

Regulation of protein kinase TAK1

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REGULATION OF PROTEIN KINASE TAK1

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This thesis has been examined and approved by Drs. Gan Yunn Hwen (National University of Singapore), Simon Rousseau (McGill University) and Koh Cheng Gee (Nanyang Technological University).

As well as by Dr. Richard Sugrue, Head, Departement of Molecular and Cell Biology, School of Biological Sciences, NTU.

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To my family.

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Summary

This study was focused on the exploration of new regulators of TAK1, a critical kinase lying near the top of multiple pro-inflammatory signaling cascades, by identifying novel interacting proteins of TAK1, which hopefully will facilitate development of potential therapeutics for chronic inflammatory diseases such as rheumatoid arthritis.

Firstly, TAK1 was identified as Hsp90 client kinase using endogenous and exogenous systems. Hsp90 directly binds to kinase domain of TAK1, while the kinase activity of TAK1 as well as autophosphorylation of TAK1 at its activation loop was not required for the binding. TAB1/2/3, the binding partners of TAK1, did not interact with Hsp90, however, interaction of TAK1-Hsp90 was found to be significantly attenuated by TAB1 regardless of TAK1 activation states whereas TAK1-TAB1 binding was not affected by over-expression of Hsp90 in HEK293 cells. This result was supported by TAB1 knockdown experiment which led to increased binding of Hsp90 to TAK1. Hsp90 was not required for TAK1 activation since phosphorylation of TAK1 activation loop was unaffected when Hsp90-TAK1 interaction was disrupted by using Hsp90 inhibitor in a short term. Nonetheless, it was required for TAK1 stability because prolonged inhibition of Hsp90 resulted in down-regulation of endogenous TAK1 but not its binding partners (TAB1, TAB2 and TAB3). Moreover, Hsp90 inhibitors abrogated the pro-inflammatory signaling pathways upon IL-1, TNF and LPS stimulation, collectively suggesting that inhibition of Hsp90 may provide an alternative route to treat inflammatory diseases in addition to its anti-cancer potential. To expand the results obtained on TAK1, a

number of other kinases involved in inflammation signaling were also examined regarding to interaction with Hsp90 and responsiveness to Hsp90 inhibitor. Several new kinase clients of Hsp90 were identified, which implied that Hsp90 inhibition exerted anti-inflammation effect by targeting many related kinases rather than TAK1 alone.

Secondly, CHIP, a U-box E3 ubiquitin ligase, was found to interact *in vivo* with TAK1, TAB2/3, but not TAB1 by using GST pull-down assay and was detected in immunoprecipitated endogenous TAK1 and TAB3 complex. Through *in vitro* ubiquitination assay, purified TAK1 in complex was ubiquitinated by CHIP and UbcH5b, leading to decrease of TAK1 activity as assessed in kinase assay. This modification was further confirmed with *in vitro* translated TAK1 complex as well as immunoprecipitated endogenous TAK1 complex. On the other hand, CHIP-UbcH13/Uev1A mediated ubiquitination made little difference to TAK1 activity; nonetheless it did cause an obvious band shift of TAB2/3 with yet an unknown function. When overexpressed in HEK293 cells, CHIP was able to significantly downregulate exogenous TAK1 level and slightly reduced the endogenous TAK1 level. Heat shock protein was involved in this downregulation base on two observations: TAK1 level was unchanged in the presence of CHIP [K30A] mutant which could not bind Hsp70/90; and overexpression of Hsp70 could partially reverse the CHIP-mediated decrease of TAK1. Additionally, CHIP was found to interact with TRAF6 and attenuate the binding of TRAF6 to TAB2/3 which is critical for IL-1-TRAF6-TAK1-IKK signaling. This interaction may constitute another layer of regulation on TAK1 activity.

Abbreviations

17-AAG	17-allylamino-17-demetoxygeldanamycin
AP-1	activator protein
CD14	myeloid cell-specific leucine-rich glycoprotein
Cdc37	cell division cycle protein 37
Cdk	cyclin dependent kinase
CFTR	cystic fibrosis transmembrane conductance regulator
CHIP	carboxyl-terminus of HSC70 interacting protein
CRDs	cysteine-rich domains
CREB	c-AMP responsive element binding protein
CUE	coupling of ubiquitin conjugation to endoplasmic reticulum degradation
CYLD	cylindromatosis associated gene
DAPK	death associated protein kinase
DD	death domain
DLK	dual leucine zipper-bearing kinase
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
E1	ubiquitin activating enzyme 1
EGFRvIII	epidermal growth factor receptor variant III
FCS	fetal calf serum
FLAG	FLAG epitope tag
GST	glutathione-S-transferase
GR	glucocorticoid receptor
h	hour
HA	hemagglutinin
HECT	homologous to E6-AP C-terminus
HIP	heat shock protein interacting protein
His	6x histidine tag
HOP	heat shock protein organizing protein
HSC70	heat shock protein 70 cognate
HSP	heat shock protein

ICE	IL-1 β converting enzyme, also termed as caspase-1
IFN β	interferon β
Ig	immunoglobulin
IL-1	Interleukin 1
IL-1R AcP	IL-1R accessory protein
IL-1sRI/II	IL-1 soluble receptor type I or II
IL-1ra	interleukin 1 receptor antagonist
I κ B	inhibitor of κ B
IKK	I κ B kinase
IPTG	isopropyl- β -D-thiogalactopyranoside
IRAK	IL-1 receptor associated protein kinase
IRFs	IFN regulatory factors
JNK	c-Jun N-terminal kinase
K or Lys	lysine residue
kDa	kilo Dalton
LKB1	Peutz-Jeghers syndrome protein, officially serine/threonine kinase 11
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAL	MyD88 adaptor-like protein
MAPK	mitogen activated protein kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MBP	maltose binding protein
MD2	lymphocyte antigen 96 precursor
MEFs	mouse embryonic fibroblasts
min	minute
MLK3	mixed lineage protein kinase 3, officially MAP3K11
MKK	mitogen activated protein kinase kinase
Myc	myc epitope tag
MyD88	myeloid differentiation primary-response protein 88
NEMO	NF κ B essential modulator, or IKK γ
NF κ B	nuclear factor - κ B
NLK	Nemo-like kinase

NPM-ALK	nucleophosmin-anaplastic lymphoma kinase
NZF	nuclear protein localization 4 zinc finger
p38	also called stress activated protein kinase 2
PAF	platelet-activating factor
PAMPs	pathogen-associated molecular patterns
PGE	prostaglandin E
PMSF	phenylmethylsulfonyl fluoride
proIL-1 β	proto-IL-1 β
Raf1	murine leukemia viral oncogene homolog 1
RING	really interesting new gene
RIP1	receptor interacting protein kinase 1
RNA	ribonucleic acid
SMAD	SMA and MAD related protein
SODD	the silencer of death domain
TAB1/2/3	TAK1 binding protein 1/2/3
TACE	TNF alpha converting enzyme
TAK1	transforming growth factor- β -activating kinase 1
TIP	Type-2A phosphatase interacting protein
TIRAP	TIR domain-containing adaptor protein
TLR	Toll-like receptors
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
Tollip	Toll interacting protein
TPL2	tumor progression locus 2, or COT (Cancer Osaka Thyroid oncogene)
TRADD	TNF receptor associated death-domain protein
TRAF	TNF receptor associated factor
TRIF	TIR domain-containing adaptor protein inducing interferon- β
TRAM	TRIF-related adaptor molecule
TPR	tetratricopeptide repeat
Ub	ubiquitin
Ubc	ubiquitin carrier protein
U-box	ubiquitin fusion degradation 2 box

Uev	ubiquitin-conjugating enzyme variant
XIAP	X-linked inhibitor of apoptosis

Chapter 1.

GENERAL INTRODUCTION TO INFLAMMATION AND INNATE IMMUNITY

1.1 Inflammation and related diseases

Inflammation is a basic mechanism available for repair of tissue after an injury and consists of a cascade of cellular and microvascular reactions that serve to remove damaged tissue followed by generation of new tissue (Schmid-Schonbein 2006). Normally, this process is meant to protect the host from infection and prevent any deleterious consequences if left unchecked.

Generally inflammation can be classified as either acute or chronic inflammation. Acute inflammation is a short-term process which is characterized by the classic signs of inflammation - swelling, redness, pain, heat, and loss of function - due to the infiltration of the tissues by plasma and leukocytes, and release of a variety of materials at the site of inflammation that induce pain. Often, this response is triggered by mast cell degranulation releasing a battery of inflammatory mediators, including bioactive amines (histamine, 5-hydroxytryptamine), and activation of resident macrophages leading to release of pro-inflammatory cytokines, such as TNF (tumor necrosis factor). Stepwise a “domino effect” is exerted by sequential release of lipid mediators, cytokines and chemokines that drive recruitment and activation of additional inflammatory cells. Normally it is a self-limiting process that occurs as long as the injurious stimulus is present and ceases once the stimulus has been removed. If resolution fails, a

chronic form of inflammation will arise (Larsen and Henson 1983; Karin *et al.* 2006; Schmid-Schonbein 2006).

Chronic inflammation is a pathological condition characterised by concurrent active inflammation, tissue destruction, and attempts at repair, without the classic signs of acute inflammation listed above. Instead, chronically inflamed tissue is found to be infiltrated with mononuclear immune cells (monocytes, macrophages, lymphocytes, and plasma cells). Interestingly, tissue destruction occurs along with attempts at healing, which include angiogenesis and fibrosis. With advance of medical knowledge, a growing list of diseases related to inflammation has been reported, just name a few: rheumatoid arthritis, gout and intestinal diseases; endocrinological or autoimmune diseases; as well as chronic diseases such as diabetes. More recently, some seemingly unrelated diseases have also been found to possess an inflammatory nature, like chronic arterial and venous disease, myocardial ischemia, acute cerebral stroke, Alzheimer's chronic disease, arterial hypertension and even cancer (Schmid-Schonbein 2006; van Kempen *et al.* 2006; Linde *et al.* 2007).

With such a close connection with multiple threats of human health, inflammation has been extensively investigated for decades, if not centuries. Although detailed mechanism still remains unclear, the illusive nature of inflammation has been partially unveiled, from aspect of organ to tissue, cellular to molecular level, with the current focus on molecular details. Of special interest, innate immunity has been highlighted as a link between microbial infections and chronic inflammation or even cancer. Multiple clinical investigations have shown

that host factors rather than microbial toxin or oncogene products play critical role in the pathogenesis of chronic infections. Therefore a clear understanding of host response against pathogen and molecules involved in microbial recognition and innate immune signaling will be invaluable to precise diagnosis and effective treatment of those chronic inflammatory diseases (Karin *et al.* 2006).

1.2 Innate immunity and related signaling pathways

Innate immunity detects the presence and nature of infection, provides the first line of host defense and controls the initiation and determination of the effector class of the adaptive immune response which comprises of humoral and cellular immunity relying on generation of a random and highly diverse repertoire of antigen receptors on B and T-cells. Several signal pathways have been identified to regulate innate immune response including TNF, IL-1 (Interleukin-1), and TLR (Toll-like receptor) pathways. We will then focus on the relationship between these signal pathways and inflammation.

1.2.1 TNF signaling

1.2.1.1 Basic biology of TNF and its receptor

TNF was identified three decades ago as a protein produced by the immune system that can induce hemorrhagic necrosis of solid tumors (Carswell *et al.* 1975). Extensive research since then has revealed that TNF is a major mediator of inflammation, viral replication, tumor metastasis, transplant rejection, rheumatoid arthritis, and septic shock. To date around 19 different members of the TNF superfamily have been identified, and most of them have been found to mediate a wide variety of diseases including cancer, arthritis, bone resorption,

allergy, diabetes, atherosclerosis, myocardial infarction, graft versus host disease, and acquired immune deficiency disease. The prototype of the TNF family is TNF α . TNF family ligands exert their biological functions via interaction with their cognate membrane receptors, which comprises the TNF receptor superfamily with more than 30 members (Locksley *et al.* 2001; Aggarwal 2003).

TNF α production is restricted to a relatively small subset of cells including macrophages, lymphoid cells, mast cells, endothelial cells, fibroblasts and neuronal tissues. It has two receptors: TNFR1 and TNFR2 (TNF receptor 1 & 2). TNFR1 is constitutively expressed in most tissues while TNFR2 is typically found in cells of immune system.

TNF α is produced primarily as a transmembrane protein arranged in stable homotrimers and composed of three 17kDa (kilo-daltons) protomer characterized by two antiparallel β -sheets with antiparallel β -strands, forming a “jelly roll” β -structure (Eck and Sprang 1989). Soluble homotrimeric TNF α is released from its membrane-integrated form via proteolytic cleavage of the metalloprotease TACE (TNF alpha converting enzyme) (Moss *et al.* 1997).

TNFRs are type I transmembrane proteins that adopt elongated structures by a scaffold of disulfide bridges and possess the “CRDs” (cysteine-rich domains) which are the hallmark of the TNFR superfamily. Generally three intra-chain disulfides will form between six highly conserved cysteines. The elongated receptor chains fit in the “grooves” between TNF α protomers within the ligand trimer (Gordon *et al.* 2003). Interestingly the extracellular part of TNFRs can also

be cleaved off, giving rise to soluble receptor fragments with potential neutralizing capacity as they can still bind to TNF, though at a lower affinity. TNFR2 is cleaved by TACE while the protease responsible for TNFR1 cleavage is still undefined (Moss *et al.* 2001). The intracellular domains of TNFR1 and TNFR2 that do not possess any enzymatic activity define them as representatives of two main subgroups of the TNFR family, the DD (death domain)-containing receptors and the TRAF (TNF receptor associated factor)-interacting receptors. Death domain can recruit other DD-containing proteins and couple death receptor to caspase activation and apoptosis. TRAF domain can mediate interaction between TRAF family and distinct TNFR family members or some other cell surface proteins (Arch *et al.* 1998). TNFR2 can directly recruit TRAF2, an important downstream adaptor, while TNFR1 can only indirectly interact with TRAF2. Another interesting difference between these two receptors is that membrane-bound TNF α can activate both receptors while soluble TNF α predominantly stimulates TNFR1 and has limited signaling capacities on TNFR2.

We then look at the signaling cascade initiated by TNFR1 when coupled with soluble TNF α .

1.2.1.2 TNFR1 Signaling and clinical implications

After binding of the TNF homotrimer to the pre-formed TNFR1 complex, SODD (the silencer of death domain) is released from cytoplasmic tail of the receptor and hence allows the recruitment of death domain adaptor TRADD (TNF receptor associated death-domain protein) by homophilic interaction between the death domains. TNFR1-bound TRADD then serves as a platform for binding of

TRAF2 or TRAF5 and the DD-containing serine-threonine kinase RIP1 (receptor interacting protein kinase 1). TRAF2/5 are RING domain ubiquitin ligase and can ubiquitinate RIP1 at lysine 377 with K63-linked poly-ubiquitin chain which normally does not lead to proteasome mediated degradation but acts as scaffold in signaling. K63-polyubiquitinated RIP can then recruit downstream TAK1-TAB1-TAB2/3 (Transforming growth factor- β -activating kinase 1 and TAK1 binding protein 1/2/3) complex through the binding between TAB2/3 and ubiquitin chain. It also can recruit NEMO (NF κ B essential modulator, or IKK γ) likewise. This will bring TAK1 and IKK (I κ B kinase) complex in proximity and facilitate activated TAK1 complex to phosphorylate IKK β (I κ B Kinase β) that pre-complexes with NEMO, leading to degradation of I κ B (inhibitor of κ B) and in turn the liberation and translocation of NF κ B into nucleus where it turns on a number of genes related to inflammation (Ea *et al.* 2006). Activated TAK1 complex can activate JNK (c-Jun N-terminal kinase) and p38 α (also called stress activated protein kinase 2a) via phosphorylation of their upstream kinases MKK4/7 and MKK3/6 (Mitogen activated protein kinase kinase) respectively, which subsequently result in the activation of transcription factors such as AP-1 (Activator protein), CREB (c-AMP responsive element binding protein) and induction of other cytokines like IL-2, IL-6, IL-12 (Adhikari *et al.* 2007). Also JNK and p38 α are involved in processing and stabilization of TNF α and play important role in T cell differentiation and activation (Dong *et al.* 2002). The signaling pathway is complicated and a schematic diagram is shown in Figure 1-1.

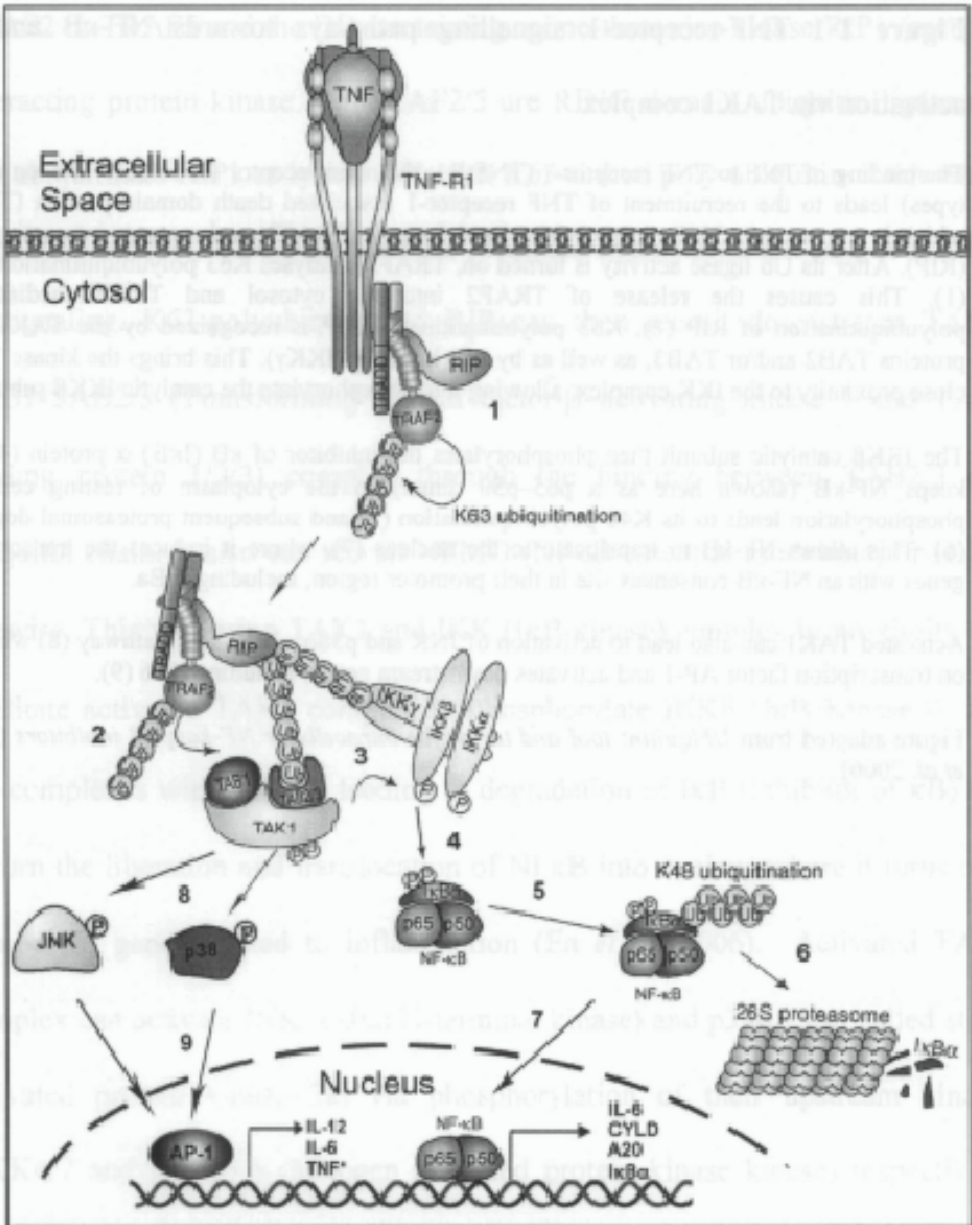
Figure 1-1 TNF-receptor-1 signalling pathways towards NF- κ B and AP-1 activation via TAK1 complex.

The binding of TNF to TNF receptor-1 (TNF-R1; the main receptor for soluble TNF on most cell types) leads to the recruitment of TNF receptor-1 associated death domain protein (TRADD), which in turn recruits TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein (RIP). After its Ub ligase activity is turned on, TRAF2 catalyses K63 polyubiquitination of itself (1). This causes the release of TRAF2 into the cytosol and TRAF2-mediated K63 polyubiquitination of RIP (2). K63 polyubiquitinated RIP is recognized by the TAK1-binding proteins TAB2 and/or TAB3, as well as by I κ B kinase γ (IKK γ). This brings the kinase TAK1 in close proximity to the IKK complex, allowing it to phosphorylate the catalytic IKK β subunit (3).

The IKK β catalytic subunit then phosphorylates the inhibitor of κ B (I κ B) α protein (4), which keeps NF- κ B (shown here as a p65–p50 dimer) in the cytoplasm of resting cells. I κ B α phosphorylation leads to its K48 polyubiquitination (5) and subsequent proteasomal degradation (6). This allows NF- κ B to translocate to the nucleus (7), where it induces the transcription of genes with an NF- κ B consensus site in their promoter region, including I κ B α .

Activated TAK1 can also lead to activation of JNK and p38 α via MAPK pathway (8) which turns on transcription factor AP-1 and activates downstream genes, including IL-6 (9).

Figure adapted from *Ubiquitin: tool and target for intracellular NF-kappaB inhibitors* (Wullaert *et al.* 2006)



Poly-ubiquitinated RIP1 can then be targeted to proteasomal degradation by A20-mediated chain shift from K63 to K48-linked poly-ubiquitination, which is proposed as a negative regulation. RIP1 can also recruit the pro-apoptotic caspase 8 in a ubiquitination independent manner, resulting in the death of cells under certain circumstances (O'Donnell *et al.* 2007).

In general, TNF may be considered to be a major pro-inflammatory mediator, with an optional capacity to induce apoptosis (Wajant *et al.* 2003). Large amount of TNF can be induced by bacterial product like LPS (lipopolysaccharide) and IL-1, in turn it can induce the production of other pro-inflammatory cytokines such as IL-1, IL-6. A locally increasing concentration of TNF will cause the cardinal signs of inflammation to occur: heat, swelling, redness and pain, whereas high concentration of TNF at a global scale can lead to shock-like symptoms in sepsis or even major organs failure (Dinarello 2003). In conjunction with other pro-inflammatory cytokines, TNF can exert a broad spectrum of biological effect in various tissues during inflammation, for instance, it can induce the acute-phase response in liver and promote the phagocytosis of macrophages, and also it is a potent chemoattractant to recruit neutrophils to the site of inflammation. Prolonged exposure to low dose of TNF results in cachexia, a symptom frequently observed in tumor patients (Matthys and Billiau 1997).

As TNF plays critical roles in the pathogenesis of inflammatory diseases, many TNF blockers have been developed and evaluated under various clinical trials for inflammatory diseases. Excitingly, several neutralizing reagents like infliximab[®] and etanercept[®] have been approved as novel therapeutic agents for Crohn's disease and rheumatoid arthritis. However, this kind of simple blockade strategy may have severe side effect since they also suppress the immune response to infection and increase the risk of cancer development (Larmonier *et al.* 2007; Mocellin and Nitti 2008). More details about the mechanism of TNF mediated signaling are required before more efficient therapeutics can be developed.

1.2.2 IL-1 signaling

1.2.2.1 Basic biology of IL-1 and its receptors

IL-1 is a major mediator of inflammation and, in general, initiates and/or amplifies a wide variety of effects associated within innate immunity and host responses to microbial invasion and tissue injury. It is mainly produced by blood monocytes, also by activated macrophages, neutrophils, endothelial cells, fibroblasts and keratinocytes. Before it was given the unifying term in 1979, IL-1 was also named variously according to its different biological functions, like endogenous pyrogen, lymphocyte activating factor, thymocyte proliferation factor, interferon (IFN)- β -inducing factor, among the others (Dinarello 1994).

There are three members of IL1 family, IL-1 α , IL-1 β and IL-1ra (interleukin 1 receptor antagonist). IL-1 α and IL-1 β are agonists and IL-1ra is receptor specific antagonist. All of them share 20-25% amino acid homology and mature forms have similar three-dimensional open barrel structures of β sheets. However, there is remarkable difference between IL-1 α and IL-1 β when examining the regulation of gene expression, mRNA stability, translation, processing and secretion. Generally IL-1 β is considered as a systemic, hormone-like mediator intended to be released from cells, whereas IL-1 α is primarily a regulator of intracellular events and mediator of local inflammation. Hence we will focus on the role of IL-1 β (Arend 1990).

The mature IL-1 β (17kDa) is processed from a 34kDa proIL-1 β (proto-IL-1 β) by cleavage of cysteine protease ICE (IL-1 β converting enzyme, also termed as caspase-1) before it is secreted. ICE itself also undergoes a maturation

process requiring cleavage, similarly under strict regulation in cells (Tocci 1997). Although proIL-1 β can also be cleaved by other proteases like trypsin, elastase, chymotrypsin which are commonly found in the inflammatory fluid, the extent of the roles which these proteases play in the *in vivo* conversion of proIL-1 β to mature form is uncertain. IL-1ra has a signal peptide and can be readily transported out of cells without proteolytic processing (Dinarello 1998).

Two types of IL-1 receptors (IL-1R) are found in cells which can bind all forms of IL-1 with different affinities: type I receptor (IL-1RI) can transduce a signal while type II receptor (IL-1RII) binds to IL-1 but does not transduce a signal. In fact IL-1RII acts as a sink for IL-1 β and thus has been termed as a “decoy” receptor (Dinarello 1991). IL-1RI can recruit a cytoplasmic adaptor protein named IL-1R AcP (IL-1R accessory protein) while IL-1RII cannot. The extracellular domain of IL-1R and IL-1R AcP are members of Ig (immunoglobulin) superfamily, they are each composed of three IgG-like domains with significant homology to each other. The cytoplasmic portion of IL-1RI contains a conserved region named TIR (Toll/IL-1 receptor) domain which is also found in Toll-like receptor and required to recruit TIR-containing adaptor proteins like MyD88 (myeloid differentiation primary-response protein 88) via homophilic interaction. IL-1 and TLR signaling share some common module and both are involved in innate immunity and host defense (Li and Qin 2005).

The extracellular portion of both receptors can be cleaved to yield the soluble form: IL-1sRI or IL-1sRII (IL-1 soluble receptor type I or II), which can bind to IL-1 and play regulatory roles. The binding affinities for the two soluble

forms are remarkably different for each of three IL-1 molecules. IL-1sRI binds to IL-1ra, IL-1 α , and IL-1 β in decreasing order of affinity, whereas for IL-1sRII, the order is IL-1 β , IL-1 α , and IL-1ra. Elevated levels of IL-1sRII are found in the circulation of patients with sepsis and in the synovial fluids of patients with active rheumatoid arthritis, which may be a counter response of body against elevated level of IL-1 β in the pathogenesis. In fact, some neutralizing agents of IL-1 β have been approved clinically for treatment of inflammatory disease (Ortiz *et al.* 2007; Voronov *et al.* 2007).

IL-1RI is found prominently on endothelial cells, smooth muscle cells, epithelial cells, hepatocytes, fibroblasts, keratinocytes, epidermal dendritic cells and T lymphocytes. IL-1RII is found predominantly on B cells and cells of myelomonocytic lineage. Therefore we will focus on the signaling mediated by IL-1 β and IL-1RI.

1.2.2.2 IL-1 signaling and physiological effect

Upon binding of IL-1 β to IL-1RI, a receptor complex is formed with addition of IL-1R AcP via interaction between IL-1 β and IL-1R AcP as well as that between IL-1RI and IL-1R AcP, following by the recruitment of adaptor protein MyD88 through a homophilic TIR-TIR interaction. MyD88 in turn brings two serine-threonine kinases into the complex, namely IRAK1 & 4 (IL-1 receptor associated kinase 1 and 4). IRAK1 exists in a preformed complex with another adaptor protein Tollip (Toll interacting protein) in its unphosphorylated state. When it binds to MyD88 through interaction of DD-DD, Tollip is released and therefore allows the phosphorylation of IRAK1 by IRAK4 which in turn recruits TRAF

family adaptor TRAF6. In an undefined manner, phosphorylated IRAK1 together with TRAF6 dissociate from receptor complex and interact with another pre-formed complex consisting of TAK1 (transforming growth factor- β -activated kinase 1) and its two partners TAB1-TAB2 (TAK1 binding protein 1 and 2) at the membrane, which induces the phosphorylation of TAB2 and TAK1. Then TRAF6 and TAK1 complex leave membrane and migrate into cytosol where two more proteins join the complex, a dimeric E2, Ubc13 (Ubiquitin conjugating enzyme 13) and Uev1A (Ubiquitin-conjugating enzyme variant 1A). In the presence of E1 and E2, TRAF6 is autoubiquitinated with a novel lysine 63-linked polyubiquitin chain, which can bind to TAB2 and TAB3 and consequently activate TAK1 (Deng *et al.* 2000; Wang *et al.* 2001; Kanayama *et al.* 2004). Similarly, as aforementioned, TAK1 then switches on the downstream IKK-NF κ B, MKK4/7-JNK and MKK3/6-p38 pathways. The diagram of IL-1 signaling is presented in Figure 1-2, part A.

IL-1 β can be induced by microbial components as LPS and also a number of non-microbial stimuli like TNF, IL-1, IL-2, GM-CSF, complement 5a, fibronectin and collagen, among the others. IL-1 β can induce the expression of many effector proteins involved in inflammatory process, such as IL-6, IL-8, nitric oxide synthetase, type 2 cyclooxygenase and matrix metalloproteases, among others (Dinarello 1996; Auron 1998; Stylianou and Saklatvala 1998).

IL-1 activates its receptor on cells with various functions, which results in the production of secondary substances that mediate inflammatory events as well as tissue remodeling. In endothelial cells, IL-1 induces the release of PAF

(platelet-activating factor), nitric oxide, and PGE (prostaglandin E), which reduce vascular resistance. Chemokines and the expression of adhesion molecules on the endothelial surface facilitate the migration of circulating leukocytes into the tissues. Lymphocytes stimulated by IL-1 release lymphocyte growth factors that activate lymphocytes and promote expansion. Chondrocytes respond to IL-1 by producing collagenases that contribute to the breakdown of cartilage. The action of IL-1 on smooth muscle results in the production of growth factors. IL-1–stimulated macrophages lead to the activation of osteoclast precursor cells and result in increased bone resorption (reviewed in Dinarello 2000).

Given the essential roles of IL-1 signaling in local and systemic inflammation, several IL-1 neutralizing agents have been developed and clinically evaluated currently in various disease models. One of them, IL-1Ra has been approved in treating rheumatoid arthritis. However, safety issue of the long term reduction of IL-1 activity has been the new concern and more caution needs to be taken in evaluation of novel intervening method (reviewed in Dinarello 2004).

1.2.3 TLR signaling

The prototype of TLR family is the Toll protein of *Drosophila* which is crucial to antifungal immunity in flies. Through database searching a number of TLR homologues are found in many other species, with vertebrates typically having 10-12 members. TLRs are type I membrane glycoproteins which are characterized by an extracellular LRR (leucine-rich repeat) domain and an intracellular TIR domain. LRRs are found in a diverse set of proteins and

responsible for recognition of PAMPs (pathogen-associated molecular patterns) which are produced by microbial pathogens and essential for the survival of these microbes and often serve as molecular signatures of a pathogen class (Medzhitov and Janeway 2000; Medzhitov 2001).

In humans the ten functional TLRs (TLR1-10) can be subgrouped according to their subcellular localization. TLR1, 2, 4, 5, 6 and 10 are expressed on the cell surface and migrate to phagosomes after activation, whereas TLR3, 7, 8 and 9 are expressed in intracellular compartments in nearly all cell types, predominantly in the endosomes and endoplasmic reticulum with the ligand-binding domains facing the lumen of the vesicles. Based on sequence homology and genomic structure TLRs can also be divided into five subfamilies: TLR3, TLR4, TLR5, TLR2 and TLR9. TLR2 subfamily consists of TLR1, 2, 6, and 10. TLR9 subfamily is composed of TLR7, 8 and 9. TLRs tend to form noncovalent dimers in the absence of ligand. TLR2 preferentially couples with TLR1 or TLR6, while the other TLRs appear to associate as homodimers (Takeda *et al.* 2003; Kanzler *et al.* 2007).

TLR can recognize specific microbial components widely expressed by bacteria, fungi, protozoa and virus. Pathogen-encoded TLR ligands fall into three broad categories: lipids and lipopeptides that are recognized by TLR2/TLR1, TLR2/TLR6, or TLR4; proteins such as flagellin recognized by TLR5; and nucleic acids (TLR3, 7, 8, 9). Besides those exogenous ligands, a lot of endogenous ligands are also identified which may expose after cell damage caused by infection or injury. This kind of self-recognition plays important role in

autoimmune reaction and chronic diseases (Marshak-Rothstein 2006; Kanzler *et al.* 2007). The details are summarized in the Table 1 (Akira *et al.* 2001; Takeda *et al.* 2003).

Ligand binding to TLR appears to result in conformational changes and possibly dimerization, leading to recruitment of downstream adaptor proteins via TIR-TIR interaction. These TIR domain-containing molecules include MyD88 as mentioned previously, TIRAP (TIR domain-containing adaptor protein), TRIF (TIR domain-containing adaptor protein inducing interferon- β) and TRAM (TRIF-related adaptor molecule) (Yamamoto *et al.* 2004). Engagement of these adaptors then activates a similar signaling pathway via IRAK-TRAF6-TAK1- IKK leading to activation of NF- κ B and MAPK, as aforementioned in IL-1 signaling. For instance, TLR4 as the first identified TLR in mammals is found to form receptor complex with two other member proteins MD2 and CD14 under stimulation of LPS, a gram-negative bacterial component, and then recruit MyD88 and Mal (MyD88 adaptor-like) via TIR homophilic interaction, which subsequently recruit IRAK1, IRAK4 and TRAF6 to the receptor complex. Similar to IL-1 signaling from here on, TAK1 complex is activated and lead to activation of downstream effectors such as NF κ B, JNK and p38 α , as shown in Figure 1-2, part B. This pathway is responsible for induction of TNF secretion by macrophages under LPS challenge (Beutler 2002; Akira *et al.* 2003; Takeda *et al.* 2003).

