)AGAGLARHTDDEMtGyVA	Phospho-p38 MAPK (Thr180/Tyr182) (28B10) Monoclonal Antibody
Phospho-MAPKAPK-2 (Thr334)	(biotin)AGAGSTKVPQtPLHTSR	Phospho-Threonine (42H4) Monoclonal Antibody
Phospho-Bad (Ser136)	(biotin)AGAGFRGRSRsAPPNLWA	Phospho-(Ser) 14-3-3 Binding Motif (4E2) Monoclonal Antibody
Phospho-Histone H3 (Ser10)	(biotin)AGAGARTKQTARKsTGGKAPRK	Phospho-Histone H3 (Ser10) (6G3) Monoclonal Antibody
Phospho-p70 S6 Kinase (Thr389)	(biotin)AGAGNQVFLGFtYVAPSV	Phospho-(Ser/Thr) PDK1 Docking Motif (18A2) Monoclonal Antibody
Phospho-ATF2 (Thr71)	(biotin)AGAGDQTPtPTRFLK	Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101)
Phospho-CaMKII (Thr286)	(biotin)AGAGVASMMHRQEtVE	Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101)
Phospho-cdc25 (Ser216)	(biotin)AGAGCGLYRSPsMPENLNRPRL	Phospho-(Ser) 14-3-3 Binding Motif (4E2) Monoclonal Antibody
Phospho-Akt (Thr308)	(biotin)AGAGIKDGATMKtFCGTP	Phospho-Threonine (42H4) Monoclonal Antibody
Phospho-SAPK/JNK (Thr183/Tyr185)	(biotin)AGAGSFMMtPyVVTRYR	Phospho-Threonine-Proline Monoclonal Antibody (P-Thr-Pro-101)
Phospho-p90RSK (Ser380)	(biotin)AGAGHQLFRGFsFVATGLM	Phospho-(Ser/Thr) PDK1 Docking Motif (18A2) Monoclonal Antibody
Phospho-p44/42 MAP Kinase (Thr202/Tyr204)	(biotin)AGAGTGFLtEyVATRWYR	Phospho-p44/42 MAPK (Thr202/Tyr204) (E10) Monoclonal Antibody
Phospho-Chk2 (Thr68)	(biotin)AGAGETVStQELYS	Phospho-Threonine (42H4) Monoclonal Antibody
Phospho-Stat3 (Ser727)	(biotin)AGAGIDLPMsPRTLDSL	Phospho-Stat3 (Ser727) (6E4) Monoclonal Antibody
Phospho-MAPKAPK-2 (Thr334)	(biotin)AGAGSTKVPQtPLHTSR	Phospho-Threonine-Proline Monoclonal Antibody

Table A-3 Result of substrate screen of Cot

Peptide Target	positive	negative	signal	SNR %
Phospho-mTOR (Ser2481)	10799	25	510	4.5015779
Phospho-DARPP-32 (Thr75)	7400	76	1783	23.306936
Phospho-C/EBPbeta (Ser105) (Rat Specific)	3890	70	133	1.6492147
Phospho-Bad (Ser112)	4742	242	506	5.8666667
Phospho-CaMKII (Thr286)	4451	23	1291	28.635953
Phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558)	636	112	627	98.282443
Phospho-GSK-3beta (Ser9)	1050	5	455	43.062201
Phospho-Chk1 (Ser345)	421	87	402	94.311377
Phospho-MEK1/2 (Ser217/221)	2827	39	2165	76.25538
Phospho-eNOS (Ser1177)	15829	225	3436	20.578057
Phospho-cdc25 (Ser216)	1181	8	489	41.005968
Phospho-VASP (Ser239)	1089	53	508	43.918919
Phospho-PKCdelta (Ser643)	5026	19	1593	31.43599
Phospho-cdc25 (Ser216)	4276	27	733	16.65487
Phospho-PKA (Thr197)	2592	11	122	4.3006587
Phospho-FKHR (Ser256)	1120	20	191	15.545455
Phospho-PLK (Ser137)	47954	462	45009	93.798956
Phospho-p90RSK (Ser380)	3994	150	3936	98.491155
Phospho-MSK1 (Ser376)	467	50	88	9.1127098
Phospho-SEK1/MKK4 (Thr261)	1242	165	248	7.7065924
Phospho-Bad (Ser155)	12062	36	576	4.4902711
Phospho-KDR (Tyr996)	20039	693	1909	6.2855371
Phospho-p53 (Ser20)	1328	54	107	4.1601256
Phospho-STAT3 (Tyr705)	728	50	126	11.20944
Phospho-4E-BP1 (Thr70)	88	9	74	82.278481
Phospho-Elk-1 (Ser383)	3262	88	981	28.134846
Phospho-eIF4G (Ser1108)	912	43	731	79.171461
Phospho-MKK3/MKK6 (Ser189/207)	642	21	435	66.666667
Phospho-c-Abl (Thr735)	1203	83	1089	89.821429
Phospho-Rb (Ser780)	3032	150	729	20.090215
Phospho-cdc2 (Thr161)	119	22	117	97.938144
Phospho-GSK-3alpha/beta (Ser21/9)	207	15	166	78.645833
Phospho-p70 S6 Kinase (Thr421/Ser424)	23319	32	418	1.6575772
Phospho-MSK1 (Ser376)	21703	83	692	2.8168363
Phospho-AMPK (Thr172)	3283	341	441	3.3990483
Phospho-Akt (Thr308)	3832	96	112	0.4282655
Phospho-Aurora 2/AIK (Thr288)	29308	549	4099	12.343962
Phospho-PLK (Ser137)	3702	76	3680	99.393271
Phospho-Bad (Ser136)	628	362	529	62.781955
Phospho-HSP27 (Ser82)	1077	588	671	16.973415