Table 1. Toll-like receptors and their ligands

TLR family	Ligands (origin)
TLR1	Tri-acyl lipopeptides (bacteria, mycobacteria) Soluble factors (<i>Neisseria meningitides</i>)
TLR2	Lipoprotein/lipopeptides (a variety of pathogens) Peptidoglycan (Gram-positive bacteria) Lipoteichoic acid (Gram-positive bacteria) Lipoarabinomannan (mycobacteria) A phenol-soluble modulins (<i>Staphylococcus epidermidis</i>) Glycoinositolphospholipids (<i>Trypanosoma Cruzi</i>) Glycolipids (<i>Treponema maltophilum</i>) Porins (<i>Neisseria</i>) Zymosan (fungi) Atypical LPS (<i>Leptospira interrogans</i>) Atypical LPS (<i>Porphyromonas gingivalis</i>) HSP70 (host)*
TLR3	Double-stranded RNA (virus/host) Poly I:C; Poly A:U (synthetic compounds)
TLR4	LPS (Gram-negative bacteria) Taxol (plant) Fusion protein (RSV) Envelope proteins (MMTV) HSP60 (<i>Chlamydia pneumoniae</i>) HSP60 (host)* HSP70 (host)* Type III repeat extra domain A of fibronectin (host) Oligosaccharides of hyaluronic acid (host) Polysaccharide fragments of heparan sulfate (host) Fibrinogen (host)
TLR5	Flagellin (bacteria)
TLR6	Di-acyl lipopeptides (mycoplasma)
TLR7	Single-stranded RNA (virus/host) Imidazoquinoline (synthetic compounds) Loxoribine (synthetic compounds) Bropirimine (synthetic compounds)
TLR8	Single-stranded RNA (virus/host) GU-rich oligoribonucleotides (synthetic compounds)
TLR9	CpG DNA (bacteria) DNA-containing immune complex (host)
TLR10	Unknown

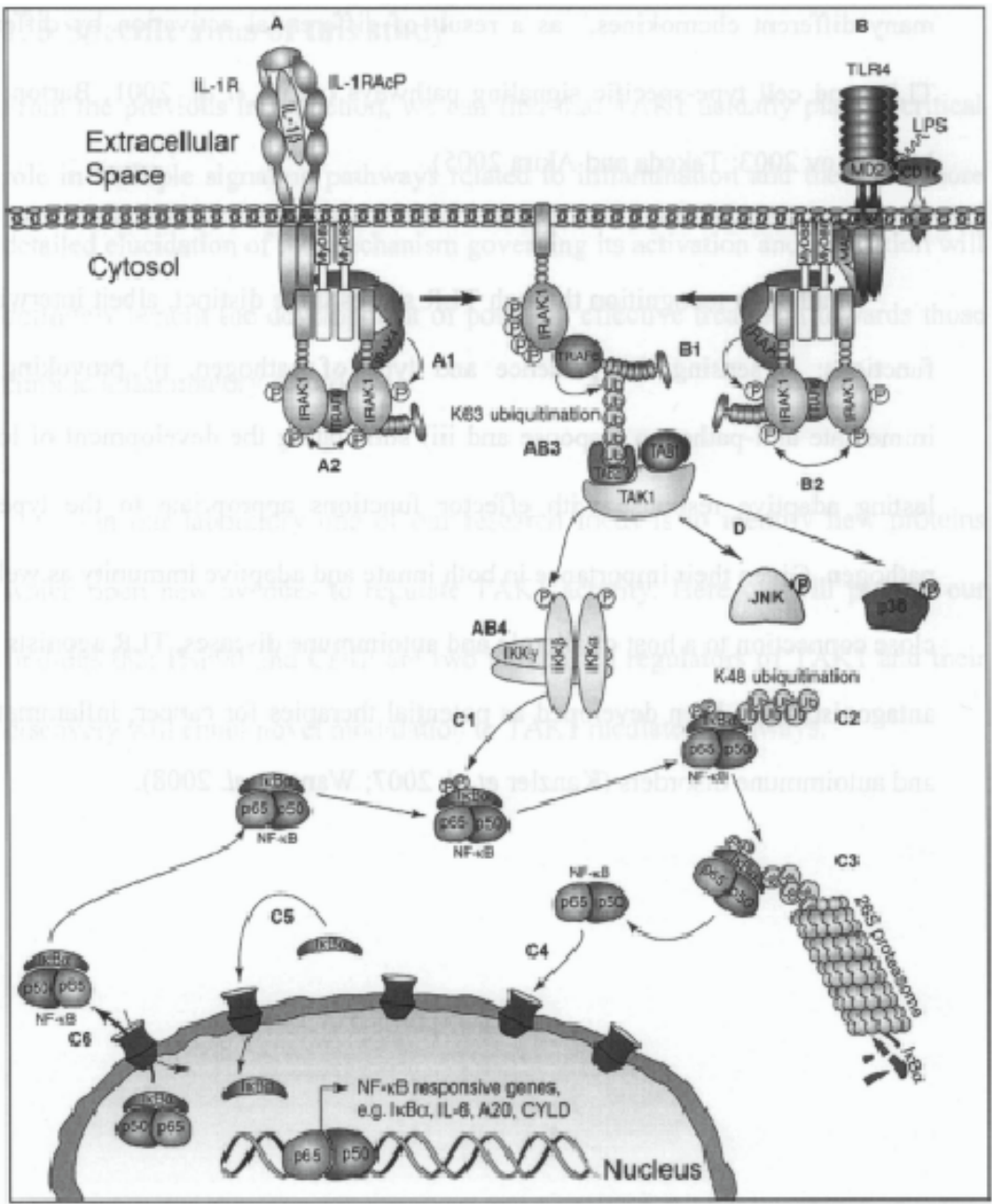
Figure 1-2 TLR4–IL-1-receptor-induced signalling pathways towards NF- κ B activation via TAK1 complex.

The binding of IL-1 β to the IL-1 receptor (IL-1R)–IL-1R accessory protein (IL-1RAcP) complex (A), or the binding of lipopolysaccharide (LPS) to the TLR4-MD2-CD14 complex (B), leads to the recruitment of myeloid differentiation factor 88 (MyD88) and MyD88 adaptor-like (MAL), which subsequently recruit IL-1 receptor-associated kinase 1 (IRAK1), IRAK4 and TRAF6 to the receptor complex. The phosphorylation of IRAK1 by IRAK4 (A1, B1) leads to the hyperphosphorylation of IRAK1 (A2, B2), followed by the dissociation of the IRAK1-TRAF6 complex from the receptor. Once released from the receptor, the Ub-ligase activity of TRAF6 mediates its K63 auto-polyubiquitination (AB3). K63-polyubiquitinated TRAF6 then recruits the TAK1-TAB1-TAB2/TAB3 complex, which subsequently phosphorylates and activates IKK β (AB4).

The IKK β catalytic subunit then phosphorylates the inhibitor of κ B (I κ B) α protein (C1), which keeps NF- κ B (shown here as a p65–p50 dimer) in the cytoplasm of resting cells. I κ B α phosphorylation leads to its K48 polyubiquitination (C2) and subsequent proteasomal degradation (C3). This allows NF- κ B to translocate to the nucleus (C4), where it induces the transcription of genes with an NF- κ B consensus site in their promoter region, including I κ B α . This newly synthesized I κ B α can establish a negative-feedback loop by moving to the nucleus (C5), where it removes DNA-bound NF- κ B complexes from their cognate sites and exports them back to the cytoplasm (C6).

Activated TAK1 can also lead to activation of JNK and p38 α via MAPK pathway (D) which turns on transcription factor AP-1 (detail not shown here).

Figure adapted from *Ubiquitin: tool and target for intracellular NF-kappaB inhibitors* (Wullaert *et al.* 2006)



A second discrete pathway used by TLR3, 7, 8 and 9, in MyD88 dependent manner, activates IRFs (IFN regulatory factors), particularly IRF-7, leading to high levels of type I IFN production. Response to TLR signaling can include cell differentiation, proliferation or apoptosis, as well as induction of many secreted mediators, prominently IFNs, $\text{TNF}\alpha$, IL-1, IL-6, IL-10, IL-12, and

many different chemokines, as a result of differential activation by different TLRs and cell type-specific signaling pathways (Akira *et al.* 2001; Barton and Medzhitov 2003; Takeda and Akira 2005).

Pathogen recognition through TLR serves three distinct, albeit intertwined functions: i) sensing the presence and type of pathogen, ii) provoking an immediate anti-pathogen response and iii) stimulating the development of long-lasting adaptive response with effector functions appropriate to the type of pathogen. Given their importance in both innate and adaptive immunity as well as close connection to a host of allergic and autoimmune diseases, TLR agonists and antagonists have been developed as potential therapies for cancer, inflammatory and autoimmune disorders (Kanzler *et al.* 2007; Wang *et al.* 2008).

1. 3 Specific aims of this study

From the previous introduction, we can find that TAK1 actually plays a critical role in multiple signaling pathways related to inflammation and therefore more detailed elucidation of the mechanism governing its activation and regulation will definitely benefit the development of potential effective treatment towards those chronic inflammatory disorders.

In our laboratory one of our research focus is to identify new proteins which open new avenues to regulate TAK1 activity. Here we will present our findings that Hsp90 and CHIP are two unreported regulators of TAK1 and their discovery will entail novel modulation to TAK1 mediated pathways.

Chapter 2.

INHIBITION OF HSP90 DESTABILIZES TAK1 AND ITS IMPLICATION IN ANTI-INFLAMMATORY RESPONSE

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2.1 Introduction

2.1.1 TAK1 and its binding partners

From last chapter, we can see that TAK1 acts as a pivotal kinase responsible for transducing the various inflammatory signals. Now we have a closer look at this kinase and its binding partners.

TAK1 (TGF β -activated protein kinase 1) was originally identified in mouse as a kinase activated by TGF β family ligands (Yamaguchi *et al.* 1995) and later was found to be a highly conserved MAPKKK (Mitogen activated protein kinase kinase kinase) that exists in different organisms like *Drosophila*, *Xenopus*, mouse and human. The human *tak1* gene is mapped to chromosome 6q16.1-6q16.3 with 17 exons spanning 71kb (Dempsey *et al.* 2000). TAK1 mRNA can be alternatively spliced, giving rise to four different isoforms of TAK1 protein: TAK1a, b, c, d. So far TAK1a is most intensively studied and the physiological significance of the rest is unclear. Here in this thesis we use TAK1 to denote TAK1a for convenience. TAK1 protein consists of 579 amino acid residues with a short N-terminal regulatory motif, catalytic domain followed by a long C-terminal extension and migrates on denaturing SDS gel electrophoresis as a 64 kDa band. Deletion of the first 22 amino acids from

TAK1 N-terminus results in constitutive activation, suggesting that N-terminal domain may engage catalytic domain to inhibit kinase activity. Its catalytic domain can then be divided into 11 subdomains based on consensus sequence alignment to protein kinase A (Yamaguchi *et al.* 1995). The activation loop resides in subdomain VIII and harbors three important sites: Thr184, Thr187 and Ser192, whose phosphorylation is essential for TAK1 activation (Singhirunnusorn *et al.* 2005). Mutation of lysine 63 which is responsible for ATP binding will render a kinase-inactive TAK1 (also called kinase dead mutant).

As implied by its name, TAK1 is a kinase involved in TGF β signaling which is more related to development (Yamaguchi *et al.* 1995; Shibuya *et al.* 1996; Yamaguchi *et al.* 1999). However, recent evidence suggests it plays important roles in TNF α , IL-1 β , bacterial LPS and osmotic stress signaling. Once activated, TAK1 can switch on the JNK pathway via MKK4/7 (Shirakabe *et al.* 1997); the p38 pathway via MKK3/6 (Moriguchi *et al.* 1996); and NF- κ B pathway via IKK (Ninomiya-Tsuji *et al.* 1999; Sakurai *et al.* 1999; Irie *et al.* 2000). These three pathways have been shown as pivotal modulators in regulating inflammatory, immune and apoptotic responses. Although some other MAPKKKs like MEKK1 (Winston *et al.* 1995; Xu and Cobb 1997), MEKK3 (Deacon and Blank 1999; Kim *et al.* 2007), Tpl2 (tumor progression loci 2) (Stafford *et al.* 2006) may also be involved in activation of these pathways, a knockout mouse study has established that TAK1 is essential for both innate and adaptive immunity (Sato *et al.* 2005).

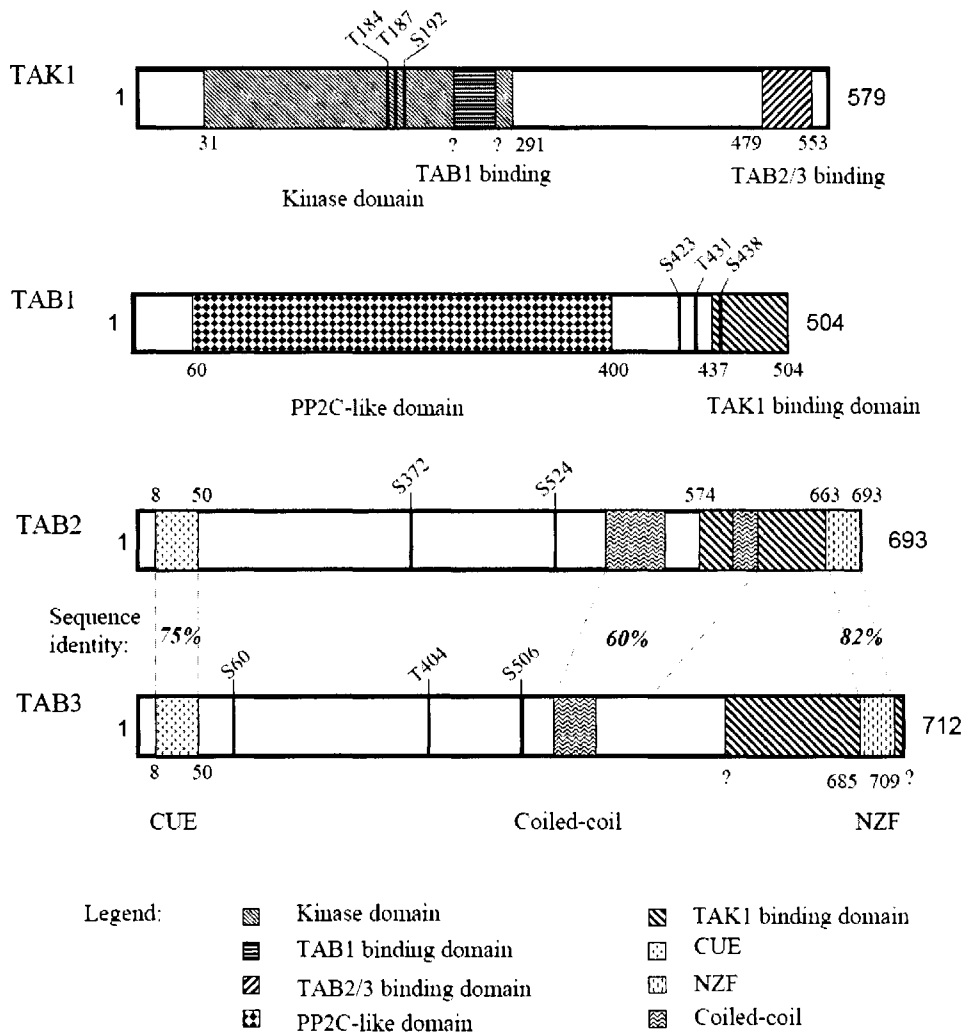
Figure 2-1 TAK1 and its binding partners

TAK1(579 aa) has an N-terminal kinase domain (31-291 aa) which harbors three important sites Thr184, Thr187 and Ser192 in activation loop, and TAB1 binding domain near the C-terminal lobe with undefined borders yet. The domain for TAB2/3 binding is located at C-terminal 479-553 amino acids.

TAB1 (504 aa) has a pseudo-phosphatase domain (PP2C-like) at its N-terminus and the C-terminal 69 amino acids fragment (437-504) is sufficient to bind and activate TAK1. It also has a domain for recruitment of p38 α (not shown here) and three sites have been found to be phosphorylated by p38 α forming a feedback loop to downregulate TAK1 activity, Ser423, Thr431 and Ser438.

TAB2 (693 aa) and TAB3 (712 aa) share considerable sequence homology at their N- and C-terminal CUE and NZF domains responsible for poly-Ub chain binding. TAK1 binding domain is at C-terminus of TAB2 (574-693 aa) and possibly the similar region on TAB3 (borders undefined). Ser372 and Ser524 on TAB2 are found to be phosphorylated upon IL-1 stimulation by a kinase other than p38 α , while Ser60, Thr404 and Ser506 on TAB3 are phosphorylated by p38 α in response to IL-1 stimulation.

The figure is just shown as diagram and not proportionally drawn strictly according to amino acid length.



What makes TAK1 peculiar is that it functions through coupling with three binding proteins. Two binding partners of TAK1 are identified by the use of the yeast two-hybrid system, a 55 kDa protein called TAB1 (TAK1-binding protein-1) (Shibuya *et al.* 1996) and a 76 kDa protein called TAB2 (TAK1-binding protein-2) (Takaesu *et al.* 2000). The third one, TAB3, is discovered by homology searching of database with TAB2, which shares 48% identity to TAB2 with especially high sequence conservation near the N- and C- termini (Cheung *et al.* 2004).

TAB1 is found to enhance the enzyme activity of TAK1 by binding directly to TAK1 N-terminus. The C-terminal 68 amino acids of TAB1 are sufficient to bind and activate TAK1 while the rest part of TAB1 acts as dominant-negative inhibitor of TGF- β -induced gene expression (Shibuya *et al.* 1996). Structural study reveals that TAB1 N-terminus harbors a domain similar to phosphatase 2C, but several key residues required for phosphatase activity are missing in TAB1 which render it inactive as phosphatase (Conner *et al.* 2006), so far the function of this pseudo-phosphatase domain is unclear. Although TAB1 can activate TAK1 directly *in vitro*, however, the endogenous TAK1 complex which contains TAB1 is inactive in unstimulated cells. Gene targeting and biochemical experiments have shown that TAB1 is dispensable for NF- κ B activation by IL-1 β or TNF α (Wang *et al.* 2001; Komatsu *et al.* 2002). However, this finding has recently been challenged by another group of researchers who found neither IL-1 nor TNF α was able to stimulate detectable TAK1 activity in TAB1 knockout MEFs (mouse embryonic fibroblasts) (Mendoza *et al.* 2008). Interestingly, TAB1 is involved in a

feedback control loop through multi-site phosphorylation of its C-terminus by TAK1 downstream MAPK p38 α (Cheung *et al.* 2003), and plays important role to recruit p38 α to TAK1 complex which allows the phosphorylation of TAB3 by p38 α (Mendoza *et al.* 2008). An isoform of TAB1, termed as TAB1 β , as a result of alternative splicing, is found to only interact with p38 α but not TAK1 due to the lack of last C-terminal 68 amino acid, TAK-binding domain (Ge *et al.* 2003).

TAB2 can bind TAK1 via interaction between their C-termini and also bind upstream adaptor TRAF6 (Takaesu *et al.* 2000; Besse *et al.* 2007). It consists of two ubiquitin binding domains, N-terminal CUE (Coupling of Ubiquitin conjugation to Endoplasmic reticulum degradation) domain and C-terminal NZF (Nuclear protein localization 4 Zinc Finger) domain. The latter can bind to K63-linked poly-ubiquitin chain conjugated to upstream adaptors or downstream effector like TRAF6, RIP, NEMO upon stimulation, which may serve as scaffold protein to assemble the signaling machinery (Kanayama *et al.* 2004). Disruption of this poly-ubiquitin binding abrogates the activation of TAK1. Notably, NF- κ B inducible deubiquitinases, such as CYLD (cylindromatosis) and A20, are identified to play a negative feedback role in NF- κ B pathway via removal of K63-linked poly-ubiquitin chains from those related molecules, for instance, TRAF6, NEMO, RIP (Zetoune *et al.* 2001; Kovalenko *et al.* 2003; Trompouki *et al.* 2003). However, the molecular detail of TAK1 activation in this novel ubiquitination-dependent manner still remains elusive. TAB3 is thought to play redundant role as TAB2 since they share high sequence homology at CUE and NZF domains. This notion is

supported by the fact that there exist two distinct complexes, one composed of TAK1-TAB1-TAB2, another composed of TAK1-TAB1-TAB3 (Cheung *et al.* 2004). Knockout of TAB2 has little effect on NF- κ B activation while simultaneous knockdown of TAB2 and TAB3 significantly suppresses NF- κ B signaling (Ishitani *et al.* 2003; Sanjo *et al.* 2003). Nevertheless, we should bear in mind that TAB2 and TAB3 are not completely redundant given the fact that p38 can phosphorylate TAB3 but not TAB2 (Mendoza *et al.* 2008).

Just recently, a protein formerly termed TIP (Type-2A phosphatase interacting protein) was renamed as TAB4 by a search group for its close connection with TAK1, it can directly bind and activate TAK1 by inducing phosphorylation of several unique sites in a linker region on TAK1 mapped between kinase domain and TAB2/3 binding domain (Prickett *et al.* 2008), however, this finding was based on over-expression study and so far the name of TAB4 has not been accepted widely.

The relation of TAK1 and its partners entails multi-layer regulation for TAK1 via the interaction between them and other proteins and thus weave an entangled complex network, which may account for how TAK1 is involved and modulated in various pathways. Canonically, TAK1 can be down-regulated by phosphatases which as implied by the name, dephosphorylate TAK1 and/or its binding protein (Hanada *et al.* 2001). Besides the abovementioned p38 α mediated feedback control via phosphorylation of its C-terminus, TAB1 is found to interact with another E3 ligase XIAP (X-linked inhibitor of apoptosis) to crosslink TGF β pathway and NF- κ B pathway

(Yamaguchi *et al.* 1999; Lu *et al.* 2007). This E3 is also reported to ubiquitinate TAK1 to downregulate JNK activation (Kaur *et al.* 2005). TAB2 and TAB3 can bind TRAF6, TRAF2/5, RIP and NEMO via K63-linked poly-ubiquitin chain and thus ushering in the novel mechanism of ubiquitination-mediated kinase regulation. TAB2 and TAB3 are reported to be sequestered by SMAD7 (SMA and MAD related protein 7), an important modulator involved in TGF β signaling, thus block the recruitment of TAB2/3 to TRAF2, adding one more layer of cross-talk between TGF β and TNF α pathways (Hong *et al.* 2007).

Since TAK1 is involved in a plethora of signaling pathways from mesoderm induction (Shibuya *et al.* 1998; Ohkawara *et al.* 2004), bone re-absorption (Kimura *et al.* 2000), energy homeostasis (Xie *et al.* 2006), to stress response (Huangfu *et al.* 2006), innate and adaptive immunity (Sato *et al.* 2005; Liu *et al.* 2006), a tight and well-orchestrated regulation is necessary to avoid any deleterious crosstalk or unchecked activation. The binding partners of TAK1 are thought to play certain roles to distinguish different stimuli, though other factors may contribute to this end with more or less significance. For instance, TAK1/TAB1 complex can phosphorylate MKKs and activate JNK and p38 but is not sufficient to activate IKK complex, while TAB2 or TAB3, but not TAB1, is required for IKK activation by TRAF6 *in vitro* (Wang *et al.* 2001). Knockout mice studies suggest that TAK1, but not its partners, is essential for multiple signaling pathways *in vivo* (Shim *et al.* 2005). The phenotype of knockout mice for TAK1, TAB1 and TAB2 is summarized in Table 2. Seemingly some controversy exists between *in vitro*

and *in vivo* studies, and until now, the detailed mechanism of TAK1 regulation still remains to be elucidated yet.

Table 2. Comparison of phenotypes of *Tak1*, *Tab1*, and *Tab2* mutant murine embryos

	<i>Tak1</i> ^{-/-}	<i>Tab1</i> ^{-/-b}	<i>Tab2</i> ^{-/-c}
Expression pattern	Ubiquitous expression at E14.5 (strong expression in nervous system, kidney, gut, testis, liver, lung, and pancreas) ^a	Ubiquitous expression through embryogenesis	Ubiquitous expression (strong expression in heart and liver)
Knockout lethality	Embryonic lethality around E10	Embryonic lethality at late stage of gestation	Embryonic lethality around E12.5
Embryo phenotype	Neural fold dysmorphogenesis	Cardiovascular and lung dysmorphogenesis	Liver degeneration and apoptosis
Knockout MEF defect in signaling	No response to TNF & IL-1 stimulation. B-cell line knockout showed impaired NF-κB but normal JNK signaling ^d . T-cell line knockout showed abolished NF-κB and JNK signaling ^e .	TAK1 activation by TNF & IL-1 was abolished while TNF/IL-1 signaling towards MAPK and NF-κB was not impaired ^f .	IL-1-IKK signaling was not impaired ^e

a: (Jadrich *et al.* 2003); b: (Komatsu *et al.* 2002); c: (Sanjo *et al.* 2003)
d: (Sato *et al.* 2005); e: (Liu *et al.* 2006); f: (Mendoza *et al.* 2008)

2.1.2 Hsp90 and kinase quality control

Hsp90 (heat shock protein 90) is a highly conserved and abundant (~1-2% of cytosolic protein) stress protein that exists in all eukaryotic cells. It is a member of heat shock protein family, also called chaperones which prevent improper associations and assist in the correct folding and maturation of other cellular proteins collectively termed as clients and substrates. In basal levels

these heat shock proteins facilitate normal protein folding and guard the proteome from the dangers of misfolding and aggregation and they are induced to higher level of expression by stress including heat, heavy metals, hypoxia and acidosis. However, Hsp90 is expressed at high level even in unstressed cells, where it performs housekeeping functions regulating the activity, turnover, and trafficking of various proteins (Pratt and Toft 2003).

In vertebrates, Hsp90 is encoded by two distinct genes as inducible and constitutively-expressed isoforms: Hsp90 α and Hsp90 β respectively, however, the functional differences between these isoforms are not clear. It resides primarily in the cytoplasm as a homodimer. The monomer is composed of three domains, an N-terminal ATPase domain which can bind to ATP, co-chaperone, and is the target domain of competitive inhibitors such as geldanamycin (its derivative 17-AAG) and radicicol (these inhibitors compete with ATP for its binding pocket and disrupt the ATP-dependent conformational change); middle domain which is responsible for client protein binding, co-chaperone binding; and C-terminal domain which mediates dimerization, and also binds to co-chaperone (Whitesell and Lindquist 2005). By using hormone nuclear receptor as model, Hsp90 is found to help its client substrates fold into functional conformation via an ATP-dependent cycling process with assistance of other chaperones such as Hsp70, Hsp40, Cdc37 (Cell division cycle protein 37), Hop (Hsp70/Hsp90-organizing protein) (Smith *et al.* 1995).

Interestingly, most of Hsp90 client proteins are involved in signal transduction such as receptors, kinases, transcription factors, deregulation of many of these molecules is responsible for oncogenesis. Chaperone function of Hsp90 can buffer genetic variation, allowing it to accumulate silently until it is released in the face of environmental stress to be acted on by natural selection (Rutherford and Lindquist 1998; Queitsch *et al.* 2002). This is especially important for tumor cell survival as many of Hsp90-client proteins are found to be mutated and require Hsp90 to exert essential proliferation role in cancer development. Inhibition of Hsp90 results in rapid proteasome-dependent degradation of those client kinases and transcription factors, which leads to growth arrest and apoptosis of tumor cells. Notably, Hsp90 in tumor cells is found in multimolecular complexes with higher affinity for 17-AAG than largely uncomplexed Hsp90 found in normal cells, providing the biochemical ground for therapeutic application of Hsp90 inhibitors in cancer treatment (Kamal *et al.* 2003). Currently, several inhibitors are quite promising and under various stages of clinical trials worldwide (Workman *et al.* 2007).

Cdc37 is a protein kinase-specific Hsp90 co-chaperone which has been identified in complex with Hsp90 and a plethora of protein kinases including Raf1 (murine leukemia viral oncogene homolog 1), Cdk4, Cdk11 (cyclin dependent kinase 4/11), EGFRvIII (epidermal growth factor receptor variant III), LKB1 (Peutz-Jeghers syndrome protein, official name: serine/threonine kinase 11), MLK3 (Mixed lineage protein kinase 3, officially MAP3K11), IKK α , β , γ . C-terminus of Cdc37 binds to N-terminal domain of Hsp90 and its N-terminus may be involved in kinase binding (Roe *et al.* 2004), therefore it is

proposed that Cdc37 may simply hold the catalytic domain of kinase to protect the entire kinase from degradation and target kinase to Hsp90. The binding of Hsp90/Cdc37 to kinase is thought to contribute to the stabilization and/or activation of kinases, although it is unclear how Hsp90-Cdc37 actually folds the kinase. Besides as a co-chaperone molecule of Hsp90, Cdc37 is also found to bind kinases in the absence of Hsp90 and express in elevated level in certain cancers suggesting that it may have similar “buffering” activity as Hsp90 in stabilizing oncogenic proteins accumulated in tumors (Pearl 2005; Vaughan *et al.* 2006; Caplan *et al.* 2007).

Hsp90 and Cdc37 both bind directly to the catalytic domain of their client kinases. Given the dynamics and diversity of kinases, no consistent sequence difference has been identified between Hsp90 client and non-client kinases, although it is suggested that Hsp90 may recognize a common surface area on kinase which is negatively charged (Citri *et al.* 2006). The binding of Cdc37 to Hsp90 client kinases is more elusive, it is not clear why only some of them can bind to Cdc37. Other chaperones and co-chaperones are also required for protein kinase folding. Unfolded kinases are bound to Hsp70/40 chaperones which appear to prepare the kinase for interaction with Cdc37 (Arlander *et al.* 2006). Hsp90 is subsequently recruited into the kinase-chaperone complex via the action of Hop and then the stable ternary complex composed of Hsp90, Cdc37 and kinase forms (Lee *et al.* 2004). How these chaperones are coordinated and regulated in detail remains unclear and so far it is established only on individual kinases, case by case, instead of a more general model.

2.2 Summary

In this study TAK1 was identified as Hsp90 client kinase using endogenous and exogenous systems. Hsp90 directly binds to kinase domain of TAK1, while the kinase activity of TAK1 as well as autophosphorylation of TAK1 at its activation loop was not required for the binding. TAB1/2/3, the binding partners of TAK1, did not interact with Hsp90, however, interaction of TAK1-Hsp90 was found to be significantly attenuated by TAB1 regardless of TAK1 activation states whereas TAK1-TAB1 binding was not affected by over-expression of Hsp90 in HEK293 cells. This result was supported by TAB1 knockdown experiment which led to increased binding of Hsp90 to TAK1. Hsp90 was not required for TAK1 activation since phosphorylation of TAK1 activation loop was unaffected when Hsp90-TAK1 interaction was disrupted by using Hsp90 inhibitor in a short term. Nonetheless, it was required for TAK1 stability because prolonged inhibition of Hsp90 resulted in down-regulation of endogenous TAK1 but not its binding partners (TAB1, TAB2 and TAB3). Moreover, Hsp90 inhibitors abrogated the pro-inflammatory signaling pathways upon IL-1, TNF and LPS stimulation, collectively suggesting that inhibition of Hsp90 may provide an alternative route to treat inflammatory diseases in addition to its anti-cancer potential. To expand the results obtained on TAK1, a number of other kinases involved in inflammation signaling were also examined regarding to interaction with Hsp90 and responsiveness to Hsp90 inhibitor. Several new kinase clients of Hsp90 were identified, which implied that Hsp90 inhibition exerted anti-inflammation effect by targeting many related kinases rather than TAK1 alone.

2.3 Materials and methods

Reagents and Chemicals

17-AAG, geldanamycin and radicicol were from AG Scientific (San Diego, CA, U.S.A.), SB203580 was from Calbiochem (La Jolla, CA, U.S.A.), and PD98059 was from Promega (Madison, USA). LPS was purchased from Sigma (St Louis, MO, U.S.A.). Instant ELISA for the quantitation of TNF α was purchased from Bender MedSystems (Vienna, Austria). Recombinant human IL-1 β and TNF α were generated in our laboratory and tested to be the same as commercial ones from Sigma. ECL reagent and Protein-G-agarose were from Pierce (Rockford, IL, U.S.A.) Secondary antibody conjugated to horseradish peroxidase was from Biorad (Hercules, CA, U.S.A.). All other chemicals were purchased from either Merck (Darmstadt, Germany), Sigma or USB (Cleveland, OH, U.S.A.). EDTA free complete protease inhibitor was from Roche (Mannheim, Germany).

Buffers

Buffer A [50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 1% (w/w) Triton X-100, 1 mM Na₃VO₄, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol plus 1 tablet/50 ml of EDTA free complete protease inhibitor cocktail]. Buffer B [50 mM Tris/HCl pH 7.5, 0.27 M sucrose and 0.1% (v/v) 2-mercaptoethanol].

Antibodies

TAK1, TAB1 and TAB3 antibodies have been described previously and were generously provided by Prof. Sir Philip Cohen, University of Dundee, UK (Cheung *et al.* 2003). TAB2 (K-20), IRAK1 (F-4), Hsp90 (H-114), Cdc37 (H-271), GST (Z-5), Myc (9E10), HA (Y-11), His-tag (H-3) and Actin (C-11) antibodies were purchased from Santa Cruz (Santa Cruz, CA, U.S.A.). Flag (M2) antibody was from Sigma. Antibodies recognising the phosphorylated form of p38 α [pThr180/pTyr182], phosphorylated form of I κ B [pSer32], phosphorylated form of TAK1 [pThr187], total p38 α , I κ B and IRAK4 were purchased from Cell Signaling (Beverly, MA, U.S.A.). Antibodies recognising the phosphorylated forms of JNK 1 & 2 [pThr183/pTyr185] were from BioSource (Carlsbad, CA, U.S.A.).

DNA Plasmids

The following constructs were used in this study: pEBG2T-TAB1, pEBG2T-TAK1, pEBG2T-TAK1 (345-579), pEBG2T-TAK1 (18-370), pEF6-TAB2-HA, pEF6-TAB3-HA, pEF6-MEKK1 mouse (1174-1493)-myc, pEF6-IRAK4-myc, pEF6-IRAKM-myc, pEF6-IRAK1-HA, pEF6-IRAK2-myc, pEF6-TPL2-myc, pEF6-MEKK2-myc and pEF-MEKK3-myc, pEF6-IKK α -HA, pEF6-IKK β -HA, pEF6-NEMO-HA, pEF6-IKK ϵ -HA, pEF6-RIP1-myc, pEF6-RIP2-myc, pEF6-RIP3-myc, pEF6-MyD88-myc, pCMV5-p38 α -myc, pCMV5-TAK1-myc, pCMV5-TAK1 [K63W]-myc, pCMV5-TAK1 [T187A]-myc, pCMV5-TAK1 [S192A]-myc, pCMV5-TAK1 [T187A&S192A]-myc, pCMV5-TAK1 Δ N22-myc, pCMV5-TAB1-His, pEF6-Hsp90 α -HA and pEF6-Hsp90 β -HA. All constructs used were human cDNA and code for the full length protein with the exception of MEKK1 which is of the mouse form and

codes for the kinase domain (1174-1493]. GST, HA and myc epitope tags are located at the N-terminus of all the proteins. pSUPER-GFP-TAK1 [88] was constructed by cloning the sequence 5'-[ccgagatcgactacaaggagattcaagagatctccttgtagtcgatctctttt]-3' into the *Bgl*II/*Hind*III site of pSUPER-GFP. pSUPER-GFP-TAB1 [218] was constructed by cloning the sequence 5'-[ccgcaaccgagtgaccaacttttcaagagaaagtggctactcggttgctttt]-3' into *Bgl*II/*Hind*III site of pSUPER-GFP (Oligoengine, Seattle, WA, U.S.A.).

Tissue Culture and Cell Stimulation

HEK293 and HeLa cells were cultured in DMEM supplemented with 10% FCS (Hyclone) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin). The cells were incubated at 37°C with a humidified atmosphere of 5% CO₂. 16 h prior to stimulation with TNFα and IL-1β, HeLa cells were incubated in DMEM plus antibiotics but without FCS. At the same time, the cells were incubated with 10 µM 17-AAG dissolved in DMSO or DMSO alone (mock). After 16 h, the cells were stimulated with 50 ng/ml of either TNFα or IL-1β. Cells were then harvested at different timepoints with buffer A and used immediately or stored at -20°C until required. RAW 264.7 macrophages were grown using the same conditions as HEK293 and HeLa cells except the FCS used was heat inactivated. At 1h or 16 h prior to stimulation with 100 ng/ml LPS, cells were treated with the following compounds dissolved in DMSO, 17-AAG, geldanamycin, radicicol, SB203580 or PD98059. Cells and media were collected at the indicated timepoints and used immediately or stored at -20°C until required. TNFα

secreted by the macrophages into the media was measured by ELISA assay according to the manufacturer's instructions (Bender MedSystems). Media was diluted so that the measured concentration of TNF α was linear with respect to the absorbance in the ELISA assay. HEK293 cells were transfected according to a modified calcium phosphate precipitation (Chen and Okayama 1987). All cell lines were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.).

GST pull-down and immunoprecipitation

Protein lysates from HEK293 cells were centrifuged at 14,000 x g for 5 min at 4°C and the insoluble debris discarded. 1 mg of the protein lysate supernatant was incubated with 10 μ l of glutathione-Sepharose beads (GE Healthcare, Little Chalfont, Bucks, U.K.) for 1 h at 4°C. The protein bound to the glutathione-Sepharose beads was separated from the supernatant by centrifugation, washed twice with 1 ml of buffer A and twice with 1 ml buffer B. Immunoprecipitations were carried out using the same method except glutathione-Sepharose beads were substituted with 2 μ g of antibody coupled to 10 μ l of Protein G-agarose beads.

Immunoblotting

Immunoblotting was carried out with standard protocol. The results shown in this thesis were the representatives of at least three times of independent experiments.

2.4 Results

2.4.1 TAK1 but not TAB1, TAB2 or TAB3 interacts with HSP90

While investigating the regulation of TAK1, we observed an unknown protein of approximately 90 kDa in size which bound to overexpressed TAK1 from HEK293. Since Hsp90 has been shown to bind to many protein kinases, we investigated further to see whether the unknown 90 kDa protein bound to TAK1 is indeed Hsp90. When affinity purified anti-TAK1 antibody was used to immunoprecipitate endogenous TAK1 from HEK293 cells, the presence of Hsp90 and the co-chaperone Cdc37 was detected by immunoblotting with anti-Hsp90 and Cdc37 antibodies. As control, a non-specific sheep antibody was used in a separate immunoprecipitation and Hsp90/Cdc37 were not detected (Figure 2-2A). This shows that TAK1 is bound to Hsp90 in cells. However, this binding is relatively weak compared to that between TAK1 and its binding partners, which is readily detectable using much less amount of cell lysates for anti-TAK1 immunoprecipitation (Cheung *et al.* 2004). Moreover, we cannot detect the presence of endogenous TAK1 in reverse immunoprecipitation with anti-Hsp90 antibody (data not shown), as Hsp90 is involved in binding of a long list of substrates and TAK1 may be just one of them therefore it is undetectable due to sensitivity limitation of immunoblotting.

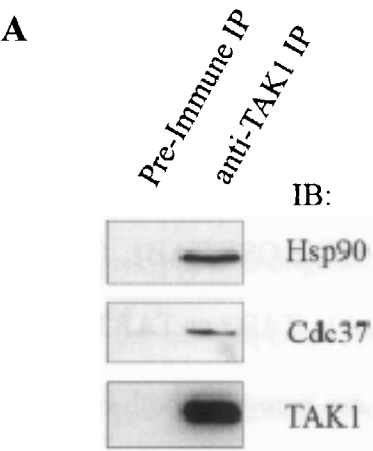
Since TAK1 exists in cells in two different functional complexes consisting of TAK1-TAB1-TAB2 or TAK1-TAB1-TAB3 (Cheung *et al.* 2004), we examined which of the four proteins (TAK1, TAB1, TAB2 or TAB3) contain the binding site for Hsp90. As shown Figure 2-2B, exogenous

GST-TAK1, GST-TAK1 (18-370), GST-TAK1 (345-579), GST-TAB1 or GST-TAK1 (345-579) together with TAB2-HA or TAB3-HA were expressed in HEK293 cells followed by GST pull-down assay to examine the presence of endogenous Hsp90 and cdc37. As TAB2 and TAB3 expressed poorly on their own they had to be coexpressed with GST-TAK1 (345-579) to enhance their expression (Figure 2-2B, lane 6&7). Endogenous Hsp90 and Cdc37 were found to bind only to GST-TAK1 and the kinase domain mutant GST-TAK1 (18-370) (Figure 2-2B, lane 2, 4), but not to GST, GST-TAB1, GST-TAK1 (345-579), or GST-TAK1 (345-579) complexed to TAB2 or TAB3 (Figure 2-2B, lane 1, 3, 5-7). Since this Hsp90 antibody can recognize both Hsp90 α and Hsp90 β , we could not tell which isoform of Hsp90 binds to TAK1. Then by overexpressing HA-Hsp90 α or Hsp90 β together with GST-TAK1, both of them could be detected in GST pull-down assay respectively, suggesting that both Hsp90 α and Hsp90 β can bind TAK1 (data not shown). From these results we can conclude that it is the TAK1 kinase itself that interacts with Hsp90/Cdc37 via kinase domain, but not via the binding partners of TAK1.

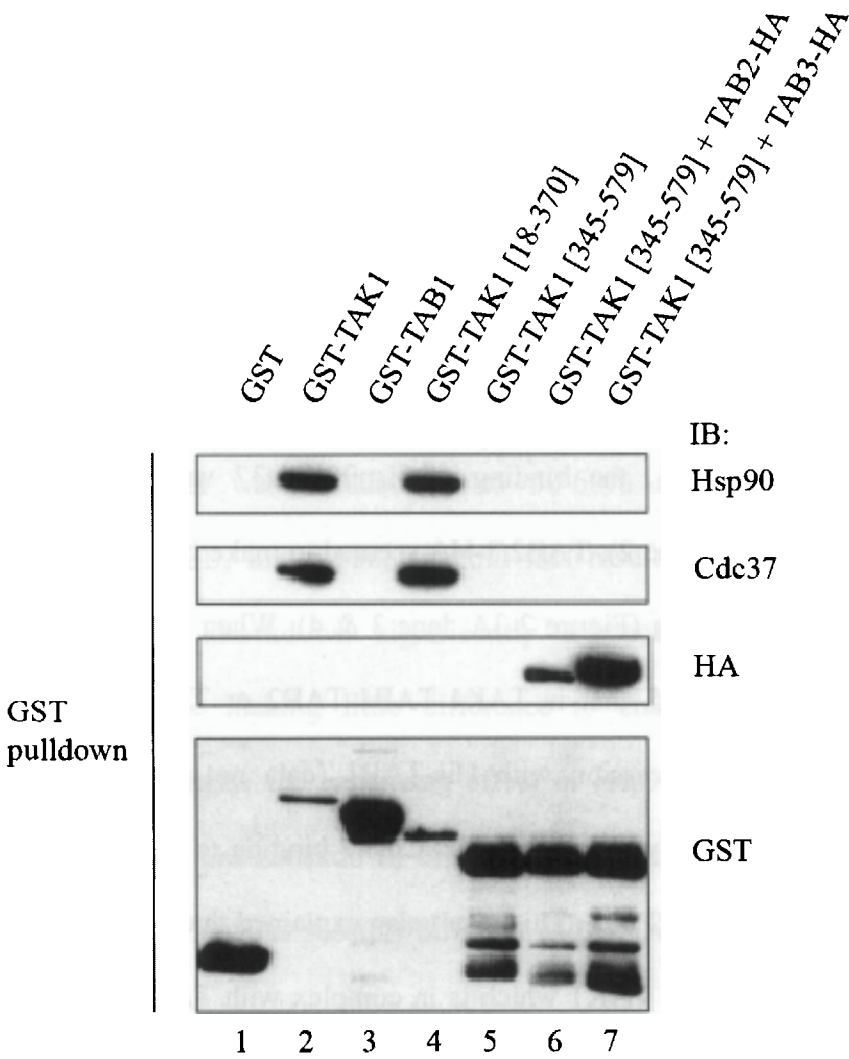
Figure 2-2 Hsp90 binds to TAK1 kinase domain but not TAB1, 2 or 3.

A) 4 mg of HEK293 cell lysates were subject to immunoprecipitation using anti-TAK1 or pre-immune antibody. After thorough wash, beads were boiled with sample buffer and resolved on SDS-PAGE followed by immunoblotting with antibody against Hsp90.

B) HEK293 cells were transfected with various plasmids encoding for proteins as indicated. 36h later, cell lysates were harvested and subject to GST pull-down followed by immunoblotting using antibodies against Hsp90, HA and GST.



B



2.4.2 TAB1 can attenuate the binding of Hsp90 to TAK1 but not vice versa

After we have found that Hsp90 binds to TAK1 but not its binding partners, we then investigate whether the binding partners of TAK1 somehow affect the TAK1-Hsp90 interaction. Exogenous GST-TAK1 was expressed on its own or in complex with His-TAB1, HA-TAB2 or TAB3. Then GST pull-down assay was performed likewise with the lysates to detect endogenous Hsp90 and Cdc37. From Figure 2-3A, we can see that when TAB1-His was co-expressed with GST-TAK1, the binding of Hsp90/Cdc37 was dramatically reduced (Figure 2-3A, lane 2). TAB2/3-HA seemed to make little difference to TAK1-Hsp90 interaction (Figure 2-3A, lane 3 & 4). When full complexes of TAK1 were investigated, that is TAK1-TAB1-TAB2 or TAB3, similar result was found as co-expression with His-TAB1 (data not shown), suggesting that TAB1 persistently reduced Hsp90/Cdc37 binding to TAK1 regardless of the presence of TAB2 or 3. This result also explained that Hsp90 interacts weakly with endogenous TAK1 which is in complex with TAB1 and TAB2/3, as we mentioned previously.

Since TAB1 can attenuate Hsp90 binding to TAK1, we then examine whether knockdown of TAB1 will increase the binding. As shown in Figure 2-3B, HEK293 cells were transfected with TAB1 siRNA encoding plasmid pSUPER-GFP-TAB1 [218] or empty vector control pSUPER-GFP. Transfection efficiency as determined by fluorescent microscopy was around 90% and cell lysates were harvested after 48h and subject to duplicate immunoprecipitation with anti-TAK1, TAB1, TAB2 and TAB3. The presence

of Hsp90/Cdc37 in anti-TAK1 immunoprecipitates was found to be more intense in TAB1 knockdown cell lysates than pSUPER-GFP control (Figure 2-3B, Hsp90 & Cdc37 IB). Meanwhile, TAK1 levels were not changed much with TAB1 knockdown and TAB1 co-precipitated with TAK1 was slightly less compared to pSUPER control (Figure 2-3B, TAK1 IP, TAK1 & TAB1 IB). We also assessed the endogenous levels of TAB2, TAB3 upon TAB1 knockdown by immunoprecipitation and immunoblotting with each antibody respectively. The result turned out that not much change occurred to TAB2/3 level when TAB1 level was reduced up to 50-60% as measured by densitometry (Figure 2-3B, lower panel). From this result we can draw a conclusion that TAB1 knockdown significantly enhanced Hsp90/Cdc37 binding to TAK1 while making little difference to endogenous TAK1, TAB2/3 levels. Interestingly, the enhancing effect of Hsp90/Cdc37 binding was not proportional to the knockdown effect of TAB1, implying that the stoichiometry of TAK1-Hsp90 is possibly different with TAK1-TAB1.

To address whether Hsp90 overexpression can make any difference to TAB1-TAK1 interaction, we co-expressed GST-TAK1 with HA-Hsp90 or His-TAB1, or both of them in HEK293 cells. The presence of HA-Hsp90 or His-TAB1 in purified GST-TAK1 was detected by immunoblotting against HA or His. As shown in Figure 2-3C, His-TAB1 or HA-Hsp90 was found if coexpressed with GST-TAK1 respectively (Figure 2-3C, lane 1 & 3), however, only His-TAB1 can be observed when GST-TAK1, His-TAB1 and HA-Hsp90 were coexpressed together (Figure 2-3C, lane 2), suggesting the preference of TAB1-TAK1 interaction over that between Hsp90 and TAK1. Notably,

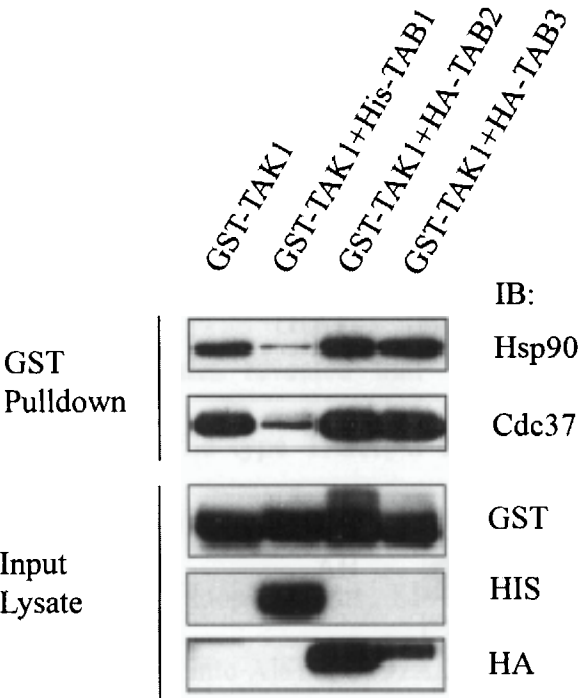
separately expressed His-TAB1 cannot disrupt preformed GST-TAK1 and endogenous Hsp90 complex (data not shown), implying that TAB1 may require certain modification in order to exert its competing ability for TAK1 or the replacement of Hsp90 by TAB1 occurs in specific temporal and spatial manner in the cells.

TAB1 is proven to bind TAK1 kinase domain and promote autophosphorylation of two threonine residues (Thr184&Thr187) and a serine residue (Ser192) in the activation loop of TAK1 (Sakurai *et al.* 2000; Ono *et al.* 2001). Coincidentally Hsp90 also binds to TAK1 kinase domain as shown previously. Therefore, TAB1 and Hsp90 possibly bind to the same part of TAK1 although with some difference which may account for different affinity of binding. Since Hsp90 along with Cdc37 is found to bind nascent protein kinases and help them fold into correct conformation, it is reasonable to hypothesize that TAB1 may substitute Hsp90 to bind TAK1 at certain stage after its synthesis in order to form a functional complex ready for signaling events, and probably activation or autophosphorylation of TAK1 may be related to Hsp90-TAB1 transition.

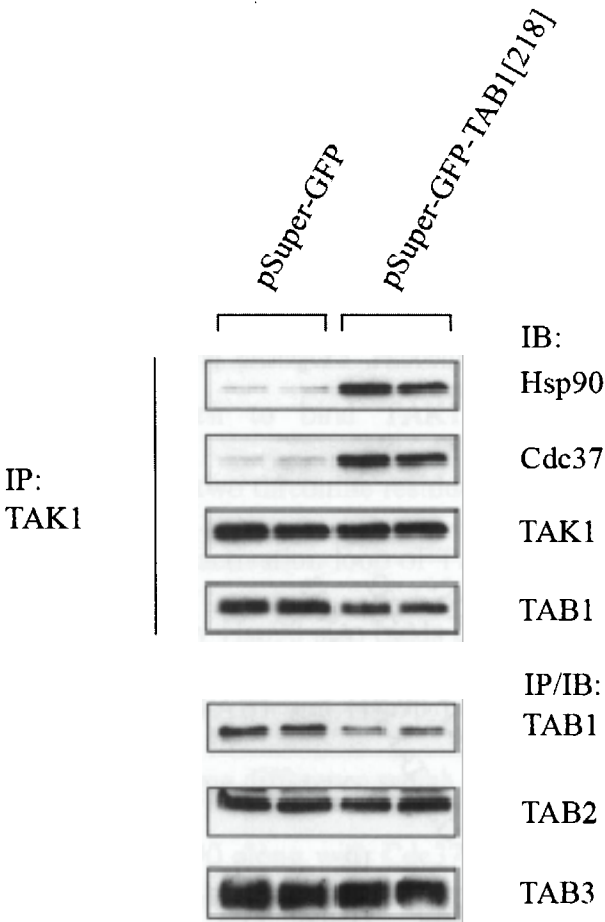
Figure 2-3 TAB1 can attenuate the binding of Hsp90 to TAK1

A) HEK293 cells were transfected with various combinations of proteins as indicated and 200 µg total protein lysates were subject to GST pull-down followed by immunoblotting with antibodies against Hsp90, Cdc37. The exogenous proteins in lysates were also immunoblotted. B) HEK293 cells were transfected with pSUPER-GFP or pSUPER-GFP-TAB1 [218] and harvested 48h later. 2 mg total cell lysates were subject to duplicate immunoprecipitation using antibodies against TAK1, TAB1, TAB2, TAB3 and immunoblotted against the respective antigen except that anti-TAK1 immunoprecipitates were also analysed using anti-Hsp90, Cdc37, TAB1 antibodies. C) HEK293 cells were transfected with different combinations as indicated, followed by GST pull-down assay using 1 mg total lysates and washed beads were resolved on gel and immunoblotted with anti-HA or His antibodies. Exogenous proteins in lysates were also immunoblotted using anti-GST, HA, His antibodies.

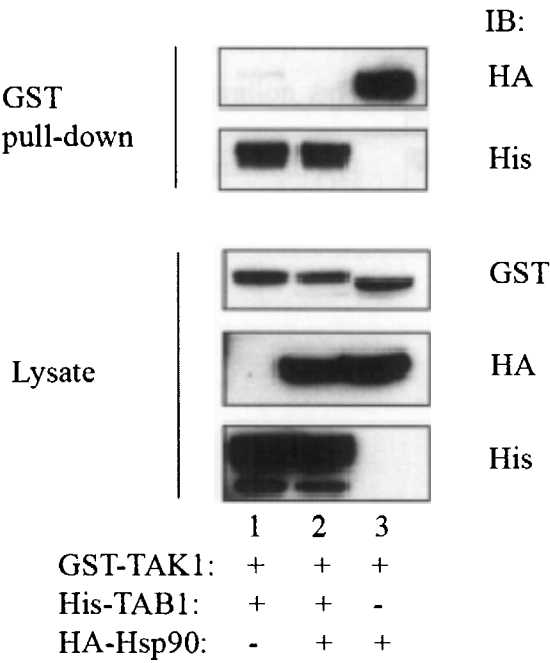
A



B



C



2.4.3 TAB1 attenuates Hsp90 binding to TAK1 irrespective to TAK1 activation state

Previous results have suggested that TAK1 binds to Hsp90 in both endogenous and exogenous conditions; however, it is not clear how this interaction is related to TAK1 activation in cells. Since TAB1 is reported to be an activator of TAK1, we then wonder if Hsp90 can only bind to inactivated TAK1 before TAB1 binds to TAK1 which activates the latter.

To address this question, wildtype myc-TAK1 (wt) and several mutants were expressed in HEK293 cells with or without His-TAB1. Endogenous Hsp90 binding to Myc-TAK1 was analysed by immunoprecipitation with anti-Myc antibody followed by immunoblotting with anti-Hsp90 antibody. As shown in Figure 2-4, levels of different Myc-TAK1 variants in lysates revealed that TAB1 can stabilize and enhance the expression of wild type TAK1, a constitutively active TAK1(Δ N22) from which 22 amino acids from N-terminus was deleted (Shibuya *et al.* 1996), three activation loop mutants, S192A (Ser192 mutated into Ala), T187A (Thr187 mutated into Ala) or S192A/T187A (both Ser192 and Thr187 mutated into Ala), while just slightly enhance the level of kinase dead TAK1 [K63W] (Figure 2-4, anti-Myc IB). Bandshift of TAK1 could be found on wildtype, constitutively active TAK1 as well as TAK1 [S192A] mutant, but not on the other mutants. Notably, TAK1 [T187A and S192A] double mutant was poorly expressed on its own while expression was massively enhanced in the presence of TAB1 (Figure 2-4, anti-Myc IB, lane 11 & 12), however, only a

marginal enhancement of expression was observed for TAK1 [K63W] mutant when it was co-expressed with TAB1 (Figure 2-4, anti-Myc IB, lane 5 & 6).

The presence of endogenous Hsp90 was found in all the immunoprecipitates of Myc-TAK1 variants, which showed that Hsp90 can bind to TAK1 despite of its kinase activity, although the intensity of Hsp90 bound to TAK1 was different. When TAB1 was present, Hsp90 was not detected to bind any of those variants, as shown in the bottom panel (Figure 2-4, anti-Hsp90 IB).

However, currently it is not clear whether TAB1 physically competes with Hsp90 to the same site or sites with allosteric effect on TAK1, or TAB1 binds to TAK1 and cause conformational change of TAK1 thus knocking Hsp90 off. It has been reported that full-length of TAB1 can promote autophosphorylation of TAK1 on Ser192, but in our experiment even TAK1 [S192A] and [T187A&S192A] mutant still showed similar pattern as wildtype with regard to Hsp90 binding in the presence of TAB1, suggesting that autophosphorylation at Ser192 or Thr187 made little difference to Hsp90 binding. Nonetheless, it is possible that autophosphorylation of these residues is the consequence rather than the cause of TAB1 induced TAK1 conformational changes, that is, TAK1 [T187A&S192A] mutant is still able to change its conformation upon TAB1 binding, but not to undergo autophosphorylation since the sites have been mutated. If this is the case, then our results can fit in perfectly. To address this possibility, a special “inactivable” TAK1 mutant that is locked at its quiescent state and irresponsive to TAB1 binding will lend

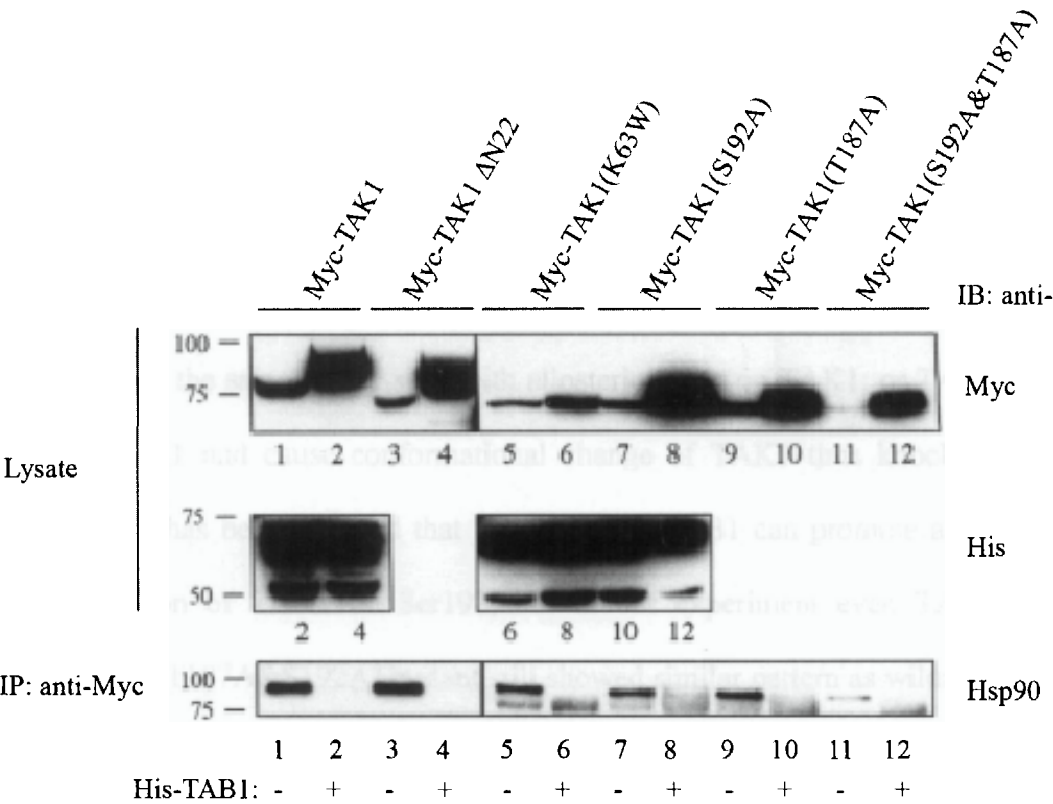
Chapter 2. TAK1-Hsp90 interaction & its implication

support to clarify this hypothesis, which will be an interesting part of future work. Also the mutant of TAK1 [T187E&S192E] which is supposed to mimic the phosphorylation of these two sites may shed more light on this mystery.

Figure 2-4 TAB1 attenuates Hsp90 binding to TAK1 despite of TAK1

activation state

HEK293 cells were transfected with different Myc-TAK1 variants with or without His-TAB1 as indicated. Cell lysates were subject to anti-Myc immunoprecipitation and subsequently immunoblotted with antibodies against Hsp90. Meanwhile, the protein expression in lysates was also immunoblotted using anti-Myc or His antibodies.



2.4.4 Hsp90 binding is required for TAK1 stability but not for activation of TAK1 in pro-inflammatory pathways

After we found and confirmed the TAK1-Hsp90 interaction, next we went on to examine the physiological significance in the cells.

As implied by previous research that Hsp90 binding is required for the stability of its client protein, firstly we examined whether inhibition of Hsp90 by 17-AAG affects the stability of TAK1 and its associated proteins TAB1, TAB2 and TAB3.

HEK293 cells were treated for 16 h with 10 μ M 17-AAG or DMSO control as shown in Figure 2-5A. Because our antibodies were not sensitive enough to detect endogenous TAK1, TAB1, TAB2 and TAB3 in protein lysates, the proteins were first immunoprecipitated using protein specific antibodies and then immunoblotted. In cells treated with 17-AAG, a dramatic decrease in the level of TAK1 was detected (Figure 2-5A, anti-TAK1 IB). With a lower dose of 17-AAG, TAK1 level was decreased to a less extent compared to that with 10 μ M 17-AAG (data not shown). However, levels of TAB1, TAB2 were unaffected by 17-AAG treatment. Seemingly band shift of TAB1 was observed with 17-AAG treatment, which migrated a little faster implying that dephosphorylation may occur. TAB3 level was decreased to a greater extent than TAB1 and TAB2 upon 17-AAG treatment and TAK1 knockdown, although still not that significant as TAK1 itself under respective conditions (Figure 2-5A, anti-TAB1, 2, 3 IB). Similar results were observed when cells were treated with radicicol, an inhibitor of Hsp90 that is

structurally different from 17-AAG (results not shown). This suggests that Hsp90 directly affects the stability of TAK1 but not TAB1, TAB2 and TAB3. Similarly, when the TAK1 protein was knocked down by RNAi, levels of TAB1, TAB2 and TAB3 were not significantly affected indicating that their stability is not dependent on TAK1 protein levels. However, this band shift of TAB1 was not observed on TAK1 knockdown, suggesting that 17-AAG may cause that effect via some unknown factor other than TAK1 only. Previously we mentioned that HA-TAB2/3 was not stable on their own and required TAK1 C-terminus to stabilize them, this controversy may be caused by overexpression which elevated TAB2/3 levels too much higher than endogenous states. Equal possibly there were some other proteins can bind and stabilize endogenous TAB2/3 when TAK1 was knocked down, but they still could not stabilize TAB2/3 in overexpression experiments. In our lab, another protein kinase NLK (Nemo-like kinase) also can stabilize TAB2/3 when overexpressed (Chan Aye Thu, Cheung, unpublished data).

To further confirm that 17-AAG treatment led to downregulation of TAK1, we sought to establish the time course and dose response pattern of TAK1 decrease upon Hsp90 inhibition by 17-AAG. HEK293 was treated with 10 μ M 17-AAG for different time (0-16 h) or different concentration of 17-AAG (0-10 μ M) for 16 h and the same amount of lysates were subjected to anti-TAK1 immunoprecipitation followed by anti-TAK1 immunoblotting. As shown in Figure 2-5B, TAK1 showed significant decrease initially observed from 4 h treatment with 10 μ M 17-AAG (Figure 2-5B, upper panel). The dose

response experiment revealed that TAK1 level was unaffected at 17-AAG concentration lower than 2.5 μ M for 16 h (Figure 2-5B, lower panel).

As we found previously, TAK1 level did not show detectable decrease within 4 h treatment with 17-AAG (Figure 2-5B). This observation then prompted us to investigate the effect of Hsp90 inhibition on a shorter term to see whether TAK1 activation in proinflammatory signaling was changed by 17-AAG treatment.

Firstly we looked at IL-1 signaling in HeLa cells. As shown in Figure 2-5C, HeLa cells were starved overnight in FCS free media to remove any basal IL-1 activation. After 1 h pre-treatment with 10 μ M 17-AAG or DMSO, cells were stimulated with 50 ng/ml IL-1 during a time course: 0, 15, 30, 45, 60 and 120 min. The same amount of lysate was subject to anti-TAK1 immunoprecipitation followed by immunoblotting with various antibodies. Here we can find that similar amount of Hsp90 bound to TAK1 during the whole time course when cells were pretreated with DMSO, while barely no binding of Hsp90 could be found when cells were treated with 17-AAG for 1 h. Similar result was shown for Cdc37 binding (Figure 2-5C, anti-Hsp90 and Cdc37 IB). Endogenous TAK1 level during the time course was not changed much (Figure 2-5C, anti-TAK1 IB). These data collectively suggested that 1h treatment with 17-AAG disrupted the interaction between TAK1 and Hsp90/Cdc37 as expected but did not affect TAK1 level significantly. We then looked at the autophosphorylation of TAK1 Thr187, an indicator of TAK1 activation. The phosphorylated Thr187 signal was not observed at non-

stimulated sample and peaked at 15 min, as reported by others (Singhirunnusorn *et al.* 2005). Interestingly, a biphasic pattern of TAK1 activation was found. The first peak of TAK1 phosphorylation occurred at 15 min and the second one appeared at 120 min. The phosphorylation of p38 α also showed the similar pattern of activation as TAK1 [pThr187] (Figure 2-5C, anti-TAK1 [pT187] and p38 α [pT180/pY182] IB). The levels of TAB1 and TAB2 bound to TAK1 were found to be the same with or without 17-AAG treatment, except that TAB1 showed band shift with IL-1 stimulation while TAB2 did not (Figure 2-5C, anti-TAB1 and TAB2 IB).

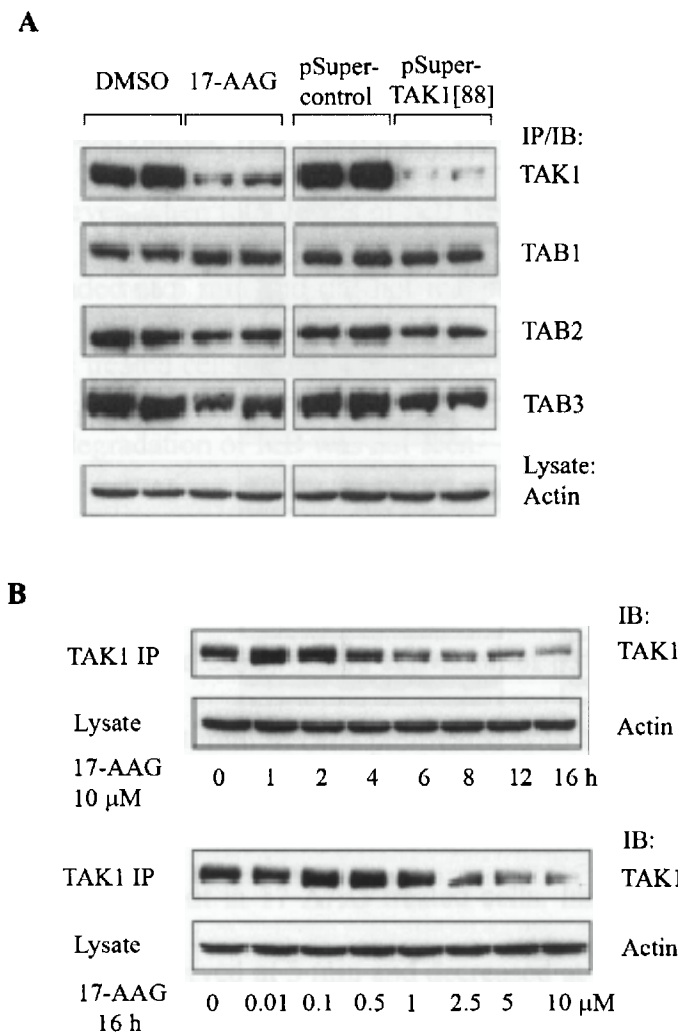
For RAW cells stimulated with LPS, basically it was similar case as what we have found in HeLa cells. As we can see in Figure 2-5D, a nearly constant level of TAK1 was found for the all time points tested with or without 17-AAG treatment (Figure 2-5D, anti-TAK1 IB). Similarly, no binding of Hsp90/Cdc37 to TAK1 was detected when 17-AAG was used (Figure 2-5D, anti-Hsp90 & Cdc37 IB). TAK1 Thr187 and p38 α phosphorylation also showed a similar pattern as IL-1 time course stimulation, peaked at 15 min and gradually dropped but regained phosphorylation at 120 min (Figure 2-5D, anti-TAK1 [pT187] and p38 α [pT180/pY182] IB). TAB1 and TAB2 co-precipitated with TAK1 also showed constant levels (Figure 2-5D, anti-TAB1 and TAB2 IB). These results collectively showed that Hsp90 is not required for TAK1 activation upon IL-1 and LPS stimulation.