Phospho-PKA (Thr197)	5530	150	546	7.3605948
Phospho-c-Jun (Ser73)	3337	100	248	4.5721347
Phospho-p44/42 MAP Kinase (Thr202/Tyr204)	17489	369	676	1.7932243
Phospho-Caspase9(ser196)	38272	137	19252	50.124557
Phospho-CREB (Ser133)	10404	12	9311	89.482294
Phospho-Cofilin (Ser3)	180	35	163	88.275862
Phospho-PTEN (Ser380)	23664	16277	19984	50.182753
Phospho-FKHR (Thr24)/FKHRL1 (Thr32)	8678	803	1142	4.3047619
Phospho-p90RSK (Ser380)	2641	15	301	10.891089
Phospho-Chk2 (Thr68)	47344	2453	18523	35.797821
Phospho-p38 MAP Kinase (Thr180/Tyr182)	1648	109	335	14.68486
Phospho-ATF2(Thr71)	2829	38	94	2.0064493
Phospho-Histone H2B (Ser32)	255	10	254	99.591837
Phospho-c-Jun (Ser63)	2632	142	238	3.8554217
Phospho-p90RSK (Thr573)	7047	856	931	1.211436
Phospho-eIF4E (Ser209)	967	42	108	7.1351351
Phospho-PKC α II (Thr633)	12587	74	180	0.847119
Phospho-SAPK/JNK (Thr183/Tyr185)	8490	64	586	6.1951104
Phospho-CaMKII (Thr286)	24364	26	346	1.3148163
Phospho-Histone H2B (Ser32)	13069	32	4805	36.611184
Phospho-Chk2 (Thr26/Ser28)	5332	69	321	4.7881436
Phospho-PAK1 (Ser144)/PAK2 (Ser141)	20295	45	322	1.3679012
Phospho-p53 (Ser15)	4183	60	781	17.487267
Phospho-Tyrosine Hydroxylase (Ser40)	11053	37	8254	74.591503
Phospho-VASP (Ser157)	9369	195	706	5.5700894
Phospho-Rad17 (Ser645)	20275	322	4830	22.593094
Phospho-(Thr) PDK1 Substrate	11984	403	883	4.1447198
Phospho-p53 (Ser15)	22216	256	277	0.0956284
Phospho-SAPK/JNK (Thr183/Tyr185)	24351	238	1511	5.2793099
Phospho-c-Abl (Thr735)	4991	168	818	13.477089
Phospho-4E-BP (Thr45)	4120	44	591	13.42002
Phospho-IkappaB-alpha (Ser32)	16741	307	1718	8.5858586
Phospho-p38 MAP Kinase (Thr180/Tyr182)	22975	354	596	1.0698024
Phospho-MAPKAPK-2 (Thr334)	5087	166	461	5.9947165
Phospho-Bad (Ser136)	12151	105	115	0.0830151
Phospho-Histone H3 (Ser10)	13594	64	10244	75.240207
Phospho-p70 S6 Kinase (Thr389)	43532	19420	19620	0.8294625
Phospho-ATF2 (Thr71)	1045	198	600	47.461629
Phospho-CaMKII (Thr286)	210	193	194	5.8823529
Phospho-cdc25 (Ser216)	21988	102	4468	19.948826
Phospho-Akt (Thr308)	4149	135	1249	27.752865
Phospho-SAPK/JNK (Thr183/Tyr185)	4805	192	575	8.302623
Phospho-p90RSK (Ser380)	34155	15054	18145	16.182399
Phospho-p44/42 MAP Kinase (Thr202/Tyr204)	13083	277	287	0.0780884

Phospho-Chk2 (Thr68)	4526	207	1692	34.382959
Phospho-Stat3 (Ser727)	7883	233	3371	41.019608
Phospho-MAPKAPK-2 (Thr334)	4697	45	65	0.4299226

Figure A-1

Figure A-2

Figure A-3

Figure A-4

Figure A-5

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Publications

Binhui Wu and Rupert C. Wilmouth (2008) Proteomics analysis of immunoprecipitated proteins associated with Cot kinase. *Mol. Cells* **25**:43-49.

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