Figure 2-5 Hsp90 binding is required for TAK1 stability but not for activation of TAK1 in pro-inflammatory pathways

A) HEK293 cells were treated with 10 μ M 17-AAG or DMSO as control for 16h and harvested. Another group of HEK293 cells were transfected with TAK1 siRNA pSUPER-GFP-TAK1 [88] or empty pSUPER-GFP vector as control. Cell lysates were subject to duplicate immunoprecipitation using anti-TAK1, TAB1, TAB2, TAB3 and immunoblotted with respective antibodies as indicated. The same amount of lysates was immunoblotted with anti-actin antibody to show equal input for each immunoprecipitation.

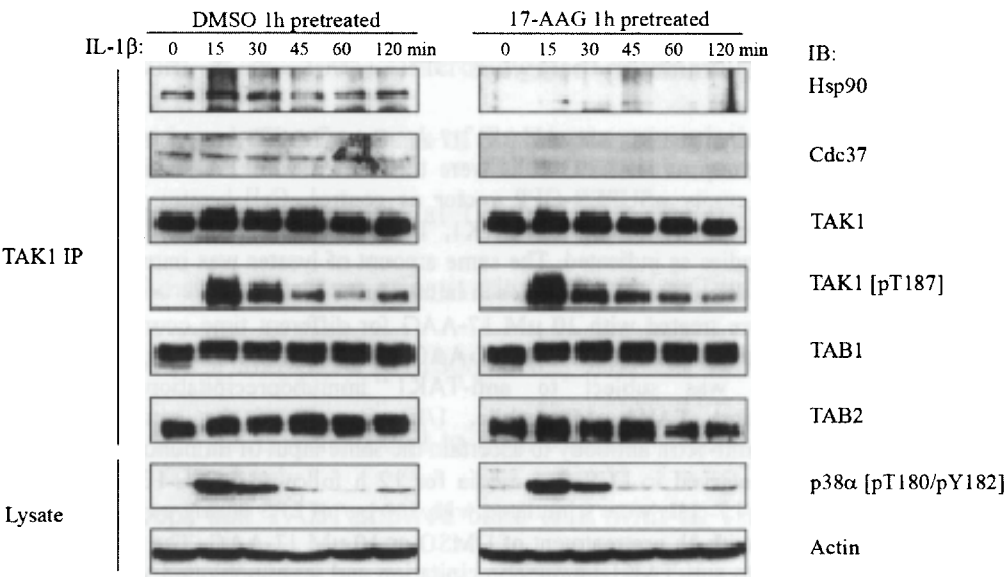
B) HEK293 cells were treated with 10 μ M 17-AAG for different time courses as indicated (upper panel) or different concentration of 17-AAG for 16 h (lower panel). Then the same amount of lysates was subject to anti-TAK1 immunoprecipitation followed by immunoblotting against TAK1. Meanwhile, 1/10 amount of the same lysates was immunoblotted with anti-actin antibody to ascertain the same input of immunoprecipitations.

C) HeLa cells were starved in FCS free media for 12 h followed by IL-1 β stimulation 50 ng/ml or **D)** RAW 264.7 cells were stimulated with 100 ng/ml LPS during a time course from 0 to 2h as indicated, with 1h pretreatment of DMSO or 10 μ M 17-AAG. The same amount of lysates were subject to anti-TAK1 immunoprecipitation and immunoblotted with anti-Hsp90, Cdc37, TAK1, TAK1 [pThr187], TAB1, TAB2 antibodies. The lysates were immunoblotted with p38 α [pT180/Y182] antibody and anti-actin IB used as loading control.

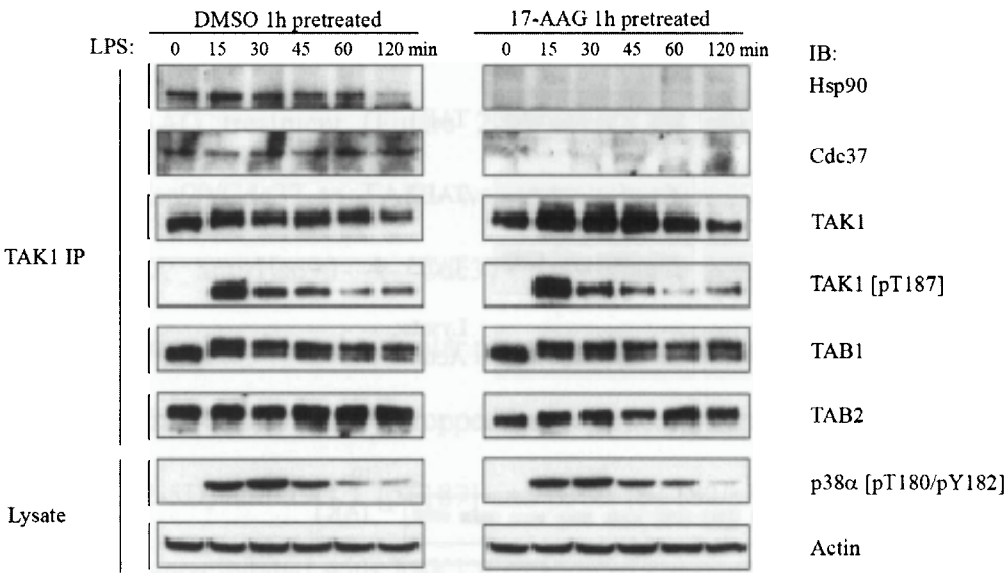


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C



D



2.4.5 17-AAG and radicicol inhibits signaling by IL-1, TNF and LPS

Given that TAK1 is a key mediator in the IL-1 β , TNF α and LPS signaling pathways, we then investigated the effect of Hsp90 inhibition by 17-AAG on the activation of NF κ B, p38 and JNK, which function downstream of TAK1. When HeLa cells were stimulated with IL-1 β (Fig 2-6A), phosphorylation on Thr180/Tyr182 of p38 α can be observed from 5 min to 20 min but pretreatment with 17-AAG caused the IL-1 β activation of p38 α to be drastically reduced. Activation of the JNK pathway was observed within 5 min by IL-1 β and peaked at 20 min. Cells exposed to 17-AAG did not exhibit any activation of the JNK pathway. Rapid phosphorylation on Ser32 of I κ B was seen at 5 min after IL-1 β stimulation. In 17-AAG treated cells, phosphorylation was decreased significantly and prolonged till 10 min. However, when total levels of I κ B were examined, protein levels of I κ B were degraded at 5 min and did not reappear till 30 min after stimulation. In 17-AAG treated cells, the total I κ B levels were readily observed at all timepoints and degradation of I κ B was not seen.

Similar results were observed when HeLa cells were stimulated with TNF α (Figure 2-6B). Rapid activation of p38 α can be seen from 10 min and peaking at 20 min. In cells pre-treated with 17-AAG, activation of p38 α was blocked completely. TNF α activation of the JNK pathway can be detected at 20 min but not in 17-AAG treated cells. Initial phosphorylation on Ser32 of I κ B was observed at 5 min and decreased at 10 min. In 17-AAG treated cells, phosphorylation on Ser32 of I κ B was not detectable. A decrease in the total protein level of I κ B was observed 10 min after TNF α stimulation but I κ B

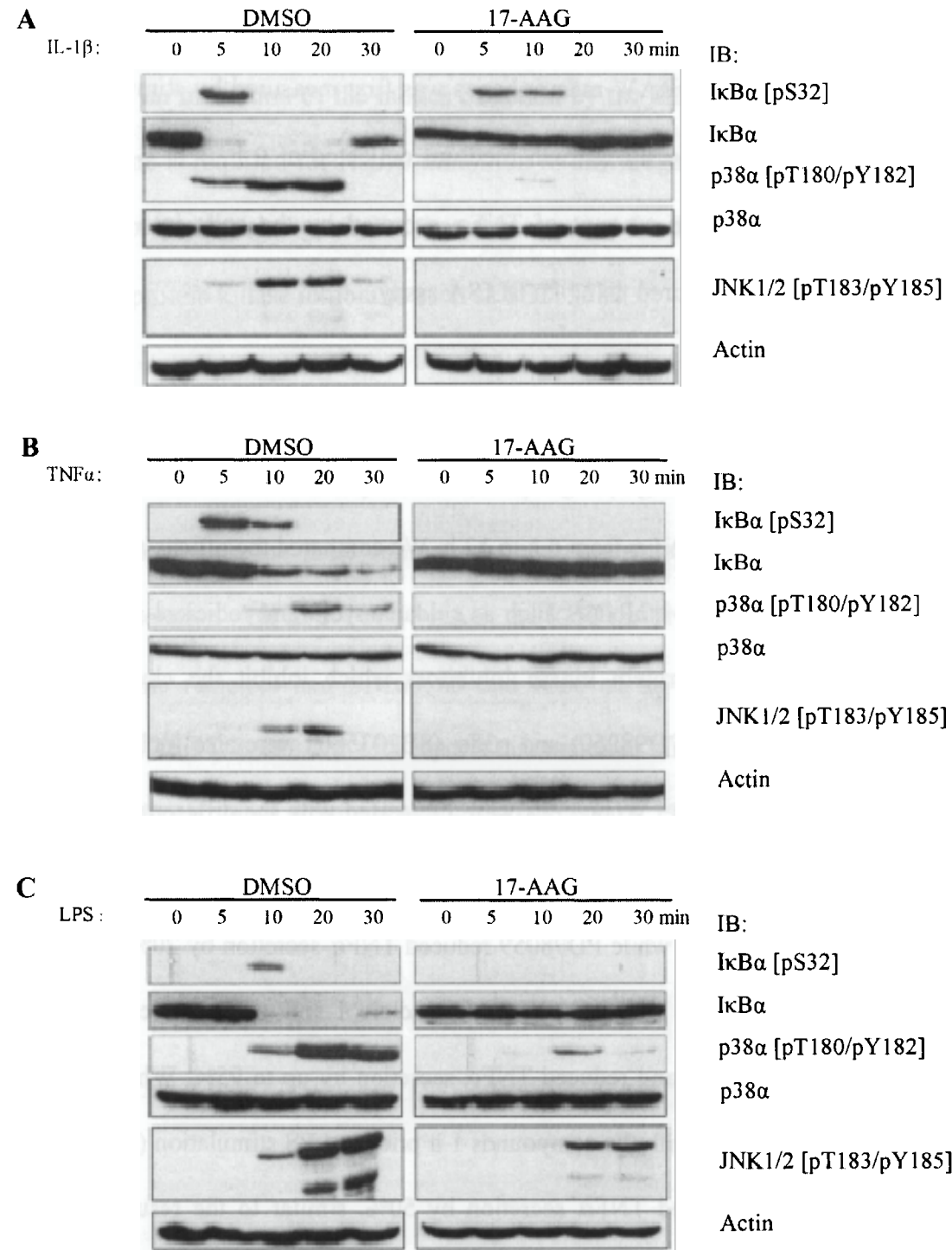
levels were unaffected in 17-AAG pre-treated cells. This shows that 17-AAG prevents the proteasomal degradation of I κ B.

We also examined the effect of 17-AAG on LPS signaling in RAW macrophages (Figure 2-6C). In DMSO control cells, 10 min after LPS stimulation, I κ B phosphorylation was detected and total I κ B decreased. Pre-treatment with 17-AAG abolished the phosphorylation of I κ B completely while total I κ B levels were unaffected. High level activation of p38 α and JNK was observed from 10 min to 30 min in DMSO control cells but markedly reduced in 17-AAG treated cells. In summary, these results show that 17-AAG is a potent inhibitor of LPS, IL-1 β and TNF α signaling and reduces markedly the activation of the NF κ B, p38 α and JNK signaling pathways.

Figure 2-6 17-AAG treatment abolished IL-1, TNF and LPS signaling.

A, B) HeLa cells were pretreated with 10 μ M 17-AAG or DMSO as control(MOCK) in FBS-free media for 16 h before stimulated with 50ng/ml IL-1, TNF respectively for the time course indicated.

C) RAW 264.7 cells were pretreated with 10 μ M 17-AAG or DMSO as control for 18h before challenged with 100 ng/ml LPS for the time course indicated. Cell lysates were harvested and subject to various immunoblotting with different antibodies as indicated.



2.4.6 Pretreatment of macrophages with 17-AAG abrogates LPS signaling and TNF α production

Since 17-AAG inhibits the activation of NF κ B, p38 α and JNK in response to LPS stimulation, we examined whether 17-AAG can suppress TNF α secretion by cells, which plays important role in igniting the inflammatory cascade. TNF α secreted by RAW macrophages was first measured by stimulation with 100 ng/ml LPS and the growth medium collected at 0 h, 3 h, 6 h and 12 h (Figure 2-7A). The amount of TNF α secreted by the cells into the growth medium was measured using an ELISA assay.

The amount of TNF α secreted increased from the basal level of 10 ng/ml to 70 ng/ml at 3 h and 110 ng/ml at 6 h. There was a small increase in the secretion of TNF α from 6 h to 12 h. We compared the efficacy of 17-AAG with other Hsp90 inhibitors such as geldanamycin and radicicol in inhibiting LPS signaling. Protein kinase inhibitors which inhibit the classical MAP kinase pathway (PD98059) and p38 α (SB203580) were also included in this study for reference. When cells were pretreated with the different compounds at 16 h prior to stimulation with LPS (black bars), SB203580 reduced TNF α secretion by 50% while PD98059 reduced TNF α secretion by 20% (Figure 2-8C). The Hsp90 inhibitors 17-AAG, radicicol and geldanamycin were all equally effective and reduced TNF α secretion by up to 95%. When the cells were pretreated with the compounds 1 h prior to LPS stimulation (black bars), SB203580 reduced TNF α secretion by 50%, similar to the result observed when the cells was pre-treated at 16 h (Figure 2-7B). At 1 h pre-treatment,

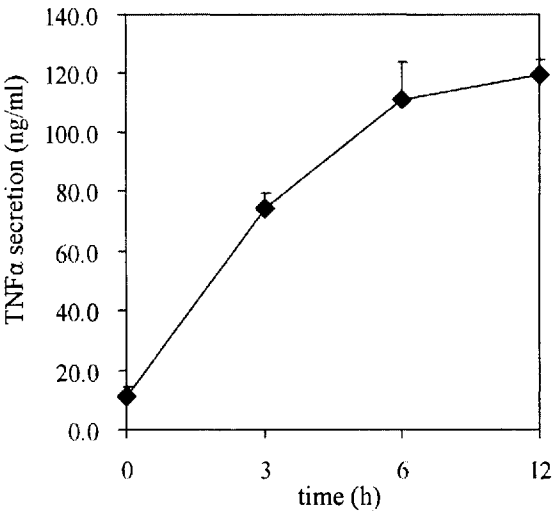
PD98059 did not significantly affect TNF α secretion. The discrepancy of PD98059 effect on TNF α secretion may due to the lower concentration of PD98059 used in our study. All the Hsp90 inhibitors reduced TNF α secretion by only 50%, compared to a 95% reduction observed for 16 h pre-treatment. The difference in TNF α secretion when the cells were pretreated at 1 h and 16 h is perhaps an indication of the modes of action by the different compounds. A longer time is required for Hsp90 inhibition to downregulate protein kinase activity by proteasomal degradation than that required for the direct inhibition of p38 α by protein kinase inhibitors such as SB203580.

We then compared the dose response of 17-AAG, radicicol and SB203580 on TNF α secretion by RAW macrophages (Figure 2-7D). The cells were pretreated with the three compounds at different concentrations 16 h prior to stimulation with LPS. For 17-AAG, the IC₅₀ for TNF α secretion was 0.6 μ M while radicicol and SB203580 had IC₅₀ of 50 nM and 0.5 μ M respectively. This is consistent with reports that radicicol binds to and inhibits Hsp90 with much higher affinity than 17-AAG (Roe *et al.* 1999).

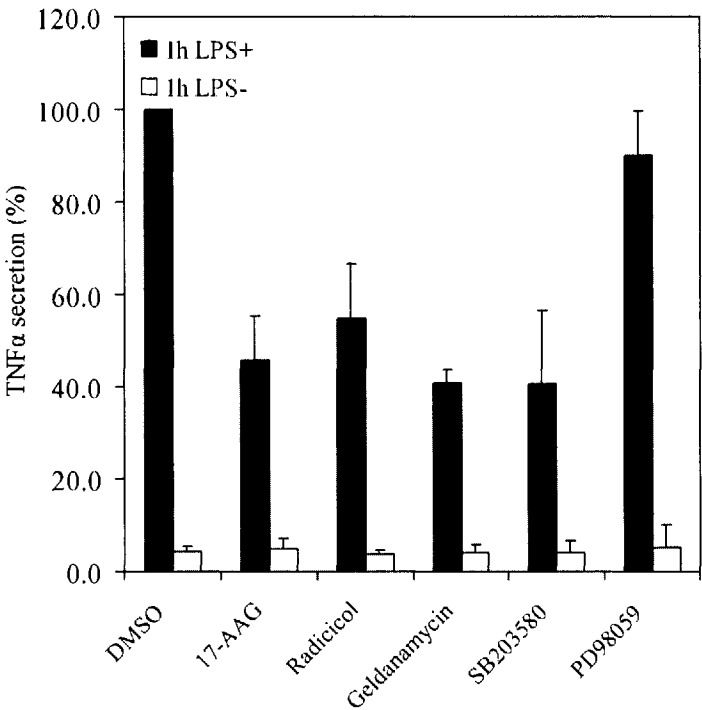
Figure 2-7 17-AAG treatment abolishes LPS signaling and TNF α production

A) RAW 264.7 cells were stimulated with 100 ng/ml LPS for time course indicated. TNF secreted into media was measured by ELISA and shown as ng/ml. **B, C).** RAW264.7 cells were pretreated with 10 μ M different compounds or DMSO for 1h (B) or 16h (C) as indicated, followed by stimulation with 100 ng/ml LPS for 6 h in continued presence of those compounds. TNF secreted into media was measured by ELISA and shown as percentage against DMSO control. **D)** RAW 264.7 cells were pretreated with different concentrations of compounds for 16 h and stimulated with 100 ng/ml LPS for 6 h in continued presence of those compounds. TNF secreted into media was measured by ELISA and shown as percentage against DMSO control. Assays were done in triplicate and the results shown are the mean \pm SEM from three independent experiments.

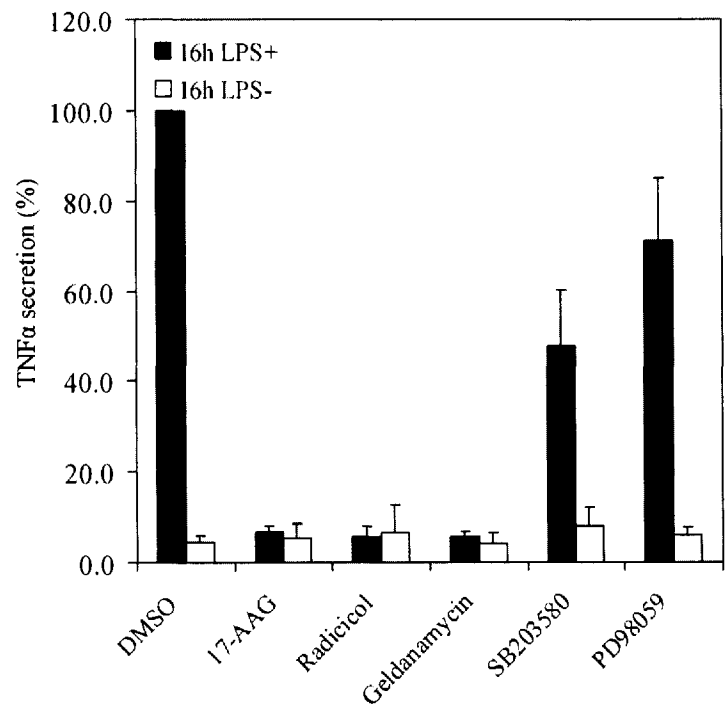
A



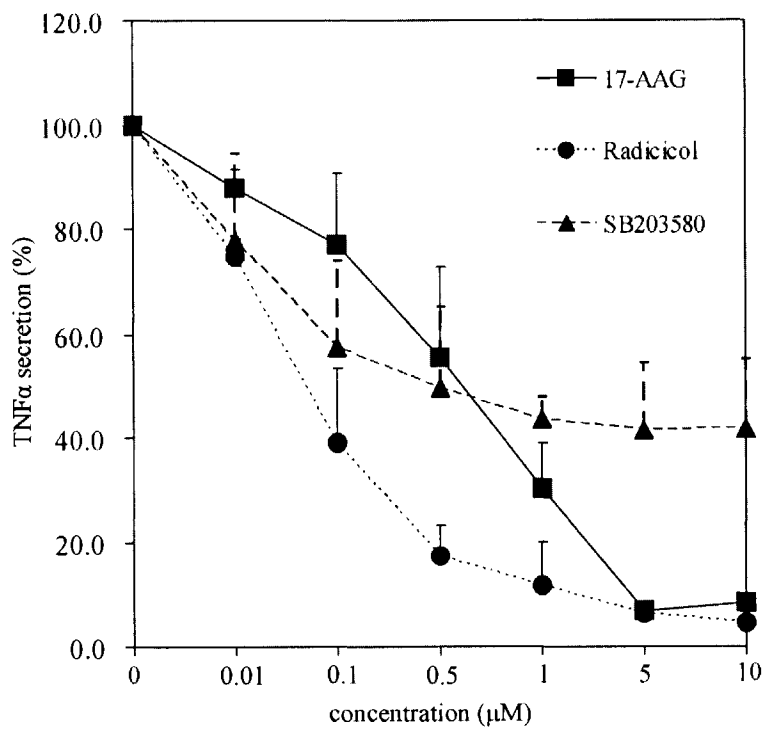
B



C



D



2.4.7 17-AAG downregulates a number of protein kinases in the pro-inflammatory pathway

Although TAK1 has been shown to be a key molecule in the pro-inflammatory pathway, we also examined the requirement of Hsp90 for the stability of other protein kinases which have been reported in the literature to function in this pathway. These include IRAK1 (Cao *et al.* 1996), IRAK2 (Muzio *et al.* 1997), IRAK4 (Li *et al.* 2002; Suzuki *et al.* 2002), IRAK-M (Wesche *et al.* 1999), MEKK1 (Xia *et al.* 2000), MEKK3 (Huang *et al.* 2004), TPL2 (also known as COT) (Stafford *et al.* 2006), RIP1 (Lee *et al.* 2003), RIP2 (also known as RICK or CARDIAK) (McCarthy *et al.* 1998), RIP3 (Yu *et al.* 1999), IKK α (DiDonato *et al.* 1997), IKK β (Mercurio *et al.* 1997; Zandi *et al.* 1997), IKK γ (also known as NEMO) (Yamaoka *et al.* 1998) and IKK ϵ (also known as IKKi) (Shimada *et al.* 1999). We also included p38 α (Raingeaud *et al.* 1995) and the adapter protein MyD88 (Muzio *et al.* 1997) as controls (Figure 2-8B).

All the proteins were transiently expressed in HEK293 cells and treated with 17-AAG or DMSO control for 16 h prior to harvesting. Their expression levels were then determined by immunoblotting analysis. Of the IRAK family members, expression of IRAK1, IRAK2 and IRAKM protein were found to be markedly lower when treated with 17-AAG. Surprisingly, the protein level of IRAK4 was unaffected by treatment with 17-AAG. Differences were found in the expression levels of the RIP family after treatment with 17-AAG. RIP1 and RIP3 were expressed at lower levels but RIP2 was even enhanced marginally. Of the IKK family, protein expression was reduced for IKK α , IKK β and IKK ϵ but not for IKK γ . We also examined

various MAPKKK family members and observed that MEKK1 was unaffected by 17-AAG. However, TPL2 and MEKK3 were sensitive to treatment with 17-AAG and their levels were reduced significantly. As negative controls, 17-AAG did not affect the stability and expression of MyD88 and p38 α . We also examined endogenous IRAK1 and IRAK4 from HEK293 and HeLa cells treated with or without 17-AAG (Figure 2-8A). Levels of IRAK1 was decreased significantly while IRAK4 was unaffected by 17-AAG thereby confirming the results shown in Figure 2-8B and also validating that 17-AAG does not downregulate protein kinase levels non-specifically.

The responsiveness to 17-AAG treatment of all those kinases was further consolidated by assessing physical interaction between them and endogenous Hsp90/Cdc37, as shown in Figure 2-8C. Those kinases were expressed with Myc tag except that IRAK1 and IKK α were tagged with HA as Myc-tagged version could not express well. The expression of each kinase was determined by immunoblotting and normalized amount of lysates were subject to immunoprecipitation with anti-Myc or HA antibodies and presence of endogenous Hsp90 and Cdc37 were detected by immunoblotting against Hsp90 or Cdc37. Similar to what we found previously in lysate treated with 17-AAG, strong binding of Hsp90 was detected on immunoprecipitation of TAK1, IKK ϵ , Tpl2, RIP3 (Figure 2-8C, panel II, lane 13, 14, 15), moderate binding was found on immunoprecipitation of RIP2, IRAK1, IRAK2, IRAKM, IKK α , IKK β , MEKK1 (Figure 2-8C, panel II, lane 2, 4, 5, 7, 10, 11, 16), weak binding was found on immunoprecipitation of RIP1, MEKK3 (Figure 2-8C, panel II, lane 1, 17). As expected, no interaction was detected on IRAK4,

IKK γ , MyD88 and p38 α (Figure 2-8C, panel II, lane 6, 8, 9). Most of these binding results were consistent with previous 17-AAG responsiveness experiment except for MEKK1, RIP2. Cdc37 was found to bind RIP3, IRAK1, IKK α , TAK1 (Figure 2-8C, panel II, lane 3, 4, 10, 14) and weakly interact with Tpl2 (Figure 2-8C, panel II, lane 15), but not to the others. Since MEKK1 construct used in our study just encodes kinase domain of full-length MEKK1, it may account for the discrepancy observed. For RIP2, we are not clear why it was not altered by 17-AAG treatment while it still bound to Hsp90.

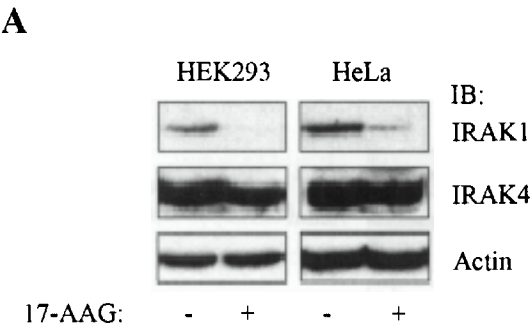
From these results, we can see that the 17-AAG is able to downregulate many kinases involved in pro-inflammatory signaling, which supports our previous results that 17-AAG can abrogate the IL-1, TNF and LPS signaling. It exerted the effect by targeting a number of kinases in the signaling instead of TAK1 alone, although TAK1 is a critical kinase involved, since a specific TAK1 inhibitor, 5Z-7-oxozeaenol, greatly inhibited the TNF α secretion in macrophages induced by LPS in our experiment (data not shown) as well as other inflammatory pathways as reported by others (Ninomiya-Tsuji *et al.* 2003).

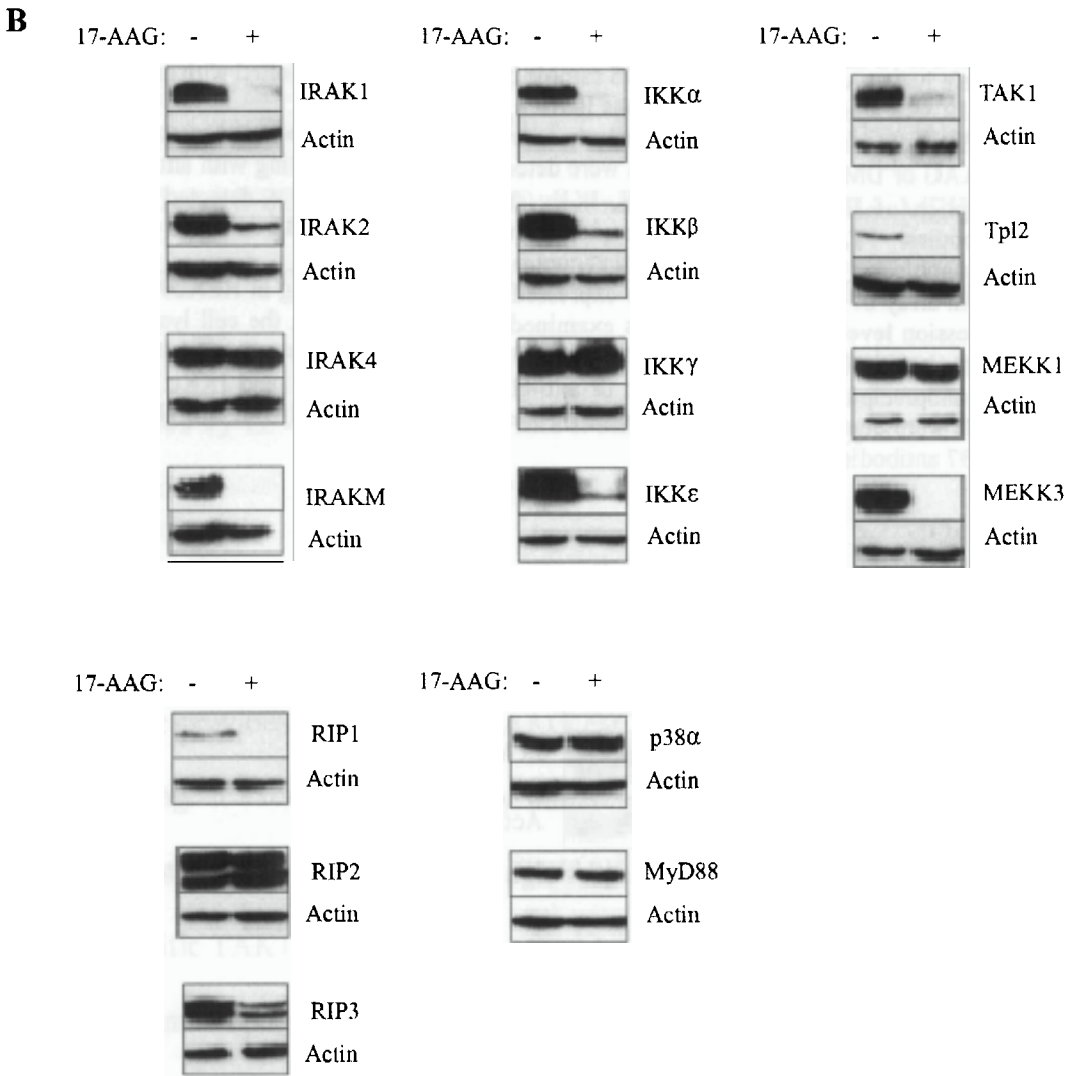
Figure 2-8 Protein kinases in pro-inflammatory pathway are downregulated by 17-AAG

A) HEK293 cells and HeLa cells were treated with 10 μ M 17-AAG or DMSO control and the protein lysates were denatured in SDS sample buffer and immunoblotted with anti-IRAK1 and anti-IRAK4 antibodies for endogenous IRAK1 and IRAK4

B) The indicated proteins were transiently expressed in HEK293 cells and treated with 10 μ M 17-AAG or DMSO. All the proteins were detected by immunoblotting with anti-myc with the exception of IRAK1, IKK α , IKK β , IKK γ and IKK ϵ which were detected with anti-HA antibodies. *TAK1 was co-expressed with TAB1 and TAB2. Protein lysates were immunoblotted for total actin as loading control.

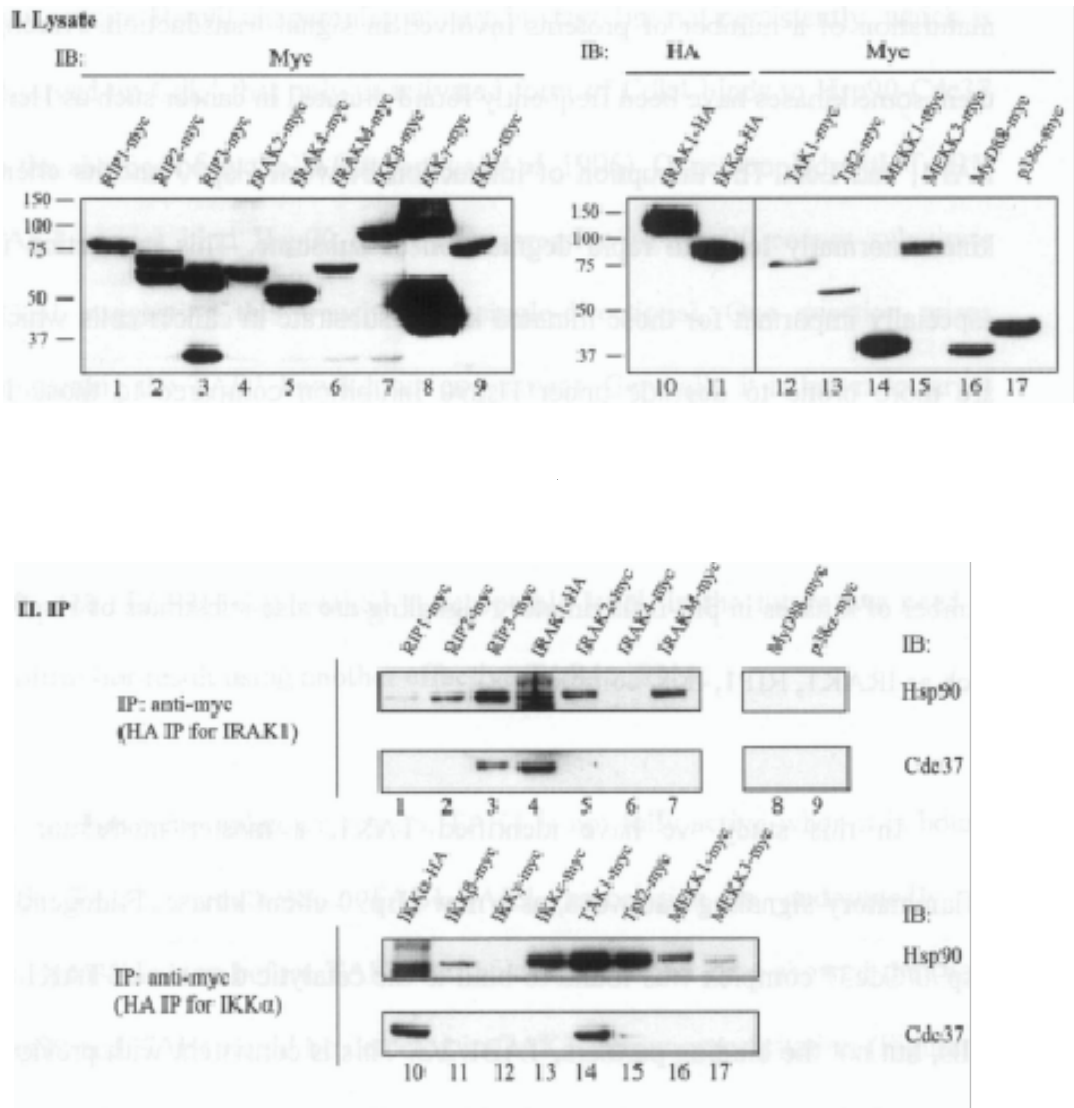
C) An array of protein kinases were expressed with Myc or HA tag in HEK293 cells and the expression level of each kinase was examined by immunoblotting the cell lysates, shown in upper panel (I). Normalized amount of those protein lysates were subjected to immunoprecipitation with anti-Myc or anti-HA (only for IRAK1 and IKK α). The washed beads were denatured with SDS sample buffer and immunoblotted with anti-Hsp90 and anti-Cdc37 antibodies (panel II).





Chapter 2. TAK1-Hsp90 interaction & its implication

C



2.5 Discussion and future work

Hsp90 together with its co-chaperones has been found to bind and assist in the maturation of a number of proteins involved in signal transduction. Among them some kinases have been frequently found mutated in cancer such as Her2, RAF1, and Lck. The disruption of interaction between Hsp90 and its client kinase normally leads to rapid degradation of substrate. This interaction is especially important for those mutated kinase substrate in cancer cells which are more prone to degrade under Hsp90 inhibition compared to those in normal cells. Therefore, Hsp90 inhibitor, geldanamycin-derived 17-AAG, is under phase II clinical trials currently for treatment of cancer. Interestingly, a number of kinases in pro-inflammatory signaling are also substrates of Hsp90, such as IRAK1, RIP1, IKK complex.

In this study we have identified TAK1, a master modulator of inflammatory signaling pathways, as a new Hsp90 client kinase. Endogenous Hsp90/Cdc37 complex was found to bind to the catalytic domain of TAK1 in cells, but not the binding partners, TAB1/2/3. This is consistent with previous finding that Hsp90 and Cdc37 specifically bind to kinase domain of their client kinase to facilitate the correct folding of these kinases.

Interestingly, the binding of Hsp90 to TAK1 was much weaker in the presence of TAB1 over-expression compared to control. This result was further supported by the RNAi of endogenous TAB1 which significantly enhanced TAK1-Hsp90 interaction (Figure 2-3). TAB2 or TAB3 seemed to make no difference. Moreover, TAK1 can bind to Hsp90 in spite of its kinase

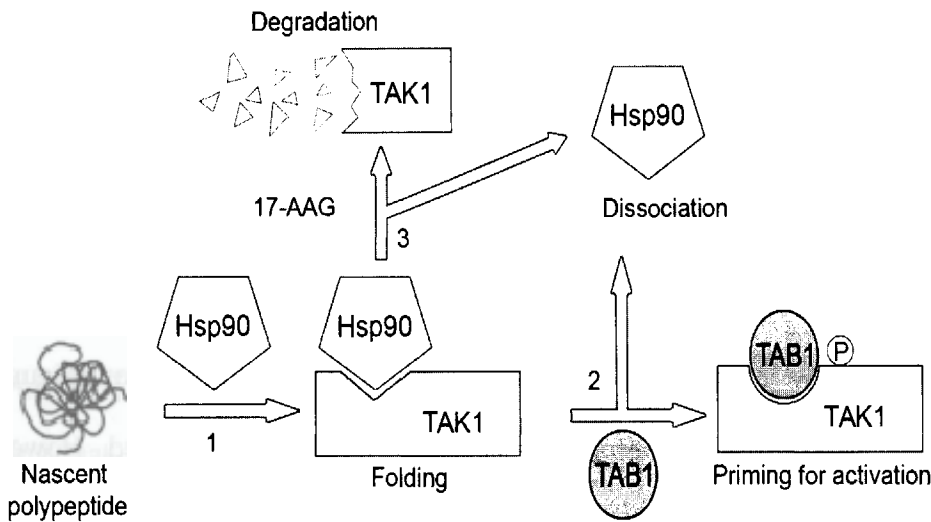
activity or activation states. This binding was reduced by over-expression of TAB1 in all the conditions we tested (Figure 2-4). We then propose that TAK1 may require Hsp90 chaperoning at certain stage but not persistently, which is observed on Cdk4 that only unactivated form of Cdk4 binds to Hsp90-Cdc37 in the absence of cyclin D (Stepanova *et al.* 1996). Once coupled with TAB1, TAK1 cannot bind Hsp90 and over-expression of Hsp90 cannot substitute TAB1 suggesting this transition is single-directional. One question arises concerning the TAB1 knockdown experiment. Generally it is better to target the same protein with at least two different siRNA to eliminate any potential off-target effect. We did try to use three different siRNAs initially, however, only one (TAB1[218]) worked at acceptable level. In the future, we need to confirm our result using another effective TAB1 siRNA.

For some unknown reason, TAK1 is not fully active when it is bound with TAB1; nonetheless, TAB1-TAK1 association is undoubtedly an indispensable step before TAK1 can be activated. Our data showed that both Hsp90 and TAB1 could be detected in TAK1 immunoprecipitation (Figure 2-5), which seemingly contradicts with the model we presented. However, immunoprecipitation as a method cannot distinguish different forms of TAK1, coupled with Hsp90 or with TAB1, it is reasonable that a mixture of TAK1 complexes is captured by immunoprecipitation. In overexpression of TAK1-TAB1-TAB2/3 complex, no Hsp90 was detected in affinity purified proteins with His-tag on TAB1 (data not shown), further supporting our previous finding. Hence we propose a model as shown in Figure 2-9. After TAK1 is synthesized, Hsp90/Cdc37 chaperone complex is involved to assist in the

initial folding of TAK1 nascent poly-peptide. If at this stage, Hsp90-TAK1 complex is disrupted by Hsp90 inhibitor, then TAK1 is subject to degradation possibly through proteasome dependent pathway. To prime TAK1 for activation in signaling, TAB1 forms functional complex with TAK1 by replacing Hsp90 which results in conformational change of TAK1 and phosphorylation at certain sites.

Figure 2-9 Schematic model of Hsp90-TAB1 substitution in complex with TAK1

After synthesis, TAK1 poly-peptide folds into initial conformation with assistance of Hsp90 chaperone machinery which binds to N-terminal kinase domain of TAK1 (1). TAB1 replaces Hsp90 and binds to kinase domain of TAK1 which induces conformational change of TAK1 and primes it for activation under external stimulation (2). 17-AAG treatment results in dissociation of Hsp90 from TAK1 and targets TAK1 to degradation (3).



TAK1 binding domain of TAB1 that has been mapped to the last 36 amino acids on its C-terminus. Surprisingly, it was found to show certain homology with a stretch of sequence on middle segment of Hsp90 that was supposed to bind client protein, although the similarity is not very high. According to the structural model of TAK1 kinase domain and TAB1 C-

terminus (Brown *et al.* 2005), TAB1 C-terminus resided into a pocket on TAK1 kinase domain via extensive lipophilic interactions mediated by several aromatic amino acid residues among with others on TAB1. These corresponding aromatic residues were missing in the segment of Hsp90, as shown in Figure 2-10. It will be interesting to investigate the exact binding domain of TAK1 on Hsp90 and whether those sequence variations account for the weaker binding affinity or TAB1-induced TAK1 conformational change causes dissociation of Hsp90. More work is required to dissect the stoichiometry between TAK1 and Hsp90, although normally Hsp90 forms dimer to bind its client proteins. The detailed mechanism can only be solved by crystallography study of TAK1-Hsp90 complex along with site-directed mutagenesis approach.

Figure 2-10 Sequence alignment of Hsp90 and TAB1 (468-504)

Amino acid sequence of TAB1 (468-504) was aligned against Hsp90 α . The identical residues were highlighted and the critical aromatic residues of TAB1 responsible for TAK1 binding were labeled by asterisks as below.

	363	399
HSP90	CDELIP EW LGFVKG V VDSEDLPLNISREVLQONKILKVI	
TAB1	-HSLPPGEDGRVEPYVDFAEFYRLWSVDHGEQSVVTAP-	
	468	504
		* *

The physiological relevance of Hsp90-TAB1 transition in TAK1 complex is unclear. We can only assume that this is required for TAK1 activation, that is, activated TAK1 does not form complex with Hsp90, but only with TAB1. The immunoprecipitation with TAK1 antibody presented in Figure 2-5 still revealed an assortment of different TAK1 complexes. To clarify whether Hsp90 bind to activated TAK1, a phosphorylation-specific antibody of TAK1 should be used in immunoprecipitation which we will carry

out in near future. Our data clearly show that Hsp90-TAK1 interaction is unaffected upon stimulation with IL-1 or LPS and short-term disruption of Hsp90 binding makes little difference to TAK1 activation in those signaling pathways. Combined with other reports on Hsp90 and kinases, it suggests that Hsp90 is required to maintain correct folding and facilitate maturation of TAK1, supported by our later results.

Although, Hsp90 was previously found to co-purify with TAK1 in a large scale tandem affinity purification study, no further characterization was carried out by the authors (Bouwmeester *et al.* 2004). At the beginning of our study, we were even unaware of this research since we could not find it by literature searching with TAK1 and Hsp90 as keywords. By inhibiting Hsp90 with 17-AAG and radicicol, we found that levels of endogenous TAK1 protein were downregulated significantly. As our data suggest, TAK1 initially associates with Hsp90/Cdc37 after synthesis and later forms functional complex with TAB1 readily for stimulation. One question is raised that 17-AAG targets Hsp90 and downregulates TAK1 level as other Hsp90 kinases, then why TAK1-TAB1 complex which is supposed to be immune to Hsp90 inhibition also drops under the same condition? We hypothesize that TAK1 requires Hsp90 after synthesis to adopt certain conformation before TAB1 can recognize the binding site to form TAK1-TAB1 complex. If Hsp90 inhibited, TAK1 is unable to fold into correct conformation and subject to degradation. The pre-formed TAK1-TAB1 complex is then downregulated by normal protein turnover, which may be accelerated by stress condition caused by Hsp90 inhibition. It has been reported that 17-AAG can induce cell cycle

arrest and apoptosis in multiple cancer cell lines (Georgakis *et al.* 2006; Garcia-Morales *et al.* 2007; Johnson *et al.* 2007). To prove whether TAK1 is susceptible to apoptosis-induced downregulation, more investigation is required.

To address the impact of Hsp90 inhibition in various signaling pathways, we tested IL-1, TNF and LPS stimulation with or without 17-AAG treatment. As expected, those pro-inflammatory pathways were nearly abrogated by Hsp90 inhibition. Moreover, LPS-induced TNF secretion by macrophage, an initial event in infection associated inflammation, was also abolished. These results collectively suggest that 17-AAG and radicicol may serve as anti-inflammation agents to combat those chronic inflammatory diseases. Excitingly, it has been reported that 17-AAG attenuates inflammation in animal model (Poulaki *et al.* 2007), lending support to our data.

To expand our finding on TAK1, we also show that IRAK1, IRAK2, IRAKM, IKK α , IKK β , IKK ϵ , TPL2, MEKK3, RIP1 and RIP3 are downregulated in cells when Hsp90 is inhibited. Those kinases have been implicated to play a role in inflammatory pathways. Previous reports have shown that RIP1 is subject to regulation by Hsp90 and treatment of cells with geldanamycin led to its degradation by the proteasome (Fearn's *et al.* 2006). Surprisingly, RIP2 expression was slightly enhanced by 17-AAG treatment while RIP3 expression was moderately decreased by 17-AAG, although both of them showed binding with Hsp90.

IRAK1 but not IRAK4 has been shown to bind to Hsp90 and the expression of IRAK1 but not IRAK4 was reduced by Hsp90 inhibition which is consistent with our results (De Nardo *et al.* 2005). We show that the two other members of the IRAK family, IRAK2 and IRAKM, both catalytically inactive are sensitive to 17-AAG. However, there is some confusion over whether IKK α and IKK β are downregulated when Hsp90 is inhibited. Our results confirm three previous findings (Vanden Berghe *et al.* 2003; Bouwmeester *et al.* 2004; Broemer *et al.* 2004) but contradicts an earlier report by Chen *et al.*, which shows that both IKK α and IKK β are unresponsive to Hsp90 (Chen *et al.* 2002). As we can find, they used NEMO antibody to co-immunoprecipitate endogenous IKK α and IKK β from HeLa cells as proof to show both of them were not changed upon 17-AAG treatment, while we just examined the over-expressed proteins separately which more likely caused some artifact. However, Broemer *et al.* in a well-designed biochemical study showed that inhibition of Hsp90 led to downregulation of endogenous IKK α/β in Namalwa cells by examining the total lysates with 17-AAG treatment in a time course. A more detailed mechanism was proposed through the pulse chase experiment that they carried out with overexpressed and ³⁵S-labeled Flag-IKK α/β in COS-7 cells. It clearly showed that Hsp90 was required for stabilizing nascent IKK α/β during biosynthesis, while the stability of more mature molecules was not impaired by Hsp90 inhibition to the same extent (Broemer *et al.* 2004). It is possible that IKK complex is stabilized by each other after initial synthesis in endogenous state and therefore it is not sensitive to 17-AAG treatment, as Chen *et al.* had shown in their paper. Also we should

bear on mind that different cell lines and methods used in those studies may contribute to the discrepancy.

In contrast to a previous report (Citri *et al.* 2006), we found that MEKK3 binds with Hsp90 and its stability is affected by 17-AAG exposure. Surprisingly, although MEKK1 can bind Hsp90, the stability is not affected by 17-AAG. As mentioned, the MEKK1 used encodes just kinase domain of mouse MEKK1, it is unclear whether this accounted for the unexpected result. Previously Vasilevskaya and co-workers mentioned that MEKK1 expression was not affected by 17-AAG which is in line with our result (Vasilevskaya and O'Dwyer 1999).

Various knockout animals and cells available further lend support to our results. TAK1^{-/-} (Sato *et al.* 2005), IKK α ^{-/-}, IKK β ^{-/-} (Sizemore *et al.* 2002), MEKK3^{-/-} (Yang *et al.* 2001), RIP1^{-/-} (Kelliher *et al.* 1998) MEFs all showed impaired TNF-induced NF κ B activation. Combined with our results that they were destabilized by 17-AAG, it is possible that 17-AAG abrogates NF κ B activation through downregulating those kinases simultaneously. NEMO^{-/-} MEFs also showed impaired NF κ B activation (Rudolph *et al.* 2000), although in our study it was not sensitive to 17-AAG treatment. This could be explained that NEMO, as scaffolding protein, is indispensable for assembly of functional IKK complex. TPL2^{-/-} macrophages are not sensitive to LPS and showed strongly reduced TNF secretion (Das *et al.* 2005), which may contribute to the effect observed in our ELISA assay (Figure 2-7C). MEKK1^{-/-} MEFs showed normal TNF- and IL-1-induced activation of JNK and NF κ B (Yujiri *et al.*

2000), in line with our result that MEKK1 was not affected by 17-AAG. IRAK1^{-/-} and IRAK4^{-/-} MEFs showed severely impaired TLR/IL-1 signaling, while IRAKM^{-/-} MEFs showed enhanced response to LPS (Janssens and Beyaert 2003). Therefore, our observation of suppressed IL-1 and LPS signaling by 17-AAG is likely an overall effect of downregulation of multiple protein kinases.

Hsp90 is a chaperone protein that is required for the initial folding process following polypeptide chain synthesis and for the refolding of proteins exposed to environmental stress to prevent aggregation. For some kinases Hsp90 is required for maturation but other kinases exhibit persistent binding to Hsp90 throughout their activation/deactivation cycle (Caplan *et al.* 2007). A point that needs addressing is why closely related kinases in particular family groupings show differential sensitivity to Hsp90 inhibition. For example, even though IRAK1 was highly susceptible to degradation when treated with 17-AAG, IRAK4 was unaffected. Similarly, although TAK1, TPL2 and MEKK3 were susceptible to 17-AAG, MEKK1 was unaffected.

In this study we show that three different Hsp90 inhibitor compounds, geldanamycin, its derivative 17-AAG and the structurally different compound radicicol all inhibited TNF α release after stimulation by LPS. This points to the importance of Hsp90 to the pro-inflammatory pathways. Although previous reports have noted that radicicol has anti-inflammatory properties, they have incorrectly suggested that it inhibits directly p38 α (Jeon *et al.* 2000) or protein tyrosine kinases (Chanmugam *et al.* 1995). More recently, others

have shown that geldanamycin abolishes TNF induced activation of JNK and p38 α (Vanden Berghe *et al.* 2003). Geldanamycin also blocks TNF induced IL-6 secretion in L929sA cells (Vanden Berghe *et al.* 2003) and the LPS induced expression of TNF α and IL-1 β mRNA (De Nardo *et al.* 2005). Our results are in line with these studies but contradict that of Chen and coworkers who showed that geldanamycin did not affect TNF activation of JNK (Chen *et al.* 2002). The discrepancy may originate from different methods employed by Chen *et al.* and our group. In their paper, JNK1 was firstly immunoprecipitated and assayed *in vitro* using bacterial recombinant GST-Jun (1-79) as substrate. The amount of immunoprecipitated JNK1 was the same with 17-AAG or DMSO treatment. This result was consistent with other reports that JNK itself is not sensitive to 17-AGG. However, if the input of protein kinase is more than enough in *in vitro* assay, the phosphorylation of substrate is likely to be saturated. Moreover, only JNK1 was examined in their paper. In our study, we directly assayed the phosphorylation of JNK1/2 in endogenous lysate, which was more accurate and error-free. It is highly possible that JNK1/2 levels are unaffected by 17-AAG, but their activation is dramatically suppressed as shown in Figure 2-6, as a number of upstream protein kinases were downregulated by 17-AAG.

As 17-AAG is currently in phase II clinical trials for the treatment of cancer, it may be prudent to take into account the results presented in this study. Elevated NF κ B activity have been detected in some types of cancer (Aggarwal *et al.* 2006) and inhibition of NF κ B signaling by 17-AAG would be good, as NF κ B is frequently found to play a role in regulation of cell

proliferation, control of apoptosis, promotion of angiogenesis, and stimulation of invasion/metastasis in certain types of cancer. However, since many protein kinases in the pro-inflammatory pathway are downregulated by Hsp90 inhibitors leading to loss of signaling, administration of 17-AAG may leave recipients vulnerable to infection which requires extra cautions, similarly to the case that patients taking TNF blockers for rheumatoid arthritis treatment were accompanied with high chance of infection (Dinarello 2004).

On the other hand, 17-AAG and other Hsp90 inhibitors may potentially be a new class of drugs for the treatment of pro-inflammatory diseases. A likely scenario in which a “wholesale” shutdown of the pro-inflammatory pathway is required may be in acute inflammatory disorders such as sepsis where activation of the pro-inflammatory pathway is up-regulated uncontrollably leading to multi-organ failure and finally death. It may even be advantageous to have a co-ordinate decrease in activity of a number of these protein kinases in the pro-inflammatory pathway since it avoids the pitfalls of negative feedback loops that are prevalent in signal transduction. This is certainly the case for p38 α where its inhibition may lead to the enhanced activation of the parallel NF κ B and JNK pathways (Cheung *et al.* 2003). Another protein kinase, IKK α was predicted to be a good drug target for the treatment of inflammatory diseases but a recent study has questioned whether inhibition of IKK α would be beneficial as an IKK α knockout mouse was found to have enhanced NF κ B activation (Greten *et al.* 2007).

Chapter 2. TAK1-Hsp90 interaction & its implication

In summary, this report has identified TAK1 as a client substrate of Hsp90. We show for the first time that the stability of TAK1, RIP3, IKK ϵ , TPL2, IRAK2 and IRAKM requires Hsp90. Furthermore, we provide evidence to support previous reports that inhibition of Hsp90 abrogates IL-1, TNF α and LPS signaling.

Chapter 3.

CHIP-MEDIATED REGULATION OF TAK1

3.1 Introduction

3.1.1 Ubiquitination

Ubiquitin is a highly conserved small protein (76 amino acids) with a compact globular structure which is also seen in other ubiquitin-like proteins and defined as ubiquitin fold. Ubiquitin is quite stable over a wide range of pH and temperature values and it is extremely resistant to trypsin digestion although it has 7 lysine (Lys, or K) residues and 4 arginine (Arg, or R) residues (Pickart and Eddins 2004).

Ubiquitination, by definition, is a biochemical reaction in which the C-terminus of ubiquitin (Gly76) is covalently conjugated to an ϵ -NH₂ group of lysine on target protein, or in rare cases to the N-terminal NH₂ of the protein. Generally, ubiquitination of a protein is carried out by an enzymatic cascade composed of E1 (Ubiquitin activating enzyme), E2 (Ubiquitin conjugating enzyme, Ubc), and E3 (Ubiquitin protein ligase). Ubiquitin is first activated by E1 in the presence of ATP to form a high energy thiol ester bond between ubiquitin C-terminal glycine and E1 active site cysteine (Cys) residue. The activated ubiquitin is then transferred to an active site Cys residue on E2, forming the second thiol ester likewise. Subsequently the ubiquitin-loaded E2, in conjunction with E3, transfers ubiquitin onto a Lys residue of substrate protein to form an isopeptide bond, or a peptide bond in the case that the N-terminal residue of substrate is the target of conjugation. By successively adding activated ubiquitin to one of the internal Lys residues on previous conjugated

ubiquitin molecule, a poly-ubiquitin chain is formed, which is termed as poly-ubiquitination (Hershko and Ciechanover 1998; Pickart 2001). Different patterns of poly-ubiquitin chains have been reported with Lys48-linked and Lys63-linked poly-ubiquitin chains as the most intensely investigated, which means an isopeptide bond is formed between the C-terminus of next ubiquitin and the Lys48 or Lys63 residue of the previous attached ubiquitin. The former type of ubiquitination typically targets the substrate protein to proteasome mediated degradation while the latter type does not lead to degradation of protein *in vivo*. Instead it plays a critical role in a variety of processes including endocytosis of surface receptors, post-replication DNA repair or kinase activation as aforementioned. Other types of poly-ubiquitin chains like Lys6, Lys11, Lys29-linked are also identified, though with unclear functions (Glickman and Ciechanover 2002). In some cases, only one ubiquitin molecule is conjugated to the substrate and termed as mono-ubiquitination which is involved in vesicular trafficking and virus budding (Hicke 2001).

A hierarchical pyramid-shape structure can be found in the ubiquitin system. There is only one E1 in most organisms including humans. The high efficiency of E1 allows the production of sufficient activated ubiquitin for the downstream conjugation reactions, even though the E1 concentration is usually lower than the total E2 concentration (Hershko *et al.* 1983). A number of E2s have been identified. There are eleven E2s in yeast and significantly more E2s in humans, each of which may serve several E3s. All E2s share a conserved core domain of around 150 amino acids, within which an active site Cys residue resides. Of interest, a special dimeric E2, consisting of UbcH13 and Uev1A, can

only catalyze the formation of Lys63-linked poly-ubiquitin chain as defined by their structural configuration which only allows the relatively extended Lys63 residue of the acceptor ubiquitin to reach the active site Cys residue that preforms a thiol ester bond with donor ubiquitin Gly76 (Moraes *et al.* 2001; Pickart 2001; VanDemark *et al.* 2001).

The substrate specificity is determined to a certain extent by E2s and to a larger extent by E3s which comprise a large and diverse family including the HECT (Homologous to E6-AP C-terminus) domain E3s, the RING (Really Interesting New Gene) finger domain E3s and the U-box (Ubiquitin fusion degradation 2 box) domain E3s. Each E3 may interact with several E2s, making the pyramid structure more complicated. HECT domain proteins contain a 350-amino acid residue sequence homologous to the C-terminal domain of prototypical member of the family E6-AP (E6-associated protein). A conserved Cys residue is harbored in this domain to which the activated ubiquitin is transferred from E2 and thus a third thiol ester forms (Wang *et al.* 2006). RING finger E3s have a hallmark Zn finger motif consisting of conserved Cys and His residues that form a cross-brace structure that probably binds two Zn cations. It does not possess chemical catalytic ability but acts as a scaffold to recruit both E2 and substrate thus facilitating the direct transfer of activated ubiquitin from E2 to substrate without the formation of thiol ester intermediate. Most RING domain E3s are single-subunit proteins, while some exist as multiple-subunit proteins when the RING finger and substrate recognition domains are located on different subunits, such as Skp1-Cullin-F box family protein that is responsible for the degradation of I κ B and β -catenin (Deshaies 1999). U-box

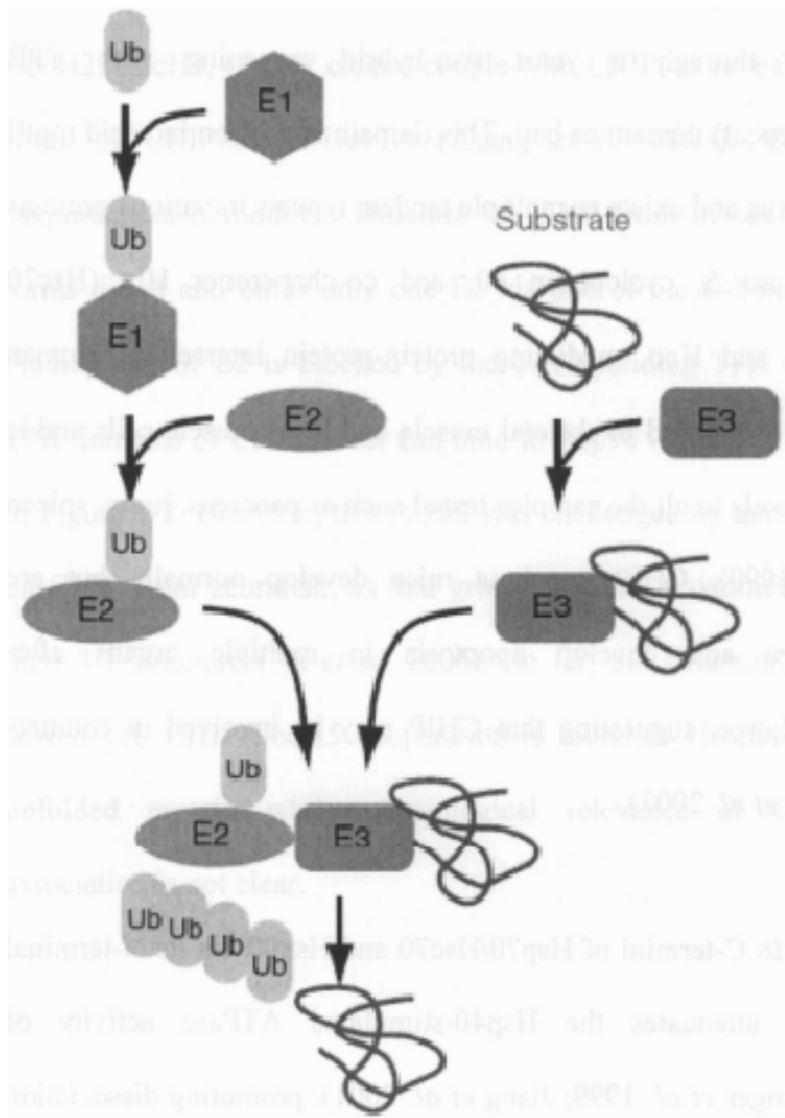
domain is structurally similar to RING finger, though lacking the conserved metal-chelating residues such as Cys and His. It is stabilized by hydrogen bonds and salt bridges instead. The prototype of this family, Ufd2, is found originally to elongate the poly-ubiquitin chain with HECT domain E3 and thus being designated as E4, ubiquitin chain elongation factor, however, the later identified U-box protein can catalyze the chain formation independent of other E3s and actually behaves as an authentic E3 (Aravind and Koonin 2000; Hatakeyama *et al.* 2001; Patterson 2002). There are around 500 E3s in the cell, whose diversity to certain extent explains the complexity of substrate-E3 interaction. However, our knowledge about signals in proteins that mark them for ubiquitination is still limited. So far no consensus sequence has been identified to define the site of ubiquitination although certain rules exist for special protein substrate, for instance, the N-end rule for erroneously transported or compartmented proteins, and destruction box for mitotic cyclin degradation. Interestingly, phosphorylation of substrate and/or ubiquitin enzymes is frequently observed in various processes, triggering different or even opposite effects to the ubiquitination reactions (Glickman and Ciechanover 2002; Hunter 2007).

Canonically, protein conjugated with Lys48-linked poly-ubiquitin chains is recognized by a specialized apparatus: 26S proteasome which is a ~2.5MDa complex made up of two copies each of at least 32 different subunits that are highly conserved among all eukaryotes. The overall structure can be divided into two major subcomplexes: the 20S core particle that possesses proteolytic activity and 19S regulatory particle that recognizes poly-ubiquitin chain and regulates the function of the former. The barrel-shaped 20S core particle is

capped by one or two regulatory particles at either end, forming the functional holoenzyme. The proteasome can degrade the targeted substrate into amino acids while recycling the ubiquitin for later use via its deubiquitinating enzyme activity. It has been found to regulate a variety of important cell proteins besides those unfolded or damaged, such as cyclins and CDKs, NF- κ B and I κ B, p53 and β -catenin, and plays a critical role in development, apoptosis and antigen presentation. Specific inhibitors of the proteasome have proven to be useful research tools, dissecting the structure and function of the proteasome and establishing the involvement of the ubiquitin-proteasome pathway in the degradation of specific proteins (Hershko and Ciechanover 1998).

Figure 3-1 The ubiquitination reaction.

The addition of ubiquitin (Ub) to a substrate occurs in a stepwise manner. Ubiquitin is first attached to a ubiquitin-activating enzyme (E1). Activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2). The substrate is recognized by a ubiquitin ligase (E3), which also recruits the E2-ubiquitin complex. Through a reaction that is not entirely understood, ubiquitin residues are transferred to the substrate. Adapted from Patterson, 2002.



3.1.2 Introduction on CHIP

Here we report that a U-box E3 ligase, CHIP (C-terminus of Hsc70 interacting protein), regulates TAK1 activity via a two-fold mechanism.

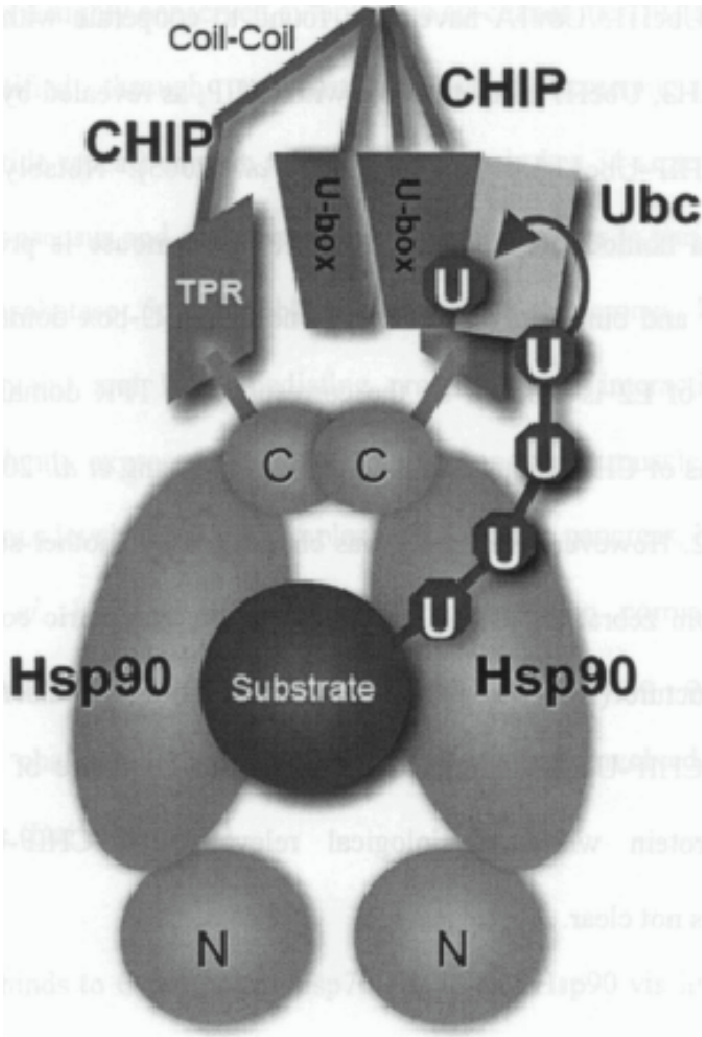
CHIP is a highly conserved cytoplasmic co-chaperone (303 amino acids) initially identified through a yeast two-hybrid screening with TPR (Tetratricopeptide repeat) domain as bait. This domain is a 34-amino acid motif with a loose consensus and exists as multiple tandem repeats in various proteins including phosphatase 5, cyclophilin 40, and co-chaperones Hip (Hsc70 interacting protein) and Hop, mediating protein-protein interaction. Human CHIP is abundantly expressed in skeletal muscle and heart muscle cells and is present at various levels in all the samples tested such as pancreas, brain, spleen (Ballinger *et al.* 1999). CHIP knockout mice develop normally but are temperature-sensitive and develop apoptosis in multiple organs after environmental challenge, suggesting that CHIP may be involved in counter-stress response (Dai *et al.* 2003).

CHIP binds to C-termini of Hsp70/Hsc70 and Hsp90 via its N-terminal TPR domain and attenuates the Hsp40-stimulated ATPase activity of Hsp70/Hsc70 (Ballinger *et al.* 1999; Jiang *et al.* 2001), promoting dissociation of p23 (a co-chaperone of Hsp90) from Hsp90 thus destabilizing the Hsp90 client proteins (Connell *et al.* 2001). Additionally CHIP contains a C-terminal U-box domain connected to TPR domain via an intermediate coil-coil domain which is responsible for dimerization (Nikolay *et al.* 2004). CHIP is known to be a bona fide ubiquitin ligase dependent on the integrity of U-box domain and

becomes a potential link between chaperone and ubiquitin system (Jiang *et al.* 2001; Murata *et al.* 2001; McDonough and Patterson 2003). As an E3, CHIP can couple with more than one E2, based on both biochemical and structural studies. UbcH4/5 family (Hatakeyama *et al.* 2001; Jiang *et al.* 2001; Murata *et al.* 2001) , UbcH13/Uev1A have been found to cooperate with CHIP, while UbcH2, UbcH3, UbcH7 cannot couple with CHIP, as revealed by the structural study of CHIP-Ubc13/Uev1A (Zhang *et al.* 2005). Notably, an unusual asymmetrical homodimer structure of CHIP from mouse is proposed: CHIP forms dimer and binds only one E2 via one of the U-box domains since one binding site of E2 is blocked by the corresponding TPR domain, while both TPR domains of CHIP dimer can bind to Hsp90 (Zhang *et al.* 2005), as shown in Figure 3-2. However, this model was challenged by another structural study on CHIP from zebrafish, as that group reported a symmetric conformation in their 3D structure (Xu *et al.* 2006). So far, the structural discrepancy is not solved yet. CHIP-UbcH5 cooperation is found in clearance of short-lived or unfolded protein while physiological relevance of CHIP-Ubc13/Uev1A association is not clear.

Figure 3-2 Schematic model of CHIP-mediated ubiquitination of an Hsp90 bound client protein

The client substrate bound to Hsp90 dimer is poly-ubiquitinated with addition of poly-ubiquitin chains by a ubiquitin-conjugating enzyme(Ubc) bound to one of the U box domains of CHIP dimer, which functions as a “Y” adaptor coupling a single ubiquitination system to a dimeric chaperone. Adapted from Zhang, Windheim *et al.* 2005.



A delicate regulatory mechanism is established in CHIP-mediated chaperone induction and downregulation: CHIP can activate HSF1 (heat shock factor 1), a special transcription factor for heat shock protein gene expression, and upregulate the level of cellular Hsp70 in response to stress, conferring cells resistance against apoptosis (Dai *et al.* 2003). Sequentially CHIP preferably ubiquitinates the chaperone-bound misfolded proteins and targets them to proteasome degradation. After depletion of those substrates, CHIP then facilitates the turnover of Hsp70 during stress recovery stage (Qian *et al.* 2006). CHIP has since been proposed to play such a critical role in protein quality control that it may decide whether to fold or to degrade its protein substrate under stress conditions, although the detailed mechanism is still obscure (Hohfeld *et al.* 2001; Cyr *et al.* 2002).

In addition to nonselective degradation of unfolded proteins, CHIP shows certain substrate-specific ubiquitination activity. A number of proteins involved in diverse cell functions are reported to be ubiquitinated by CHIP in a chaperone-dependent manner, including Hsp70/Hsc70 (Jiang *et al.* 2001), CFTR (cystic fibrosis transmembrane conductance regulator) (Meacham *et al.* 2001), GR (glucocorticoid receptor) (Connell *et al.* 2001), microtubule-associated protein tau (Petrucelli *et al.* 2004; Shimura *et al.* 2004), nitric-oxide synthase (Peng *et al.* 2004), Epsin (Timsit *et al.* 2005), p53 (Tripathi *et al.* 2007; Muller *et al.* 2008). Interestingly, many Hsp90 client kinases are reported to be degraded by CHIP upon inhibition of Hsp90 with geldanamycin, such as ErbB2 (Xu *et al.* 2002; Zhou *et al.* 2003), NPM-ALK (nucleophosmin-anaplastic lymphoma kinase) (Bonvini *et al.* 2004), DLK (dual leucine zipper-bearing

kinase) (Daviau *et al.* 2006), DAPK (death associated protein kinase) (Zhang *et al.* 2007) . This phenomenon has aroused great interest to investigate the role of CHIP in TAK1 regulation given the hint that CHIP is found to interact with TAB2 in a yeast two-hybrid screening (Cheung, unpublished data).

3.2 Summary

CHIP, a U-box E3 ubiquitin ligase, was found to interact *in vivo* with TAK1, TAB2/3, but not TAB1 by using GST pull-down assay and was detected in immunoprecipitated endogenous TAK1 and TAB3 complex. Through *in vitro* ubiquitination assay, purified TAK1 in complex was ubiquitinated by CHIP and UbcH5b, leading to decrease of TAK1 activity as assessed in kinase assay. This modification was further confirmed with *in vitro* translated TAK1 complex as well as immunoprecipitated endogenous TAK1 complex. On the other hand, CHIP-UbcH13/Uev1A mediated ubiquitination made little difference to TAK1 activity; nonetheless it did cause an obvious band shift of TAB2/3 with yet an unknown function. When overexpressed in HEK293 cells, CHIP was able to significantly downregulate exogenous TAK1 level and slightly reduced the endogenous TAK1 level. Heat shock protein was involved in this downregulation base on two observations: TAK1 level was unchanged in the presence of CHIP [K30A] mutant which could not bind Hsp70/90; and overexpression of Hsp70 could partially reverse the CHIP-mediated decrease of TAK1. Additionally, CHIP was found to interact with TRAF6 and attenuate the binding of TRAF6 to TAB2/3 which is critical for IL-1-TRAF6-TAK1-IKK signaling. This interaction may constitute another layer of regulation on TAK1 activity.

3.3 Materials and methods

Reagents and Chemicals

[$\gamma^{32}\text{P}$]-ATP (150mCi/ml), cold ATP and Glutathione sepharose 4B were from Amersham (GE Healthcare, Little Chalfont, Bucks, U.K.); pure protein of ubiquitin variants (wild type, K48R, K63R, K63 only, >95%) were from Boston Biochem (Boston, MA, U.S.A.). ECL reagent and Protein-G-agarose were from Pierce (Rockford, IL, U.S.A.). Secondary antibody conjugated to horseradish peroxidase was from Biorad (Hercules, CA, U.S.A.). TNT[®] T7 coupled Transcription/Translation reticulocyte lysate system purchased from Promega (Madison, WI, U.S.A.). All other common chemicals and reagents were purchased from Sigma or Merck.

Antibodies

Ubiquitin (P4D1) and TRAF6 (D-10) were from Santa Cruz (Santa Cruz, CA, U.S.A.); GST, TAB2, HA, Flag, Myc, His, TAK1, TAB1, TAB3 antibodies have been described previously in chapter 2.

DNA plasmids

pEBG2T-TAK1, pEBG2T-TAB1, pEBG2T-TAB2, pEBG2T-TAB3, pEBG2T-TAK1 (18-370), pEBG2T-TAK1 (345-579), pEBG2T-TAB2 (1-400), pEBG2T-TAB2 (401-693), pEBG2T-TAB3 (1-429), pEBG2T-TAB3 (430-712), pEBG2T-CHIP, pEBG2T-TRAF6 (1-291)-gyrase, pEBG2T empty vector, pCMV5-CHIP [wt/H260Q/K30A]-FLAG, pEF6-Hsp90 α -HA, pEF6-Hsp70-HA, pCMV5-HOP-myc, pCMV5-XIAP-myc, pCMV5-TRAF6-FLAG, pCMV5-

TAK1-myc, pCMV5-TAK1 [K63W]-myc, pEF6-TAB2-HA, pEF6-TAB3-HA, pCMV5-TAB1-His, pCDNA3-TAK1, pCDNA3-TAB1 were used for mammalian cell expression. GST, HA, myc and FLAG epitope tags are located at the N-terminus of all the proteins. pET21-UBE1, pET21-UbcH13, pET21-Uev1A with C-terminal His-tag, pET16b-UbcH5b with N-terminal His-tag, pGEX6P1-CHIP were used for *E.coli* (BL21) expression system. All constructs used were human cDNA and code for the full length protein unless indicated.

Tissue Culture and Transfection

The same as described in the previous chapter.

Protein expression and purification in bacterial expression system

Full-length human proteins: E1, UbcH13, Uev1A, UbcH5b were cloned into pET16b or pET21 vectors (Novagen) for His-tag and CHIP were inserted into pGEX6P-1 vector (Amersham) for GST fusion protein expression. After confirmation by sequencing, those plasmids were transformed into BL21 *E.coli*, grown to A_{600 nm} of 0.6 and induced for 1 h at 37°C with 200 mM IPTG (isopropyl-β-D-thiogalactopyranoside). The cells from 1 litre culture were harvested by centrifugation and resuspended in extraction buffer [50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 150 mM NaCl, 0.03% (w/v) Brij-35, 5% (v/v) glycerol, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM PMSF (phenylmethylsulfonyl fluoride), 1 mM benzamidine and 0.5% (v/v) Triton X-100]. The cells were freeze-thawed once and sonicated for 2 min on ice. Triton X-100 was added to a final concentration of 1% (v/v) and the suspension left on ice for 30 min. The cell debris was cleared by centrifugation for 30 min at 75 000 g and the

supernatant (termed lysate) decanted. The lysate (30 ml) was incubated for 1 h at 4°C with 3 ml of a 50% slurry of nickel-agarose (Qiagen) if recombinant protein is tagged with 10xHis, or Glutathione sepharose 4B (GE healthcare) if recombinant protein is tagged with GST (glutathione S-transferase). The pellet was washed twice with 50 mM Tris-HCl pH 7.5, 0.27 M sucrose, 0.5 M NaCl, 0.1% (v/v) 2-mercaptoethanol, followed by two washes with 50 mM Tris-HCl pH 7.5, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol. The bound His-tag protein was eluted in 0.2 M imidazole in 50 mM Tris-HCl pH 7.5, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol, and GST-tag proteins were eluted with 20 mM reduced glutathione in the same solution. Imidazole or glutathione was removed by dialysis in 50 mM Tris pH 7.5, 10 mM NaCl, 0.27 M sucrose, 0.1% (v/v) 2-mercaptoethanol. For GST-CHIP, GST tag was cleaved off with PreScission[®] protease (GE healthcare) according to manufacturer's instruction. The mixture of GST, CHIP and low amount of protease was loaded onto Mono-Q ion exchange column in ÄKTA[®] FPLC system (GE healthcare) and eluted with a gradient of Buffer B [500 mM NaCl in 50 mM Tris-HCl pH 7.5, 0.27M sucrose, 0.1%(v/v) 2-mercaptoethanol] as named in FPLC system, ranging from 0% to 100%. The fractions containing pure CHIP were pooled and concentrated.

***In vitro* ubiquitination assay**

Ubiquitination was carried out with 1 µM bacterially expressed His-E1, 2.5 µM E2, 5 µM E3, 50 µM Ub (WT or K48R, K63R mutant) with 2 mM ATP, 5 mM MgCl₂ in the buffer of 50 mM Tris-HCl (pH 7.5), 0.27 M sucrose, 0.1% (v-v) 2-mercaptoethanol, incubated at 30°C for 1 h with shaking. Reactions were

stopped by addition of sample buffer. Samples were resolved on SDS-PAGE and analyzed by immunoblotting with appropriate antibodies.

***In vitro* Transcription & Translation**

In vitro translation was carried out with TNT[®] T7 coupled Transcription/Translation reticulocyte lysate system purchased from Promega. pCDNA3-TAK1, pCDNA3-TAB1, pEF6-TAB2-HA and pEF6-TAB3-HA obtained from mini-prep were used as templates in different combinations. In a 25 µl-volume reaction, 9 µl of template was mixed with other components according to manufacturer's instruction. ³⁵S-methionine (1 µl in 25 µl, stock: 1000 Ci/mM at 10 mCi/ml) was used to label translated protein. After 90 min incubation at 30°C, the reaction product was stored at -20°C before it was used.

***In vitro* TAK1 kinase activity assay**

The TAK1 complex (TAB1-His, TAB2/3-HA, TAK1-myc) purified from HEK293 cells was assayed by its ability to activate MKK6. TAK1 complex (0.2-1 µg was incubated with various ubiquitination reactions (10 µl volume, 1 µM E1, 2.5 µM E2, 5 µM E3, 2 mM ATP, 5 mM MgCl₂) at 30°C for 1hr. Then 1 µl of each reaction was removed and incubated at 30°C with 24 µl of 50 mM Tris-HCl (pH7.5), 0.1%(v/v) 2-mercaptoethanol, 4 µg of unactivated MBP-MKK6, 10 mM MgCl₂, 0.1 mM [γ -³²P] ATP (3x10⁶c.p.m./nmol). After 30 min, the reaction was terminated by the addition of 6 µl sample buffer and resolved on SDS-PAGE. Gel was stained and dried overnight at room temperature and then subjected to autoradiography for 3 h or overnight at -80°C depending on the intensity of signal.

GST pull-down and immunoprecipitation

The protocol used was as described in the previous chapter.

Immunoblotting

Immunoblotting was carried out with standard protocol. The results shown in this thesis were the representatives of at least two times of independent experiments.

3.4 Results

3.4.1 CHIP can bind to TAK1, TAB2 and TAB3, but not TAB1

To confirm the yeast two-hybrid screening preliminary result and examine the interaction between CHIP and TAB2 or TAB3 in mammalian cells, we carried out co-transfection experiment followed by GST pull-down assay. Since TAB2 or TAB3 is found to associate with TAK1 and TAB1 to form a complex, to discern which protein interacts with CHIP directly, different combinations of CHIP-TAK1 complex were tested at the same time. As shown in Figure 3-3A, Flag-tagged CHIP was found to strongly interact with full length TAK1 (Figure 3-3A, lane 2). Both N- and C-terminal fragments of TAK1 (Figure 3-3A, lane 3 & 4) showed strong binding of CHIP, and N-terminus seemed to have higher affinity than C-terminus, taking into account of the relatively lower level of N-terminus compared to C-terminus of TAK1. No binding of CHIP was detected with full length TAB1 (Figure 3-3A, lane 5), or the control GST alone (Figure 3-3A, lane 1). Weak interaction was detected between full length TAB2 or 3 and CHIP, although this may be due to low expression levels of GST-TAB2/3.

However, N-termini of TAB2 and TAB3 were stabilized by CHIP and showed higher binding of CHIP consequently.

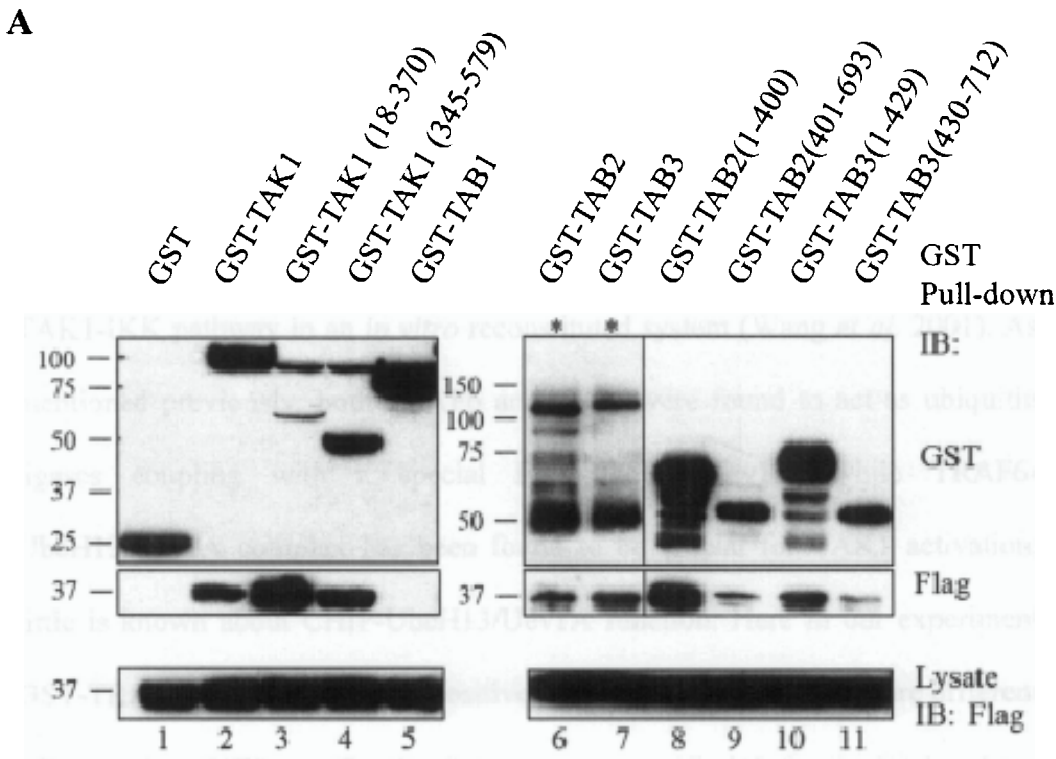
To confirm this interaction with another method, Flag-CHIP was expressed in HEK293 cells and binding to endogenous TAK1 or TAB3 was examined. Here we included heat shock stimulation to see if there is any difference, given that CHIP was a co-chaperone involved in heat shock response. As shown in Figure 3-3B, overexpressed Flag-CHIP was found in immunoprecipitation of TAK1 and TAB3. Heat-shock treatment made little difference to the interaction. Since TAB1 always couples with TAK1 in the cells as revealed by co-immunoprecipitation experiments (Cheung *et al.* 2004) and TAK1 may serve as bridging molecule to link TAB1 and CHIP, we cannot show that TAB1 does not interact with CHIP by immunoprecipitating endogenous TAB1. Without a specific and sensitive antibody against endogenous CHIP, it is difficult to detect the interaction between CHIP and TAK1 under endogenous condition. Therefore our results show that the interactions between CHIP and TAK1 complex are merely well-established with over-expression system, and more work is required to investigate them in endogenous conditions.

Figure 3-3 CHIP binds to TAK1, TAB2, TAB3 but not TAB1.

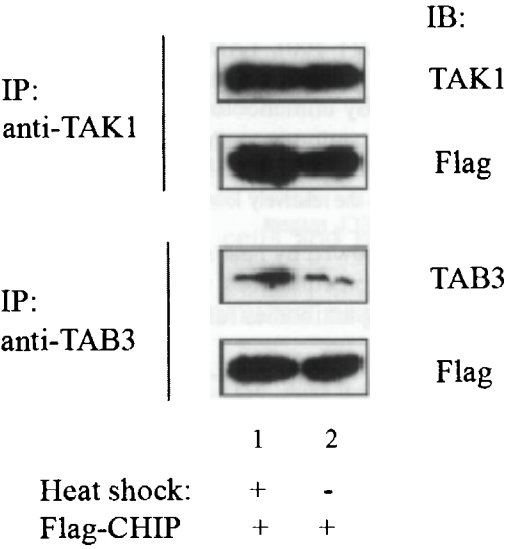
A) GST fusion protein encoding plasmids, empty vector pEBG2T or pEBG2T-TAK1, TAB1, TAB2, TAB3, TAK1(18-370), TAK1(345-579), TAB2(1-400), TAB2(401-629), TAB3(1-429), TAB3(430-702) were transfected into HEK293 cells together with pCMV5-CHIP-Flag. 1mg total lysates were subject to GST pull-down followed by immunoblotting against GST and Flag antibodies. 40 µg lysates were also analysed with Flag antibody to show the expression of Flag-CHIP with all GST-tag proteins.

* Lane 6 and 7 were processed with ECL Advance® reagent due to the relatively lower expression levels of full length GST-TAB2/3 while the rest part of blots was analyzed with normal ECL reagent.

B) HEK293 cells were transfected with Flag-CHIP followed by heat shock treatment at 42°C for 2h or left untreated. Anti-TAK1 and TAB3 immunoprecipitations using the same amount of lysate were immunoblotted with TAK1/TAB3 and Flag antibodies respectively.



B



3.4.2. Establishment of *in vitro* ubiquitination assay

After the confirmation of CHIP-TAK1 complex interaction, next we went on to examine the significance of these interactions in TAK1 mediated pathways. Given the known function of CHIP as a ubiquitin ligase and the established role of TRAF6-mediated ubiquitination in TAK1 activation, we aimed to find out whether the ligase activity of CHIP would likewise affect TAK1 function.

Firstly we sought to establish an *in vitro* ubiquitination system with purified protein E1-His, His-UbcH5b, UbcH13-His, Uev1A-His, CHIP and a chimera protein GST-TRAF6-Gyrase which was shown to successfully activate TAK1-IKK pathway in an *in vitro* reconstituted system (Wang *et al.* 2001). As mentioned previously, both TRAF6 and CHIP were found to act as ubiquitin ligases coupling with a special E2, UbcH13/Uev1A, while TRAF6-UbcH13/Uev1A complex has been found to be crucial for TAK1 activation, little is known about CHIP-UbcH13/Uev1A function. Here in our experiment GST-TRAF6-gyrase serves as a positive control and we could compare different effects using CHIP or TRAF6 if there was any. UbcH5 family E2 has been reported to cooperate with CHIP in ubiquitination (Jiang *et al.* 2001) and it is obviously a different E2 compared to UbcH13/Uev1A. Hence we chose UbcH5b to check whether any difference could be found between UbcH13/Uev1A and UbcH5 in TAK1 regulation.

Recombinant proteins E1-His, UbcH13-His, Uev1A-His, His-UbcH5b, GST-CHIP were expressed and purified from *E.coli* using pET21, pET16b or pGEX6P1 vectors followed by affinity purification. The result shown in Figure

3-4A was optimized after several attempts using different vectors, and different induction conditions for all the proteins of interest. As shown in Figure 3-4A, near homogenous proteins were obtained after affinity purification. For GST-CHIP, PreScission protease was applied to cleave the fusion protein and the GST tag removed by anion exchange chromatography. The fractions containing CHIP were pooled and concentrated (Figure 3-4A, lower panel). GST-TRAF6-*gyrase* were expressed in HEK293 cells and purified through affinity purification using glutathione sepharose 4B, since it was not stably expressed in bacteria.

With those purified proteins and purchased ubiquitin variants, we then set up an *in vitro* ubiquitination system. Different concentrations of E1, E2, E3, Ub and MgCl_2 -ATP were tested and optimized as described in methods. As shown in Figure 3-4B, when UbcH13/Uev1A were used as E2, judged by the hallmark-like smear in anti-ubiquitin IB ranging from low to high molecular weight, poly-ubiquitin chain was formed to a substantial extent using wildtype ubiquitin and K48R ubiquitin, but not K63R ubiquitin (Figure 3-4B, lane 1-3). This result suggested that UbcH13 and Uev1A can catalyse K63-linked poly-ubiquitin chain formation without any E3, as reported earlier by other researchers (Hofmann and Pickart 2001), and our preparation of these enzymes was successful. When CHIP was included in the reactions, massive increase of poly-ubiquitin smear was observed indicating that purified CHIP was active and significantly enhanced the reactivity of ubiquitination (Figure 3-4B, lane 4-6). However, there was still no chain formed using K63R ubiquitin mutant, in line with the report that the specificity of K63-lined chain formation was

determined by the unique structure of UbcH13 and Uev1A (VanDemark *et al.* 2001). When UbcH5b was used as E2, we barely detected any poly-ubiquitin chain formation in the absence of E3 (Figure 3-4B, lane 7-9), but a drastic enhancement of chain formation was observed with input of CHIP as E3 (Figure 3-4B, lane 10-12), consistent with other research. Interestingly, reaction with K63R ubiquitin mutant showed slightly weaker smear compared to the one with K48R mutant (Figure 3-4B, lane 11 & 12). This result showed that CHIP was active as an E3 and could cooperate with both UbcH13/Uev1A and UbcH5b *in vitro*.

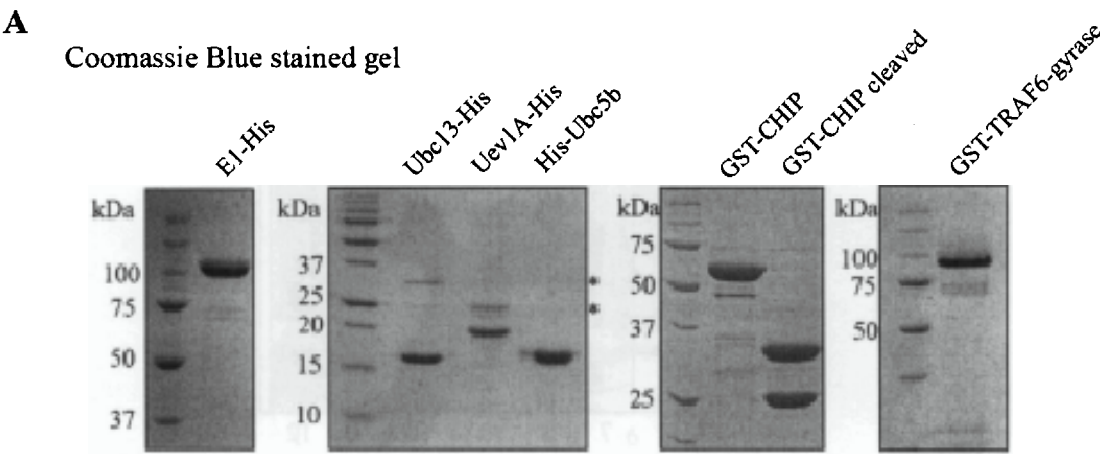
We then examined another E3, TRAF6 in the same system. As indicated by the previous research, the swap of C-terminal TRAF domain with N-terminal domain of gyrase B does not affect the TRAF6 ubiquitin ligase activity which requires the N-terminal RING domain. This chimera protein is supposed to undergo coumermycin A-induced polymerization which is important for the activation of TAK1 (Wang *et al.* 2001). After purification of GST-TRAF6-gyrase from HEK293 cells, we examined its function as E3 ligase *in vitro*. As shown in Figure 3-4C, GST-TRAF6-gyrase was able to enhance the reactivity of UbcH13/Uev1A and UbcH5b in poly-ubiquitin chain formation (Figure 3-4C, anti-Ub IB, lane 1-6), with greater enhancement observed in reactions with UbcH5b as E2. When the reactions were probed with anti-TRAF6 antibody, stronger smear was found on UbcH5b-used lanes compared with UbcH13/Uev1A used ones, but not on the lanes with only TRAF6 (Figure 3-4C, anti-TRAF6 IB, lane 3-8), which suggested strong auto-ubiquitination of TRAF6 occurred with UbcH5b as E2. Auto-ubiquitination of TRAF6 in conjugation of

UbcH13/Uev1A has been found to be essential for NF- κ B signaling (Lamothe *et al.* 2007); however, TRAF6-UbcH5b ubiquitination and the modification of TRAF6 was not reported until recently when it was mentioned in an *in vitro* study of TRAF6 and CHIP (Windheim *et al.* 2008). Currently the physiological significance is still unclear.

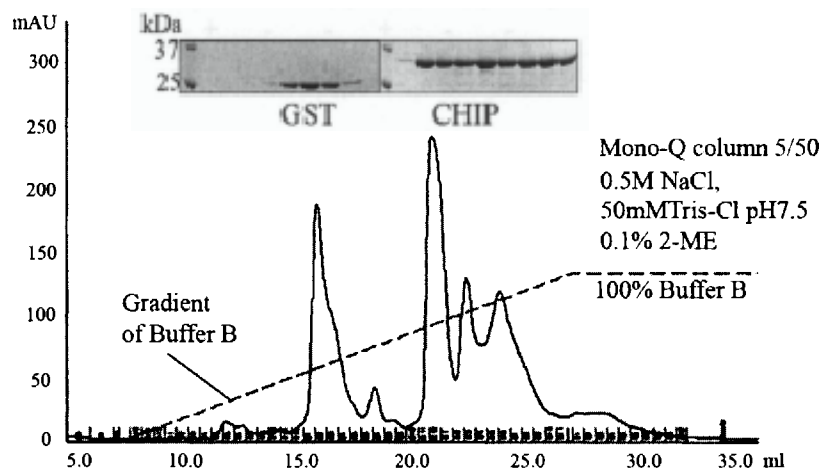
Figure 3-4 Establishment of ubiquitination system *in vitro*.

A) E1, UbcH13, UbcH5b, Uev1A were purified from *E.coli* using pET vectors and subsequent Ni-NTA affinity purification. 4 µg eluates were analysed on SDS-PAGE as shown herein. CHIP was cloned into pGEX6P1 vector and purified from *E.coli* using Glutathione sepharose 4B. Eluated protein was cleaved by PreScission® protease and subject to anion exchange chromatography. The fractions containing CHIP were pooled and concentrated. Purified GST-CHIP, cleaved GST-CHIP as well as fractions containing GST or CHIP were analysed on SDS-PAGE. GST-TRAF6-gyrase was purified from HEK293 cells via transfection and affinity purification. Eluates were analysed on SDS-PAGE.

B, C) Ubiquitination was carried out in 10 µl total volume with 1 µM E1, 2.5 µM E2, 5 µM CHIP, 1 µg or 2 µg GST-TRAF6-gyrase, 50 µM Ub (wt, K48R or K63R) in different combinations. Reaction mixtures were incubated at 30°C for 1 h with 2 mM ATP, 5 mM MgCl₂. Half of the reaction mixture was subject to immunoblotting with antibody against Ub or TRAF6.



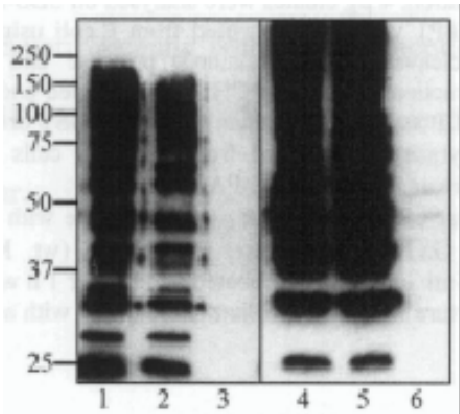
Anion Exchange Chromatography



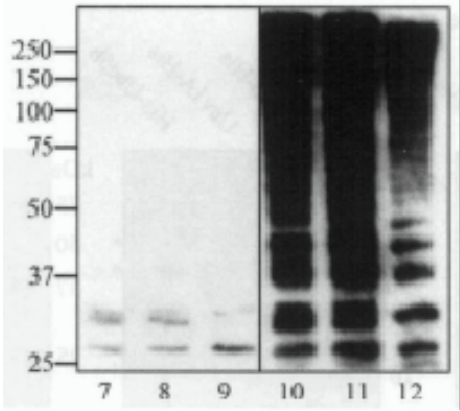
B

IB: Ub

I. Ubc13-Uev1A as E2



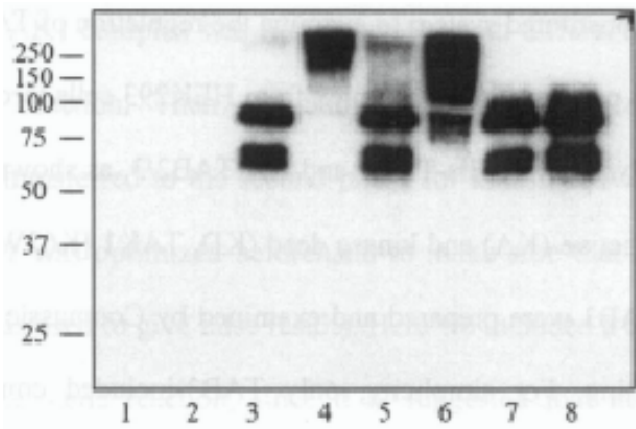
II. Ubc5b as E2



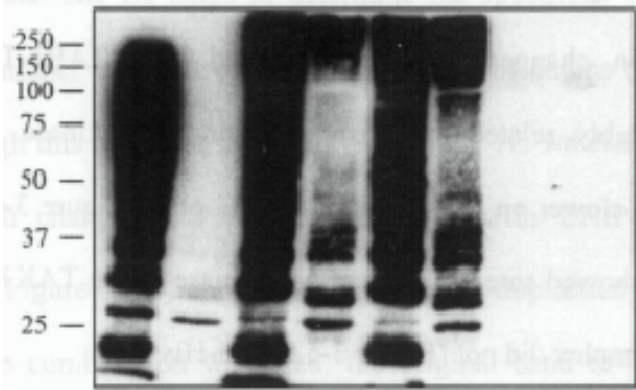
E1+E2+Mg-ATP:	+	+	+	+	+	+
CHIP:	-	-	-	+	+	+
Ub(wt):	+	-	-	+	-	-
Ub(K48R):	-	+	-	-	+	-
Ub (K63R):	-	-	+	-	-	+

C

IB:
TRAF6



Ub



	1	2	3	4	5	6	7	8
E1+Ub+Mg-ATP	+	+	+	+	+	+	+	+
UbcH13/Uev1A	+	-	+	-	+	-	-	-
UbcH5b	-	+	-	+	-	+	-	-
GST-TRAF6-ubiquitinase	-	-	1μg	1μg	2μg	2μg	1μg	2μg

3.4.3. Effect of CHIP mediated ubiquitination on TAK1 complex *in vitro*

To establish a reconstituted system to examine the regulation of TAK1 by CHIP, we expressed and purified TAK1 complex from HEK293 cells through transient transfection of Myc-TAK1, His-TAB1 and HA-TAB2/3, as shown in Figure 3-5A. Both kinase active (KA) and kinase dead (KD, TAK1 [K63W]) complexes with TAB2 or TAB3 were prepared and examined by Coomassie blue staining and immunoblotting. For simplicity, only TAB3-included complexes were shown here with gel staining. From the result we could see that TAK1 underwent certain changes when co-expressed with TAB1-TAB2/3. This change was possibly related to phosphorylation since kinase active TAK1 migrated slightly slower on gel than kinase dead ones (Figure 3-5A, anti-Myc IB). TAB1 also showed smear-like band with kinase active TAK1 while TAB1 in kinase dead complex did not (Figure 3-5A, anti-His IB).

Next we then examine what would happen to TAK1 when incubated with CHIP-mediated ubiquitination. Shown in Figure 3-5B, a strong long-ranged smear of TAK1 was found when it was incubated with CHIP-UbcH5b reaction, but not CHIP-UbcH13/Uev1A reaction. Similar pattern was seen for TAB2/3 (data not shown). This modification of TAK1 was dependent on a special ubiquitination reaction because no smear was found if there was no ubiquitin input (Figure 3-5B, lane 4), or no E1, E2, CHIP, Mg-ATP input (data not shown).

To address if TAK1 activity was changed due to CHIP-UbcH5b mediated ubiquitination of TAK1, a kinase assay coupled to ubiquitination

reaction was carried out with unactivated MBP-MKK6 as substrate. In this two-phase assay, TAK1 complex was first incubated with different combinations of ubiquitination reaction. Then one-tenth of the reaction containing TAK1 complex was transferred to the second phase for kinase activity measurement. Input of TAK1 was optimized beforehand to make sure that not too much or little kinase was used to give false results. Here we included a combination with two E2s in the same reaction, since it is suggested that multiple E2s may cooperate so that one E2 helps to determine the specificity of poly-ubiquitin chain while another one is involved in elongation of chains (Windheim *et al.* 2008), although this model is not widely accepted. As we can see in Figure 3-5C, not much change of TAK1 can be seen with CHIP-UbcH13/Uev1A combination (Figure 3-5C, anti-TAK1 IB, lane 2, duplicated here), but when CHIP-UbcH5b combination was used, the original band of TAK1 at around 75kDa totally disappeared and a long-ranged smear was found above 75 kDa till more than 250 kDa (Figure 3-5C, anti-TAK1 IB, lane 3). Similar pattern was found on reaction of CHIP with two E2s, however, some TAK1 remained at 75 kDa (Figure 3-5C, anti-TAK1 IB, lane 4). From autoradiograph we find that TAK1 complex was quite active on its own as assayed with MBP-MKK6 (Figure 3-5C, autoradiograph, lane 1). Although TAK1 and MBP-MKK6 share the similar molecular weight around 75 kDa, the signal at 75 kDa can only be that of MBP-MKK6, as the autophosphorylation of TAK1 is undetectable in this condition due to the trace amount input of TAK1 complex. CHIP-UbcH13/Uev1A combination can barely enhance or suppress its activity. In the presence of CHIP-UbcH5b, strikingly, TAK1 showed drastically lower activity, although CHIP-two E2s also caused a substantial decrease in activity, to less

extent. The reaction without E2 input caused slight decrease of TAK1 activity, suggesting that possibly there was some endogenous E2s in TAK1 complex which had been co-purified from HEK293 cells. This observation indicated that the ubiquitination of TAK1 may serve as a down-regulatory mechanism, and further investigation *in vivo* is required.

As mentioned previously, CHIP-UbcH13/Uev1A mediated ubiquitination seemed to make little difference to TAK1, both in immunoblotting analysis and *in vitro* kinase assay. Nonetheless we noticed that something occurred to its binding partner TAB2/3. As shown in Figure 3-5D, when TAK1 complex (TAK1-TAB1-TAB2 used here) was incubated with CHIP-UbcH13/Uev1A in the presence of K63 only ubiquitin mutant, TAB2 in kinase active TAK1 complex showed an obvious band shift (Figure 3-5D, lane 5) which was not seen with K63R ubiquitin (Figure 3-5D, lane 7) or kinase dead TAK1 complex (Figure 3-5D, lane 6, 8). In the absence of CHIP, a negligible shift of TAB2 was observed with kinase active TAK1 complex compared with kinase dead one (Figure 3-5D, lane 3, 4). Therefore, TAB1 band shift, possibly caused by phosphorylation, requires TAK1 activity and CHIP-UbcH13/Uev1A-mediated ubiquitination which can only utilize K63 of ubiquitin to form poly-ubiquitin chains. When CHIP was substituted by GST-TRAF6-gyrase, similar pattern of band shift could be reproduced while the extent of band shift was less than that caused by CHIP (data not shown), suggesting that K63-linked poly-ubiquitination mediated by different E3s may play different roles in the regulation of TAK1 complex via TAB2. This TAB2 band shift *in vitro* does not seem to make much difference to TAK1 activity as assessed by kinase assay;

however, this may serve as a regulatory mechanism *in vivo*, which entails further studies.

Figure 3-5 Effect of CHIP ubiquitination on TAK1 complex *in vitro*.

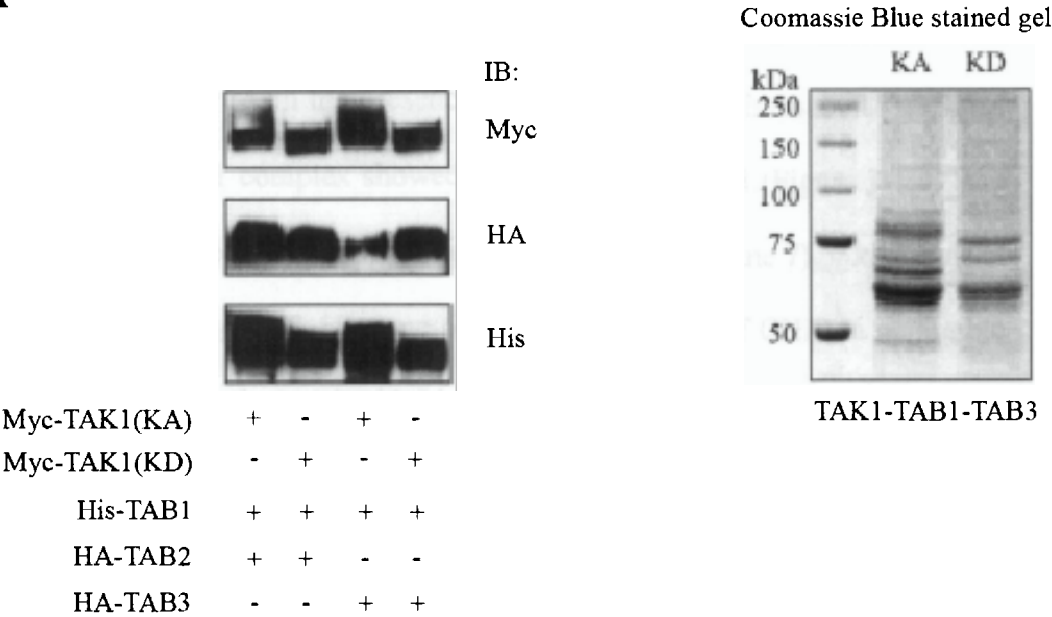
A) TAK1 complex was purified from HEK293 cells via transfection and affinity purification. Eluates were analysed on SDS-PAGE as well as subject to immunoblotting with anti-Myc, HA, and His antibodies respectively.

B) Ubiquitination assay was carried out in 10 µl total volume with 1 µM E1, 2.5 µM UbcH13/Uev1A or UbcH5b, 5 µM CHIP, 50 µM Ub (wt), 2 mM ATP and 5 mM MgCl₂ in different combinations with 1µg TAK1 complex (TAK1-TAB1-TAB2) at 30°C for 1 h. Then reaction mixtures were subject to immunoblotting with anti-Myc antibody.

C) 0.2 µg TAK1 complex (TAK1-TAB1-TAB2) were incubated with 1 µM E1, 2.5 µM UbcH13/Uev1A or UbcH5b, 5 µM CHIP, 50 µM Ub (wt), 2 mM ATP and 5 mM MgCl₂ in different combinations at 30°C for 1h. Half of the reactions were subject to immunoblotting with anti-TAK1 antibody. Meanwhile 1/10 reaction were removed to mix with 4 µg MBP-MKK6 and incubate with 1x10⁶ c.p.m/nmol [γ-³²P]-ATP (0.1 mM) at 30°C for 30 min. Reactions were stopped by adding SDS sample buffer and subject to SDS-PAGE. Gels were stained with Coomassie Blue and dried before autoradiography at -80°C for overnight.

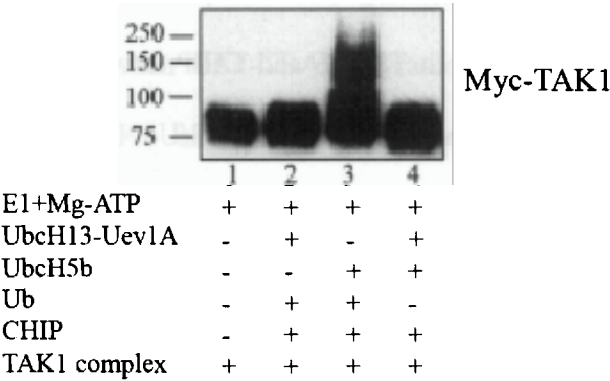
D) 1.5 µg TAK1 complex (KA for kinase active, KD for kinase dead) was incubated with 1 µM E1, 2.5 µM UbcH13/Uev1A, 5 µM CHIP, 50 µM Ub(K63 only, K63R), 2 mM ATP and 5 mM MgCl₂ in different combinations at 30°C for 1 h. Reactions were subject to immunoblotting with anti-TAB2 antibody.

A



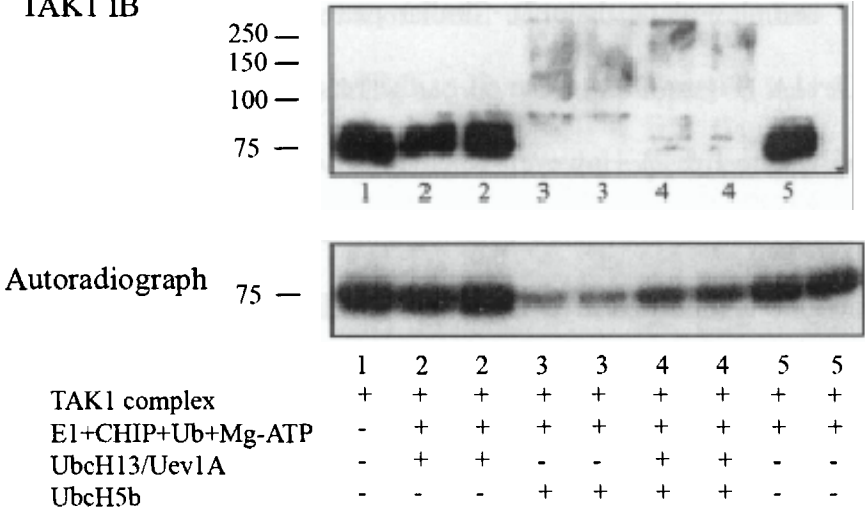
B

Myc IB



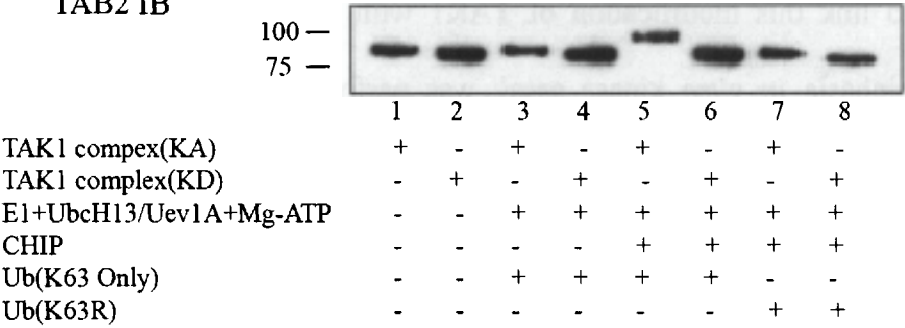
C

TAK1 IB



D

TAB2 IB



3.4.4. CHIP but not TRAF6 suppresses TAK1 activity via Ubch5b mediated ubiquitination reaction

After we have found that both TRAF6 and CHIP have the ability to catalyse poly-ubiquitin chain formation with both Ubch13/Uev1A and Ubch5b *in vitro*, we then examined whether they play similar roles by regulating TAK1 activity. As shown in Figure 3-6A, when TAK1 complex was incubated with GST-TRAF6-ubiquitinase and Ubch13/Uev1A along with other components of ubiquitination, TAK1 showed little change compared to the control (Figure 3-6A, TAK1 complex alone, lane 7). Similar pattern was observed for CHIP-Ubch13/Uev1A (Figure 3-6A, lane 5) or the reactions with only E1 and E2 (Figure 3-6A, lane 1 & 2). However, when TAK1 complex was incubated with GST-TRAF6-ubiquitinase and Ubch5b system, a moderate smear of TAK1 was found above the unmodified myc-TAK1 which still remained as a big band at 75 kDa (Figure 3-6A, lane 4), whereas in CHIP-Ubch5b used reaction, TAK1 showed a even stronger smear and the original band at 75kDa nearly disappeared (Figure 3-6A, lane 6).

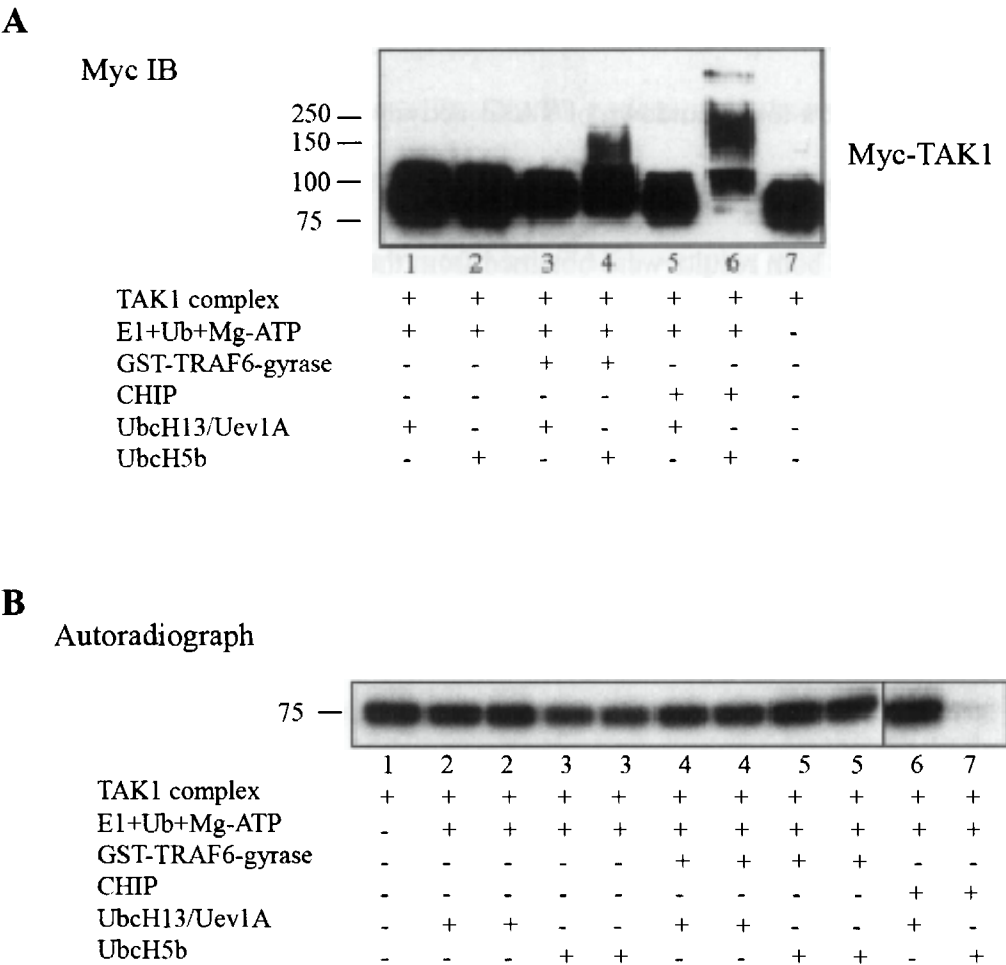
To link this modification of TAK1 with regulation of its activity, a similar biphasic *in vitro* kinase assay was performed after a ubiquitination reaction. As we can see in Figure 3-6B, TAK1 activity, as measured by phosphorylated substrate MKK6, was not changed when incubated with E1-Ubch13/Uev1A only or GST-TRAF6-ubiquitinase plus E1-Ubch13/Uev1A (Figure 3-6B, lane 1, 2, 4). CHIP-Ubch13/Uev1A ubiquitination seemed to just slightly enhance TAK1 activity (Figure 3-6B, lane 6). When E1-Ubch5b only reaction was employed, about 20% decrease of TAK1 activity could be detected as

estimated (Figure 3-6B, lane 3), which suggested there might be some endogenous E3 in TAK1 complex working with UbcH5b to suppress TAK1 activity. Interestingly, when GST-TRAF6-ubiquitinase and UbcH5b were used, a slight increase of TAK1 activity could be seen compared to TRAF6-UbcH13/Uev1A ones (Figure 3-6B, lane 5 compared to 4); whereas a total shutdown of TAK1 activity was found when CHIP-UbcH5b reaction was used (Figure 3-6B, lane 7). Here we have to explain that the difference between Figure 3-5C and Figure 3-6B was introduced by different exposure time of autoradiograph: a total shutdown of TAK1 activity was seen on lane 7 of Figure 3-6B whereas still some remaining activity could be found on lane 3 of Figure 3-5C, although both results were obtained from the experiments under the same conditions when TAK1 complex was incubated with CHIP-UbcH5b ubiquitination. This result showed that the down-regulating effect of CHIP-UbcH5b ubiquitination on TAK1 activity was unique to CHIP and there might be some endogenous E3 in TAK1 complex which can cooperate with UbcH5b to downregulate TAK1 activity.

Figure 3-6 CHIP but not TRAF6 suppresses TAK1 activity via UbcH5b mediated ubiquitination reaction.

A) 1.0 µg TAK1 complex was incubated with 1 µM E1, 2.5 µM UbcH13/Uev1A or UbcH5b, 5 µM CHIP, 50 µM Ub (wt), 2 mM ATP and 5 mM MgCl₂ in different combinations at 30°C for 1h. Reactions were subject to immunoblotting with anti-Myc antibody.

B) 0.2 µg TAK1 complex was incubated with 1 µM E1, 2.5 µM UbcH13/Uev1A or UbcH5b, 5 µM CHIP or 2 µg GST-TRAF6-*gyrase*, 50 µM Ub (wt), 2 mM ATP and 5 mM MgCl₂ in different combinations at 30°C for 1h. Then 1/10 reactions were removed to mix with 4 µg unactivated MBP-MKK6 and incubated with 1x10⁶ c.p.m/nmol [γ-³²P]-ATP (0.1 mM) at 30°C for 30 min. Reactions were stopped by adding SDS sample buffer and subject to SDS-PAGE. Gels were stained with Coomassie Blue and dried before autoradiography at -80°C for 3h.



3.4.5 Validation of CHIP-UbcH5b mediated ubiquitination of TAK1 complex *in vitro*

After we found that CHIP with UbcH5b can ubiquitinate TAK1 using the purified TAK1 complex from HEK293 cells with fusion tags, we wanted to confirm this observation using different systems.

Firstly we tried the *in vitro* transcription and translation system in which TAK1 and its partners were translated and labeled with ^{35}S -methionine in different combinations, single or in complex, followed by incubation with CHIP mediated ubiquitination, either along with UbcH13/Uev1A or UbcH5b. As shown in Figure 3-7A, TAK1 and TAB1-3 were successfully synthesized and labeled using TNT system, as autoradiograph revealed the bands at expected size of each protein (Figure 3-7A, lane 1, 8, 11, 14). The band intensity of individual protein was different, probably due to different promoters used to drive the transcription, among other reasons. When incubated with CHIP-UbcH13/Uev1A ubiquitination, those proteins seemed unaffected compared to the control (Figure 3-7A, lane 2, 9, 12, 15), and the two complexes also did not show much change either (Figure 3-7A, lane 4, 6, control not shown here). However, when these proteins or complexes were incubated with CHIP-UbcH5b mediated ubiquitination system, a stronger smear or ladder-like band pattern was detected above the original band (Figure 3-7A, lane 3, 5, 10), or the original band almost disappeared (Figure 3-7A, lane 7, 13, 16). The input of each labeled protein was supposed to be the same. These smears or ladders may be the modified or ubiquitinated species of each labeled protein since they were correlated to CHIP-UbcH5b ubiquitination.

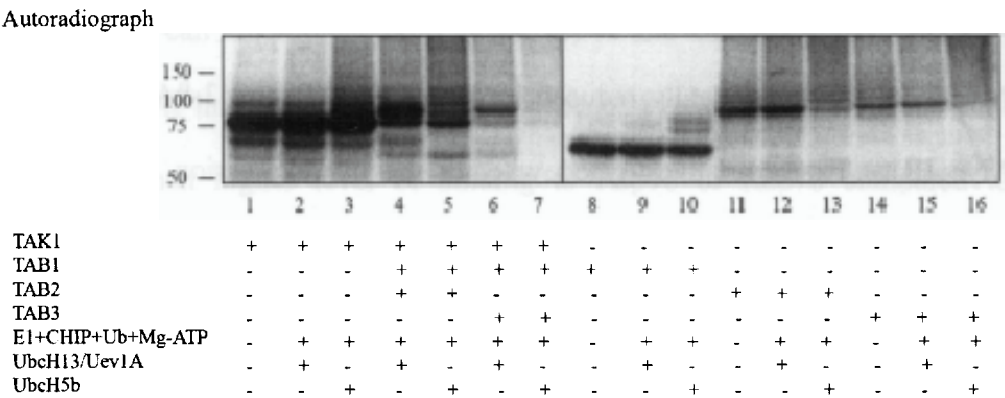
The second method adopted to validate CHIP-UbcH5b effect on TAK1 was to use endogenous TAK1 immunoprecipitates as substrate. As shown in Figure 3-7B, immunopurified TAK1 complex by anti-TAB1 immunoprecipitation was incubated with CHIP-UbcH5b ubiquitination system, including controls in which E2 or E3 was absent. The supernatant of reaction was immunoblotted with anti-ubiquitin antibody to show that strong smear of poly-ubiquitin chain can only be seen with all of the components (Figure 3-7B, anti-Ub IB, lane 4). The lower part was immunoblotted with anti-TAK1 antibody and revealed that the band of TAK1 was slightly decreased when incubated with reactions without E2 or E3 (Figure 3-7B, anti-TAK1 IB, lane 2, 3), but totally disappeared if a complete ubiquitination system was included (Figure 3-7B, anti-TAK1 IB, lane 4). This result supports the previous finding that endogenous E2 or E3 may exist and cooperate with exogenous E3 or E2 to downregulate TAK1. Due to less sensitivity of immunoblotting compared to autoradiography, we could not detect the presence of ubiquitinated endogenous TAK1. However, this result still partially dispels the argument that over-expressed TAK1 complex may give artifact since the protein is tagged and the stoichiometry of complex may be incorrect.

Figure 3-7 Validation of CHIP-UbcH5b mediated ubiquitination of TAK1 complex.

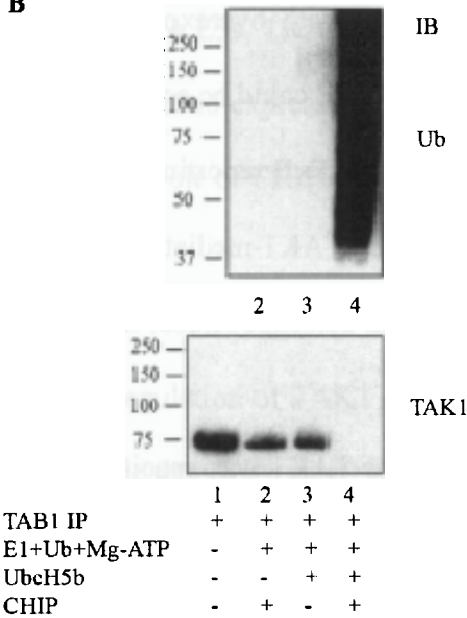
A) *In vitro* translation reactions were carried out with TAK1, TAB1, TAB2, TAB3 or TAK1-TAB1-TAB2, TAK1-TAB1-TAB3 as templates using TNT[®] T7 coupled Transcription/Translation reticulocyte lysate system. Ubiquitination reactions were carried out with 1 μ M E1, 2.5 μ M UbcH13/Uev1A or UbcH5b, 5 μ M CHIP, 50 μ M Ub (wt), 2 mM ATP and 5 mM MgCl₂ at 30°C for 1 h. 5 μ l of ubiquitination reaction was then mixed with 5 μ l *in vitro* translated protein mixture and incubated at 30°C for 1 h. Reactions were stopped by adding 10ml SDS sample buffer and subject to SDS-PAGE. Gels were stained with Coomassie Blue and dried before autoradiography at -80°C for overnight.

B) Immunoprecipitation was carried out with 3 mg total lysates of HEK293 cells adding to protein G agarose bead coupled with 2 mg TAB1 antibody. After 3 h incubation at 4°C, beads were washed thoroughly and incubated with 1 μ M E1, 2.5 μ M UbcH5b, 5 μ M CHIP, 50 μ M Ub (wt), 2 mM ATP and 5 mM MgCl₂ in different combinations at 30°C for 1 h. Beads were spin down and 1 μ l of supernatant removed to another tube. Both beads and supernatant were subject to immunoblotting with anti-TAK1 or Ub antibody respectively.

A



B



3.4.6 Overexpression of CHIP *in vivo* decreases TAK1 level in HSP-dependent manner

Previously we examined the role of CHIP-mediated ubiquitination in TAK1 regulation *in vitro*, next we investigated whether this regulation also occurs in cells.

Firstly we started with co-expression of TAK1 and CHIP in the cells, without input of E1, E2, ubiquitin, since CHIP may use endogenous E1, E2 and ubiquitin. Myc-TAK1 was expressed in HEK293 cells in the presence of Flag-CHIP or with empty vector. The level of exogenous TAK1 was examined by immunoblotting against Myc antibody. As shown in left panel of Figure 3-8A, Myc-TAK1 was drastically reduced by Flag-CHIP (Figure 3-8A, lysate, anti-Myc IB). Then we investigated whether the overexpression of CHIP and UbcH5b can make any difference to endogenous TAK1. The immunoprecipitated TAK1 was assessed to be slightly less when CHIP and UbcH5b were present (Figure 3-8A, TAK1 IP, anti-TAK1 IB), although it was not that drastic a decrease compared to that seen with overexpressed Myc-TAK1 and Flag-CHIP. Again we found that Flag-CHIP could be co-precipitated by TAK1. In line with this decrease of TAK1 level, NF κ B reporting gene assay also showed a significant reduction in IL-1-induced TAK1-mediated activation in the presence of Flag-CHIP (Figure 3-8B).

To address whether this downregulation of TAK1 was specifically due to CHIP rather than other E3s, and to dissect which function of CHIP was relevant, GST-TAK1 was expressed along with wildtype CHIP (wt), an E3

ligase inactive mutant CHIP [H260Q], or CHIP [K30A] mutant that cannot interact with Hsp90/Hsp70 (Qian *et al.* 2006), and two other E3 ligases that have been reported to interact with TAK1, TRAF6 and XIAP. As shown in Figure 3-8C, CHIP (wt) and [H260Q] mutant drastically reduces the level of GST-TAK1, but not [K30A] mutant (Figure 3-8C, lane 1-4). This result suggests that interaction with Hsp90/70 is crucial for CHIP to downregulate TAK1 level, but strikingly its ubiquitin ligase activity may be dispensable. We have ascertained that CHIP [K30A] mutant does not interact with Hsp90 or Hsp70 (Figure 3-8D, lane 3 & 6), although it was still active as E3 ligase as shown in the *in vitro* ubiquitination assay (Figure 3-8E, lane 8). Moreover, K30A mutant can ubiquitinate both kinase active and kinase inactive TAK1 *in vitro* as shown in Figure 3-8F (lane 2, 4 & 6, 8); whereas H260Q mutant showed a much higher level of binding to Hsp90 or Hsp70 compared to wildtype CHIP (Figure 3-8D, lane 1, 2 & 4, 5) but no ubiquitin ligase activity as shown in Figure 3-8E (lane 7). This result is consistent with the fact that CHIP [H260Q] is an inactive ubiquitin ligase as it is unable to bind the cognate E2. The other two E3s, TRAF6 and XIAP, were found to make little difference to TAK1 level if co-expressed with TAK1 (Figure 3-8C, lane 5 & 6), highlighting the peculiar role of CHIP in regulation of TAK1.

Our result suggested that CHIP ubiquitin ligase activity was not required for downregulation of TAK1 *in vivo*, while *in vitro* we have shown that TAK1 is downregulated by CHIP-UbcH5b mediated ubiquitination. To solve the controversy, we hypothesized that CHIP [H260Q] mutant was able to recruit endogenous CHIP via dimerization as reported (Nikolay *et al.* 2004). As shown

in Figure 3-8G, all three variants of Flag-CHIP can strongly bind to GST-CHIP. Therefore, it is possible that CHIP [H260Q] mutant may execute its downregulating effect on TAK1 via recruitment of the endogenous wildtype CHIP. To address this possibility in the future, a mutant of CHIP that is unable to dimerize and at the same time cannot bind E2 will be very useful for further investigations.

On the other hand, it is reasonable to hypothesize that CHIP may directly interact with Hsp90/70 to exert its downregulating effect on TAK1, because CHIP[K30A] cannot affect TAK1 level. As reported on its initial discovery, CHIP was found to inhibit ATPase activity of Hsp70 or Hsp90 (Ballinger *et al.* 1999), and later CHIP was proven to ubiquitinate Hsp70/Hsc70 (Jiang *et al.* 2001; Qian *et al.* 2006). In the previous chapter of my thesis, I have shown that Hsp90 is required for TAK1 stability and Hsp90 inhibition leads to the decrease of TAK1 level. Therefore the downregulation of TAK1 by CHIP may be explained that endogenous Hsp90/70 was inhibited by overexpressed CHIP. If this is the case, we should be able to rescue the TAK1 downregulation by overexpressing Hsp90 or Hsp70. As shown in Figure 3-8H, GST-TAK1 was expressed in HEK293 cells together with HA-Hsp70 or HA-Hsp90, in the presence/absence of wildtype CHIP or CHIP [H260Q]. Consistently, overexpression of CHIP could decrease GST-TAK1 level (Figure 3-8H, lane 1 & 2, 7 & 8), and CHIP [H260Q] caused an even greater decrease compared to wildtype CHIP. When HA-Hsp90 was expressed together with TAK1, level of TAK1 was not changed; while TAK1 expression was enhanced by co-expression of HA-Hsp70 (Figure 3-8H, lane 3 & 4). The similar pattern was

seen in the presence of CHIP (Figure 3-8H, lane 5 & 6, 9 & 10), showing that Hsp70 may partially rescue the down-regulating effect of CHIP, both wildtype and [H260Q] mutant, whereas Hsp90 plays a more limited role to rescue TAK1 level in the presence of CHIP. This result may imply that CHIP seems to target directly on Hsp70 to downregulate TAK1. The downregulating effect of wildtype CHIP and CHIP [H260Q] was found to correlate with their binding affinity to Hsp70: the stronger binding to Hsp70, the greater decrease could be found on TAK1 level (Figure 3-8D & H). In our experiment, we also observed some modification of Hsp70 by overexpressed wildtype CHIP but not CHIP [H260Q] (data not shown); however, this modification does not correlate with the change of TAK1 levels. It is possible that CHIP directly targets Hsp70 by inhibiting its ATPase activity instead of ubiquitinating Hsp70. To further confirm this implication, knockdown of Hsp70 or inhibitors of Hsp70 should provide valuable information in future work.

Figure 3-8 Overexpression of CHIP *in vivo* decreases TAK1 level in HSP-dependent manner

A) Left: Myc-TAK1 was expressed without or with Flag-CHIP in HEK293 cells and cell lysates were immunoblotted using anti-Myc or Flag antibodies. Anti-actin immunoblotting was used as loading control. Right: Flag-CHIP and HA-UbcH5b were co-expressed in HEK293 cells and lysates were subject to anti-TAK1 immunoprecipitation followed by immunoblotting with anti-TAK1 and Flag antibodies.

B) HEK293 IL1RA cells were transfected with pCMV5 empty vector or Myc-TAK1 with or without Flag-CHIP and NF κ B driven luciferase plasmid as well as internal control Renilla luciferase plasmid as indicated. Cells were starved in FCS-free media for 12 h prior to IL-1 β stimulation (50 ng/ml) for 6h, followed by luciferase assay using Promega Dual Luciferase kit as instructed. Three independent experiments were carried out and results were shown in \pm S.D.M.

C) GST-TAK1 was expressed together with various E3s, Flag-CHIP (wt/H260Q/K30A) mutants, Flag-TRAF6 and Myc-XIAP in HEK293 cells. Cell lysates were immunoblotted using anti-GST, Flag and Myc antibodies respectively. Anti-actin immunoblotting was used to show the same loading.

D) HA-Hsp90 or Hsp70 and Myc-HOP was coexpressed in HEK293 cells with Flag-CHIP variants (wt/H260Q/K30A) and cell lysates were subject to immunoprecipitation using anti-HA or Myc and the beads were immunoblotted with anti-HA/Myc anti-Flag antibody. Lysates were immunoblotted with anti-Flag antibody to show the levels of Flag-CHIP.

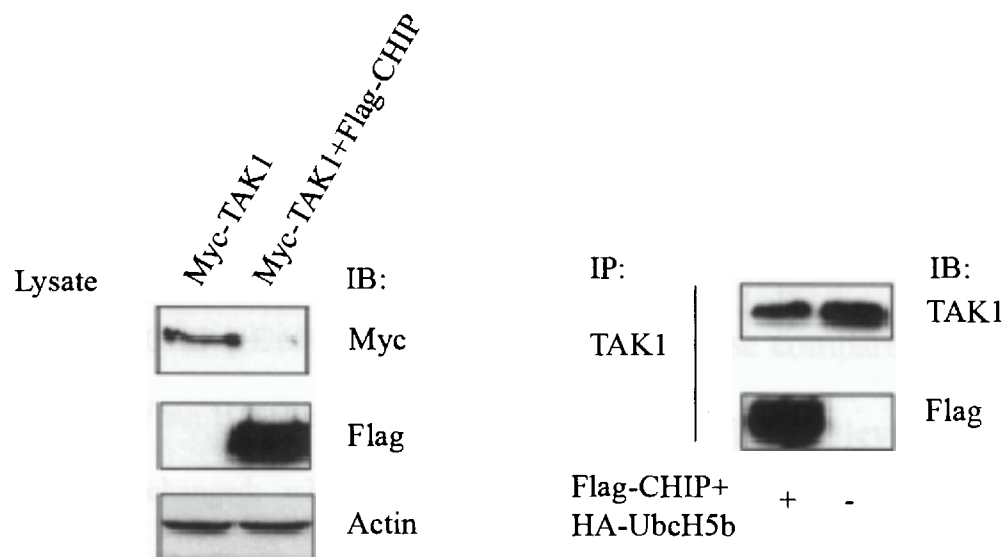
E) Flag-CHIP variants (wt/H260Q/K30A) were expressed in HEK293 cells along with an empty vector control and immunoprecipitated by anti-Flag antibody. The immunoprecipitates were subject to an *in vitro* ubiquitination assay with E1, UbcH5b, Ub and Mg-ATP as described. The reactions without E3 were used as control (lane 1-4).

F) Flag-CHIP variants (wt/H260Q/K30A) were expressed in HEK293 cells and immunoprecipitated by anti-Flag antibody. Then an *in vitro* ubiquitination assay was carried out with TAK1 complex as substrate using E1, UbcH5b along with CHIP immunoprecipitates as indicated, followed by immunoblotting against Myc antibody.

G) GST-CHIP was co-expressed in HEK293 cells with Flag-CHIP variants (wt/H260Q/K30A) and lysates were subject to GST pull-down assay followed by immunoblotting with anti-GST and Flag antibodies.

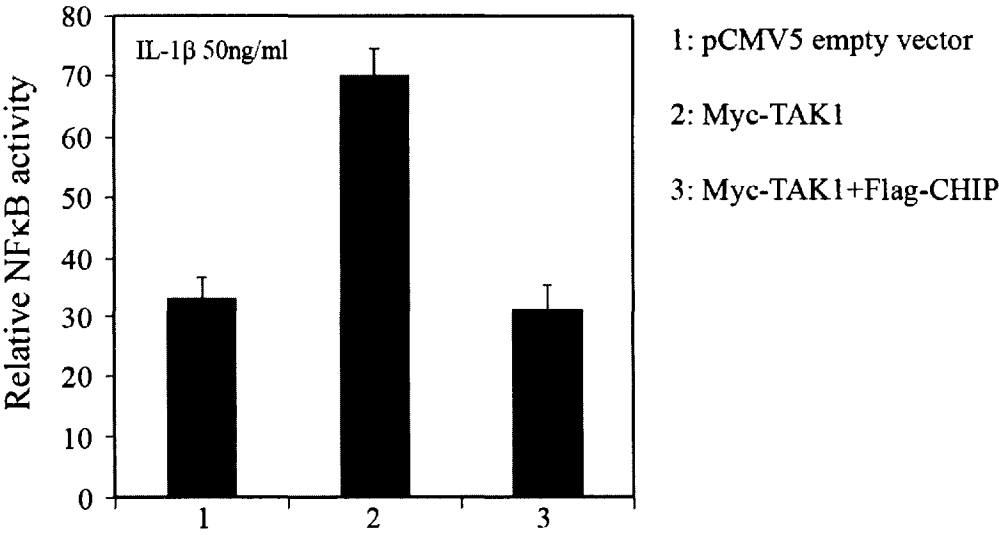
H) GST-TAK1 was co-expressed in HEK293 cells with HA-Hsp90/Hsp70, in the presence or absence of Flag-CHIP (wt/H260Q). The lysates were immunoblotted using anti-GST, HA, Flag and actin antibodies.

A

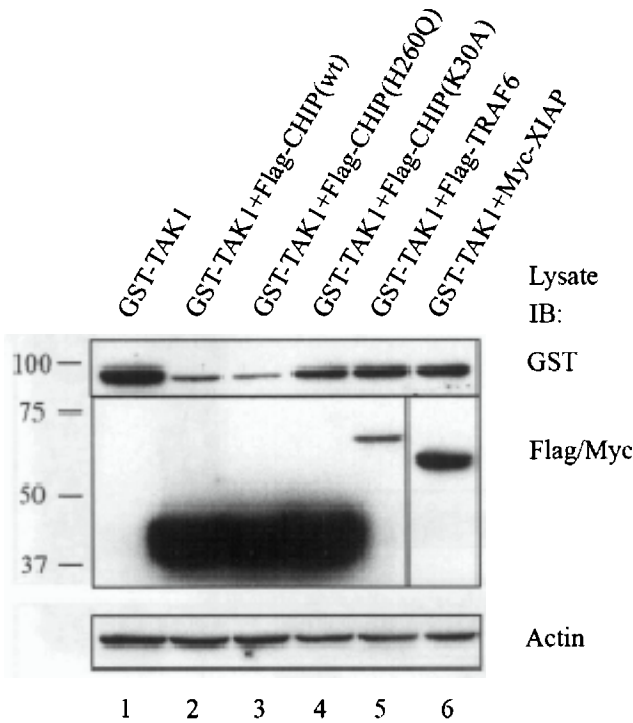


B

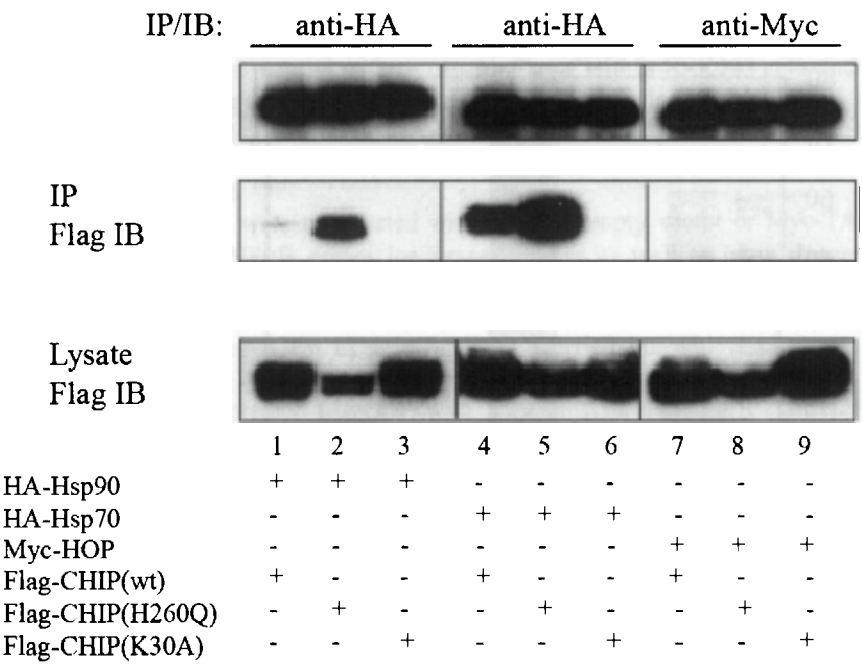
Dual luciferase assay



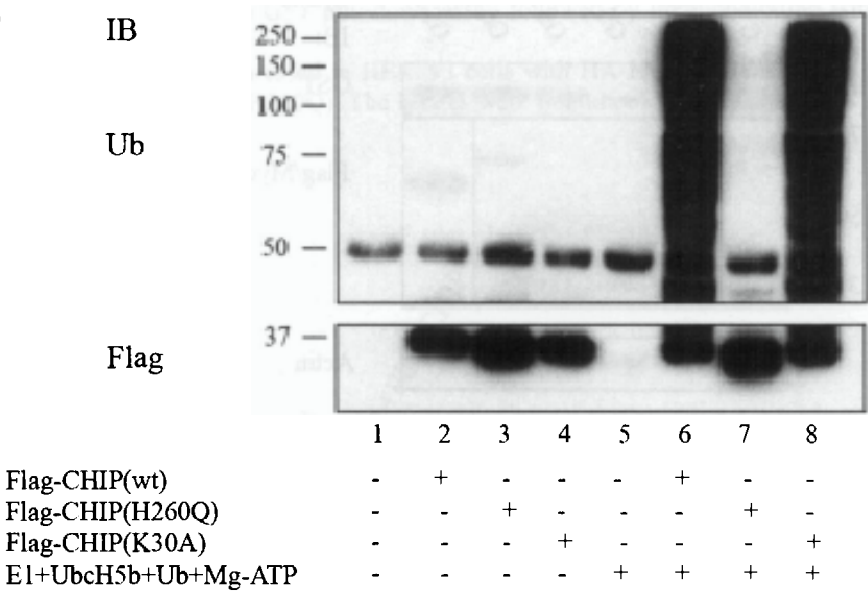
C



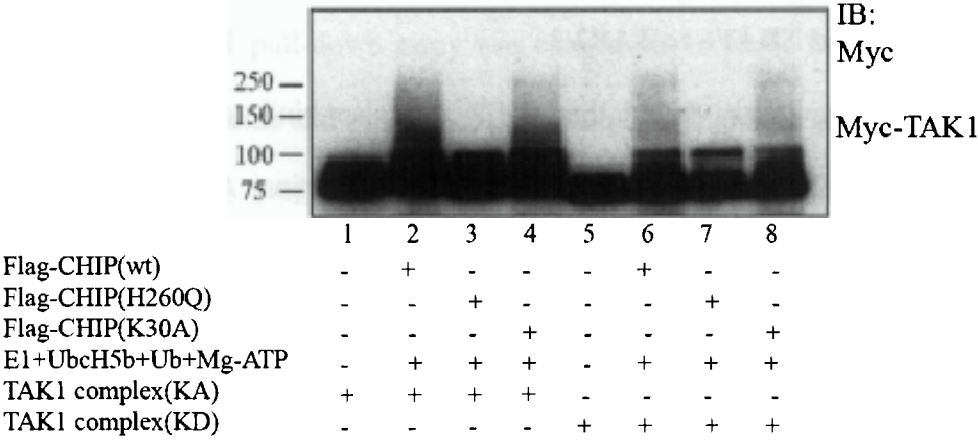
D



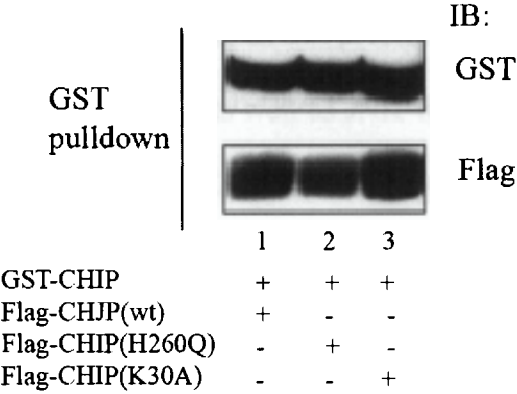
E



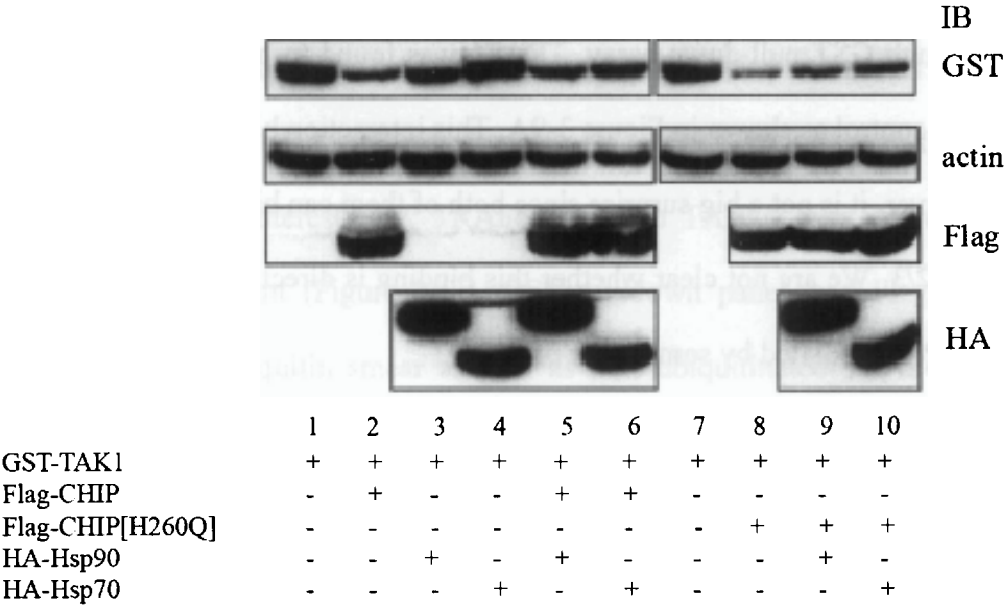
F



G



H



3.4.7 CHIP is recruited by TAB2/3 to attenuate binding of auto-ubiquitinated TRAF6 to TAB2/3

Since we previously found that CHIP can also bind to TAB2/3, we then examined the physiological relevance of this interaction. As an established E3 ligase involved in TAK1-IKK signaling, TRAF6 can undergo auto-ubiquitination via K63-linked poly-ubiquitin chain and is recruited by TAB2/3 through their NZF domains. This K63-linked poly-ubiquitination relies on a special dimeric E2, UbcH13/Uev1A. Interestingly, CHIP was reported to catalyse formation of K63-linked poly-ubiquitin chain together with UbcH13/Uev1A, by us and other researchers (Zhang *et al.* 2005). Therefore both CHIP and TRAF6 may be recruited to TAK1-TAB2/3 complex, either simultaneously or in a specific temporal and/or spatial pattern, and they may interact as well.

To examine the potential interaction between CHIP and TRAF6, we co-expressed GST control or GST-CHIP with Flag-TRAF6 in HEK293 cells. Through GST pull-down assay, TRAF6 was found to bind GST-CHIP but not GST control as shown in Figure 3-9A. This interaction has not been reported yet, however, it is not a big surprise since both of them can bind to the same proteins TAB2/3. We are not clear whether this binding is directly between TRAF6 and CHIP or mediated by some other protein.

TAK1-TAB2/3-TRAF6 complex was expressed in HEK293 cells with or without CHIP, to address the effect of CHIP on this established pathway. The lysates were immunoblotted with a number of antibodies to examine the

expression exogenous proteins and activation of downstream signaling pathways, while GST pull-down assay was carried out to probe the interaction between various proteins and binding of poly-ubiquitin chains to GST-TAB2/3. As shown in Figure 3-9B, when CHIP was present, Myc-TAK1 level was decreased (Figure 3-9B, lysate panel, anti-Myc IB), similar to TAK1-CHIP single combination previously. On the contrary, TRAF6 level seemed to be increased (Figure 3-9B, lysate panel, anti-Flag IB, upper one), suggesting that CHIP may stabilize TRAF6 in this circumstance.

Not surprisingly, in the presence of CHIP, phosphorylation of Thr184 and Thr187 on TAK1 as well as JNK was decreased (Figure 3-9B, lysate panel, anti-TAK1[pT184/T187] and JNK1/2[pT183/Y185] IB), probably due to less amount of TAK1 remained, in line with NF κ B report gene assay shown previously (Figure 3-8B). However, TAB2/3 levels did not change with or without CHIP (Figure 3-9B, lysate panel, anti-GST IB).

Next we looked at the interactions between various proteins, as GST pull-down assay revealed, CHIP was found to bind GST-TAB2/3, consistent with previous result. Interestingly, TRAF6 bound to TAB2/3 was decreased when CHIP was present (Figure 3-9B, GST pulldown panel, anti-Flag IB). Similarly, the poly-ubiquitin smear as well as auto-ubiquitinated TRAF6 co-precipitated with TAB2/3 was reduced greatly in the presence of CHIP (Figure 3-9B, GST pulldown panel, anti-ubiquitin and anti-TRAF6 IB). Of note, TRAF6 level in lysate was much higher when coexpressed with CHIP, but less TRAF6 was detected to bind TAB2/3 at the same condition. This result gave us

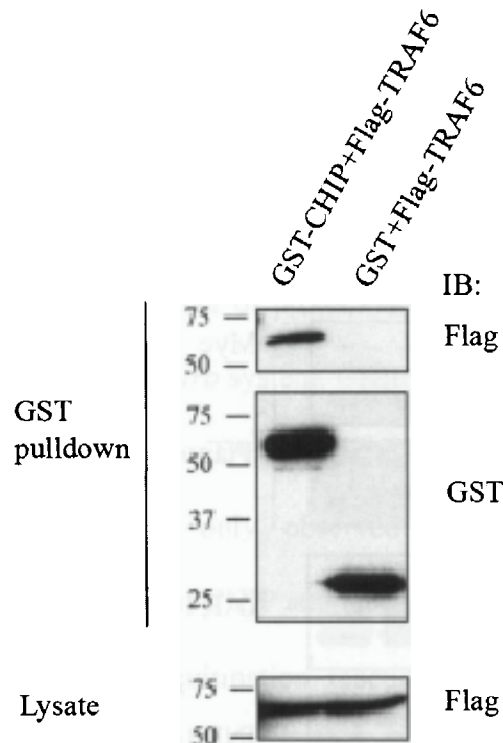
two implications: i) TRAF6 is stabilized by CHIP as they interact; ii) at the same time CHIP out-competes TRAF6 for the binding of TAB2/3, because less TRAF6 was found to bind TAB2/3 even if the level of TRAF6 was enhanced. It has been reported that TRAF6 binds to C-termini of TAB2/3 (Takaesu *et al.* 2000; Ishitani *et al.* 2003), whereas CHIP binds to both N and C-termini of TAB2/3 as we have found, they may simply compete for C-termini of TAB2/3 directly, or some allosteric effect is caused by CHIP upon its binding to TAB2/3. To address those possibilities, further investigation is required in the future.

Figure 3-9 CHIP is recruited by TAB2/3 to attenuate binding of auto-ubiquitinated TRAF6 to TAB2/3

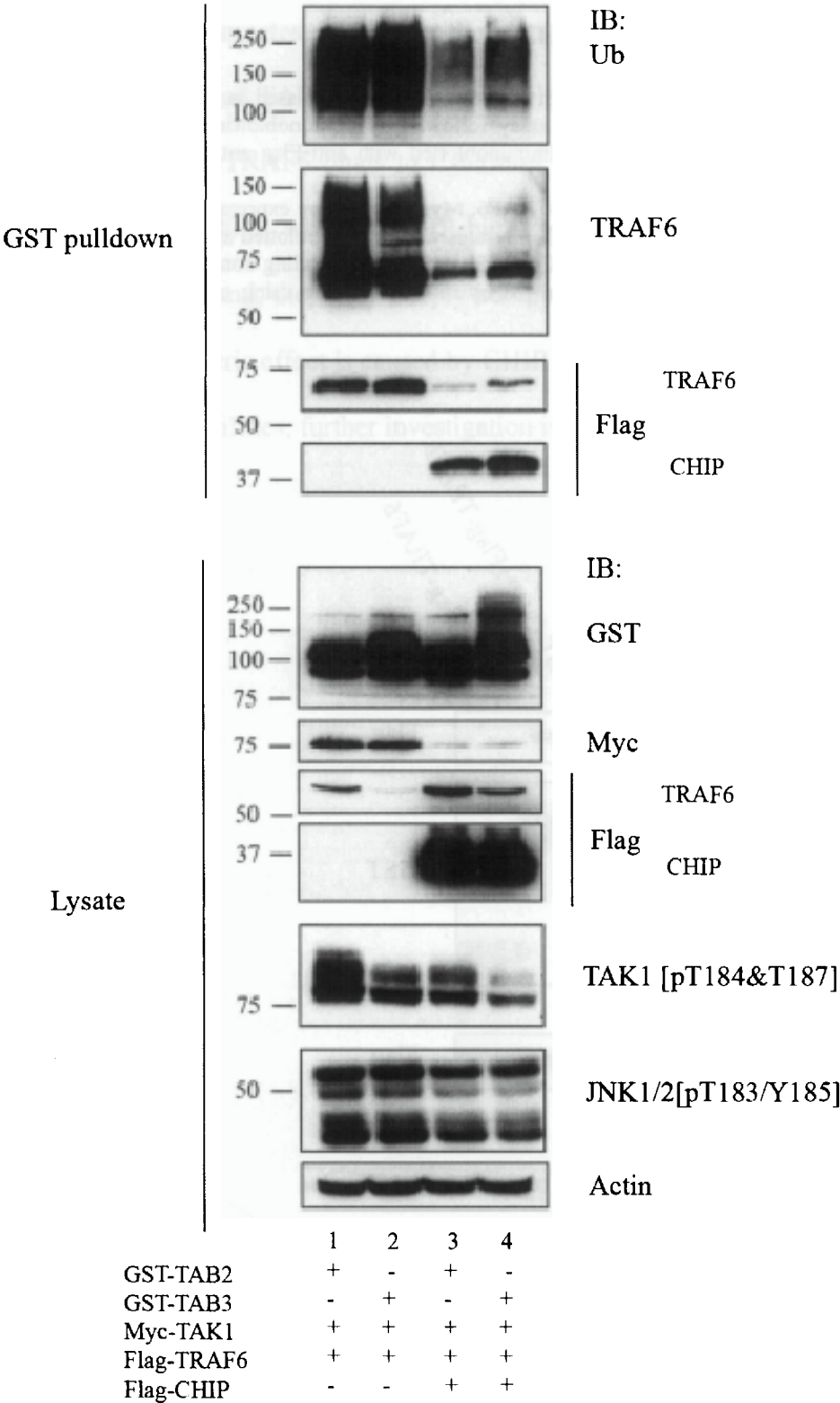
A) GST or GST-CHIP along with Flag-TRAF6 was expressed in HEK293 cells. The lysates were subject to GST pull-down assay followed by immunoblotting with anti-GST and Flag antibodies. The lysate was also immunoblotted with anti-Flag antibody to show the level of Flag-TRAF6.

B) GST-TAB2/3 along with Flag-TRAF6, Myc-TAK1 were expressed in HEK293 cells with or without Flag-CHIP as indicated. Cell lysates were immunoblotted against anti-GST, Myc, Flag, TAK1 [pT184/T187], JNK [pT183/Y185] and actin as loading control. Meanwhile, GST pull-down assay was carried out followed by immunoblotting using anti-ubiquitin, TRAF6, Flag antibodies.

A



B



3.5 Discussion and future work

Some protein kinases possess dynamic and diversified structure features which require a delicate quality control mechanism provided by Hsp90-Hsp70 chaperone system to maintain the proper folding necessary for function. TAK1 has been shown to be a substrate kinase of Hsp90 in the previous chapter and plays an important role in multiple inflammatory pathways.

In this study we found that CHIP, a co-chaperone and ubiquitin ligase, was a binding protein to TAK1 complex, confirming and extending the yeast-two hybrid screening result. It can bind to TAK1, TAB2 and TAB3 but not TAB1 as revealed by GST pull-down assay. CHIP has been reported to ubiquitinate a number of protein substrates, many being Hsp90 client kinases. Indeed, via an *in vitro* system, CHIP along with UbcH5b was found for the first time to ubiquitinate TAK1 and thus down-regulating its kinase activity. This modification was only observed in reaction with CHIP and UbcH5b combination but not CHIP and UbcH13/Uev1A, although the latter also showed highly active ubiquitination reaction *in vitro*. Moreover, overexpression of CHIP resulted in significant decrease of exogenous TAK1 in HEK293 cells, supporting our *in vitro* data, although it is still uncertain whether ubiquitin-proteasome pathway is involved. The binding of CHIP and TAB2/3 may open a novel avenue to regulate TAK1: CHIP can attenuate the binding of TRAF6 to TAB2/3, probably via an intriguing network forms among CHIP, TAB2/3 and TRAF6. In the following part, we will discuss some of these results.

When we explored the interaction of CHIP and TAK1 complex, it is interesting to find out that only TAB1 does not bind CHIP. As we know, TAB1 can attenuate the binding of Hsp90 to TAK1, and CHIP was found to be a co-chaperone molecule of Hsp90/Hsp70. Probably there is some connection between these findings, for instance, Hsp90 acts as a bridging molecule between TAK1 and CHIP. Seemingly it is not the case, as C-terminus of GST-TAK1 can bind to CHIP but not Hsp90 (see Figure 2-1B, and Figure 3-3). For the same reason, CHIP is not the molecule mediating TAK1-Hsp90 binding. Therefore, CHIP-TAK1 complex interaction is independent of Hsp90 although CHIP is initially identified as a co-chaperone binding with heat-shock proteins. These unique binding characteristics between CHIP and TAK1 complex may account for the distinct roles of CHIP in TAK1 regulation: CHIP directly downregulates TAK1 level or indirectly attenuates TAK1 activation by preventing TRAF6 from binding to TAK1 binding partners, as shown previously in our results.

More than just a co-chaperone molecule, CHIP has proven to be a bona fide E3 ubiquitin ligase characterized by its U-box domain which is structurally similar to RING domain. Besides coupling with UbcH5 family E2s, CHIP is found to cooperate with a unique E2 complex UbcH13/Uev1A *in vitro* and catalyse the formation of poly-ubiquitin chains. Considering the essential role of TRAF6-UbcH13/Uev1A in TAK1-IKK signaling (Deng *et al.* 2000), we intended to establish a similar reconstituted *in vitro* system to study the function of CHIP-UbcH13/Uev1A ubiquitination which has been unclear since its discovery. Using proteins purified from bacteria, *in vitro* ubiquitination reactions could be carried out and lead to the formation of the strong poly-

ubiquitin smear which was recognized as hallmark of ubiquitination. Interestingly, E1 and UbcH13/Uev1A can catalyse the chain formation without any E3 as reported by other research (Hofmann and Pickart 2001), but E1 and UbcH5b seemed to be less reactive, maybe due to the distinct intrinsic characteristics of these two E2s. This kind of poly-ubiquitin chain can be preformed on the active site of E2 and transferred to substrate when available, as supported by a recent research (Li *et al.* 2007), or they just simply exist as a mixture of free poly-ubiquitin chains. For the first time, TRAF6 was found to cooperate with UbcH5b in ubiquitination and could modify TAK1 *in vitro* to certain extent, although this modification seemed to make little difference to TAK1 activity *in vitro*, compared with impact exerted by CHIP-UbcH5b on TAK1. On the contrary, CHIP-UbcH13/Uev1A mediated ubiquitination did not show any effect on TAK1 activation in our system, either possibly some relevant protein was missing in our system, or as our later data suggested, CHIP may simply compete with TRAF6 to associate with UbcH13/Uev1A and thus down-regulating TAK1 activity in the cells. Excitingly, we reported for the first time that TAB2/3 undergoes band shift in the presence of CHIP-UbcH13/Uev1A mediated ubiquitination. This observation was dependent on TAK1 kinase activity, suggesting that TAK1 may be involved in the phosphorylation of TAB2/3 in this case. However, the physiological relevance of our *in vitro* data is so far unclear. More work is required.

Although the role of CHIP-UbcH13/Uev1A ubiquitination was still elusive, CHIP-UbcH5b ubiquitination did modify TAK1 complex *in vitro* as proven by different systems. In the future, we need to find out whether this

ubiquitination occurs in the cells, using multiple approaches, such as proteasome inhibition, siRNA against CHIP and UbcH5 family to investigate TAK1 modification and its physiological significance. However, we should bear in mind that *in vitro* study allows detailed investigation in a defined manner, yet the results obtained are not necessarily the truth all the time *in vivo*. Simply they may just represent a special situation that those proteins have higher probability to couple together to initiate any reactions otherwise they are sequestered separately in the cells; or they are only triggered by a specific stimulation to occur. Additionally, *in vitro* study could amplify the effect by artificially increasing the concentrations of proteins involved to much higher a degree than those in physiological conditions, which more easily results in some artifacts.

Through an over-expression approach, CHIP was found to significantly reduce the exogenous TAK1 level in HEK293 cells, but in a similar experiment, CHIP could only slightly reduce the endogenous TAK1 level (Figure 3-8A). This discrepancy suggests that maybe only a small proportion of TAK1 is subject to CHIP regulation in a specific cellular context. As we know, CHIP is reported to be responsible for protein quality control, which is supposed to remove those unfolded proteins under stress conditions or misfolded proteins in normal physiological states. Here in our experiments, the over-expressed TAK1 may tend to have folding problem, given its highly elevated level compared to that in normal state. Therefore the down-regulating effect of CHIP became more obvious than that on endogenous TAK1. Moreover, it is interesting to take into account that TAK1 is an Hsp90 client kinase and CHIP is a co-chaperone of

Hsp90-Hsp70 system which is found to downregulate some kinase substrates under Hsp90 inhibition. At the moment we are not clear whether it is the same case for CHIP mediated regulation of TAK1. In the future we need to address this possibility with a specific and efficient RNAi approach against CHIP. If TAK1 level shows less decrease in the presence of CHIP knockdown upon Hsp90 inhibition compared to the control, we will know that CHIP does play a role in Hsp90-dependent regulation of TAK1 level.

To deepen our understanding of CHIP-mediated regulation on TAK1 level in overexpression system, CHIP variants were used. We found the interaction between CHIP and heat shock proteins was disrupted by using CHIP [K30A] mutation, and TAK1 level was not significantly changed compared to the drastic decrease caused by wildtype CHIP or another variant CHIP [H260Q] which strongly bound with Hsp90/70. Since the CHIP [H260Q] mutant that is E3 ligase inactive also caused significant decrease of TAK1, one may simply conclude that E3 ligase activity is not required for this downregulation. However, such a conclusion may turn out to be a hasty one. Previous research has indicated that CHIP undergoes dimerization which is indispensable for its activity and structural study has revealed an unexpected asymmetric model: CHIP dimer can bind only one copy of E2, that is to say, CHIP dimer possesses only one functional E3. These findings support such a hypothesis that CHIP [H260Q], through dimerization, may recruit endogenous CHIP which can bind E2 and function as a normal E3 ligase. To clarify whether CHIP ligase activity is really indispensable, a double mutant of CHIP which cannot dimerize or bind E2 is required. Alternatively, we could knock down, or even knock out

endogenous CHIP and knock in different CHIP mutants to dissect their roles in this setting.

From another prospective, if CHIP just directly binds Hsp90/70 and inhibits their ATPase activity as reported, we should be able to rescue the downregulating effect on TAK1 by overexpressing Hsp90/70. Truly as we found, overexpression of HA-Hsp70 but not HA-Hsp90 could reverse TAK1 downregulation effectively. It makes sense that newly synthesized protein kinase requires Hsp90/Cdc37 to stabilize in the early stage followed by Hsp70 chaperone machinery to fold into mature conformation. The overexpression of TAK1 may mimic the condition that chaperone system is almost overloaded and cannot help substrate fold properly. In this case the quality control program is redirected into degradational branch which involves CHIP mediated ubiquitination. CHIP overexpression is likely to further promote this transition toward the degradation of unfolded protein substrates. Under normal states, we could only see marginal downregulation of endogenous TAK1 by CHIP overexpression, which may indicate that TAK1 can be folded properly by chaperone system and no involvement of CHIP is needed. Moreover, as we have found in the previous chapter, TAB1 can substitute Hsp90 to stabilize TAK1 after its synthesis, and coincidentally, TAB1 does not bind to CHIP by GST pull-down assay. This may suggest that the binding of CHIP-TAK1 is conditional and possibly only occurs in early stage after the synthesis of TAK1. To explore this possibility, biochemical study on different cellular fractions combined with cell immunostaining techniques in a time course is required.

Since Hsp70 may be involved in CHIP-mediated downregulation of TAK1, as reported in the downregulation of DLK kinase (Daviau *et al.* 2006), siRNA against Hsp70 or inhibitor of Hsp70 such as quercetin will be helpful to dissect the role of Hsp70. However, it is possible that CHIP directly inhibits Hsp70 ATPase activity but does not ubiquitinate or downregulate Hsp70 level. A dominant negative Hsp70 will shed light on this puzzle.

Currently, little is known about the significance of CHIP-UbcH13/Uev1A ubiquitination in TAK1 signaling. In this study, we presented firstly that TAB2/3 underwent high level of band shift in the presence of CHIP-UbcH13/Uev1A ubiquitination system *in vitro*. Secondly CHIP can interact with TRAF6, as well as TAB2/3, constituting another layer of CHIP-mediated regulation of TAK1 signaling. Through an unclear mechanism, CHIP can attenuate binding of TRAF6 as well as the poly-ubiquitin chain to TAB2/3, which has been found to be indispensable for TAK1 activation (Kanayama *et al.* 2004). At present it is not clear whether CHIP and TRAF6 directly compete for the same part of TAB2/3, or some allosteric effect on TAB2/3 is caused by CHIP, thus attenuating TRAF6-TAB2/3 interaction. More investigation is required to map out the specific binding sites of both TRAF6 and CHIP on TAB2/3.

It is reported that TAB2/3 also bind to NEMO via their CUE domains and poly-ubiquitin chain on NEMO (Kanayama *et al.* 2004), recruiting IKK complex to the vicinity of TAK1 catalytic site. Since CHIP affects the binding

of auto-ubiquitinated TRAF6 to TAB2/3, it is interesting to test if CHIP has the similar effect on NEMO-TAB2/3 interaction.

Last but not least, almost all the results presented in this chapter were obtained from overexpression studies from HEK293 cells. It is highly necessary to investigate the endogenous level of CHIP-TAK1 regulation in various cell lines under different stimulations to avoid any cell type specific artifacts and lend robust credibility to our findings.

In summary, we found that CHIP, as a new binding partner of TAK1 complex, can downregulate TAK1 activity via UbcH5b mediated ubiquitination *in vitro*, while band shift of TAB2/3 was detected in the presence of CHIP-UbcH13/Uev1A ubiquitination. Overexpression studies revealed that CHIP regulates TAK1 via a two-fold mechanism: i) it downregulates TAK1 level in HSP70 dependent manner; ii) it attenuates the binding of TRAF6 and poly-ubiquitin chain to TAB2/3 thus down-regulating the activation of TAK1.

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