AUP1 (Ancient Ubiquitous Protein 1) is a negative regulator of pro-inflammatory cytokine signaling

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Gießen, den 02.07.2009

Dang Quan Nguyen

CONTENTS

ABBREVIATIONS

AA/Bis-AA   : acrylamide/bis-acrylamide

AGPAT   : 1-acylglycerolphosphate acyltransferase

APS   : ammonium persulfate

ATP   : adenosine triphosphate

AUP1   : ancient ubiquitous protein 1

bp   : base pair

Bcl-2   : B-cell lymphoma 2

BCL10   : scaffolding protein B-cell leukemia/lymphoma 10

Bcl-XL   : B-cell lymphoma-extra large

BSA   : bovine serum albumin

Btk   : Bruton's tyrosine kinase

bZIP   : basic leucine zipper

^{o}C   : degree Celsius

cAMP   : cyclic adenosine monophosphate

caspase   : cysteine-aspartic acid proteases

CD14   : Cluster of differentiation 14

cDNA   : complementary deoxyribonucleic acid

C/EPB   : CAAT/enhancer-binding protein

c-FLIP   : cellular FLICE-like inhibitory protein

cGMP   : cyclic guanosine monophosphate

c-IAP1/2   : cellular inhibitor of apoptosis protein 1/2

ct   : cycle threshold

CUE   : coupling of ubiquitin conjugation to the endoplasmic reticulum degradation

DD   : death domain

DEPC   : Diethylpyrocarbonate

DMEM   : Dulbecco modified Eagle's minimal essential medium

DMSO   : Dimethyl sulfoxide

DNA   : deoxyribonucleic acid

dNTP   : deoxy nucleoside triphosphate

DPBS   : Dulbecco's phosphate buffered saline

DTT   : Dithiothreitol

ECL	: enhanced chemiluminescene
E coli	: *Escherichia coli*
ECSIT	: evolutionarily conserved signaling intermediate in Toll pathways
EDTA	: ethylene diamine tetraacetic acid
EGFP	: enhanced green fluorescent protein
ELISA	: enzyme-linked immunosorbent assay
ER	: endoplasmic reticulum
ERK	: extracellular signal-related kinase
EST database	: expressed sequence tag database
FADD	: Fas-associated death domain
FCS	: fetal calf serum
FLICE	: FADD-like IL-1β-converting enzyme
GAPDH	: glyceraldehyde 3-phosphate dehydrogenase
GFP	: green fluorescent protein
gp130	: glycoprotein 130
gp80	: glycoprotein 80
GPAT	: glycerolphosphate acyltransferase
GTPase	: guanosine triphosphatase
h	: human
HA	: hemagglutinin
HEPES	: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRD1	: hypoxia responsive domain-1
HRP	: horseradish peroxidase
IBDs	: inflammatory-bowel diseases
IFNβ	: interferon beta
Ig	: immunoglobulin
IκB	: inhibitor of NF-κB
IKK	: inhibitor of NF-κB (IκB) kinase
IL	: interleukin
IL-1RI	: interleukin 1 receptor type I
IL-1RAcP	: interleukin 1 receptor accessory protein
IL-6Rα	: interleukin 6 receptor alpha chain

IP	: immunoprecipitation
IRAK1/2/4/M	: interleukin 1 receptor associated kinase 1/2/4/M
IRF1/3	: interferon regulatory factor 1/3
JAK1	: Janus kinase 1
JNK	: c-Jun N-terminal kinase
K	: amino acid lysine
LB	: lysogeny broth
LBP	: LPS-binding protein
LPA	: lysophosphatidic acid
LPS	: lipopolysaccharide
Lys	: amino acid lysine
m	: mouse
MALT1	: mucosa-associated lymphoid tissue 1
Mal/TIRAP	: MyD88 adaptor like / TIR domain-containing adaptor protein
MAPK	: mitogen-activated protein kinase
MEKK3	: mitogen-activated protein kinase kinase kinase 3
MHC	: major histocompatibility complex
min	: minute
MKK3/4/6	: mitogen-activated protein kinase kinase 3/4/6
mnd2	: motor neuron degeneration 2
MP	: milk powder
mRNA	: messenger ribonucleic acid
MTT	: (4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide
MyD88	: myeloid differentiation factor 88
NADPH	: nicotinamide adenine dinucleotide phosphate
NEMO	: nuclear factor kappa B essential modulator
NF-κB	: nuclear factor kappa B
OD	: optical density
PA	: phosphatidic acid
PAGE	: polyacrilamide gel electrophoresis
PBS	: phosphate buffered saline
PCR	: polymerase chain reaction

PEI	:	polyethylenimine
PI3	:	phosphoinositide 3
PIP2	:	phosphatidylinositol 4,5-bisphosphate
PKC	:	protein kinase C
PlsC	:	phosphate acyltransferase domain
ProST region	:	proline-, serine-, threonine-rich region
PtdIns(3)P	:	phosphatidylinositol-3-phosphate
PtdIns(3,4,5)P	:	phosphatidylinositol-3,4,5-phosphate
PVDF	:	Polyvinylidene Fluoride
qPCR	:	quantitative polymerase chain reaction
RE	:	restriction endonuclease
RFP	:	red fluorescent protein
rhIL-1β	:	recombinant human IL-1β
rhIL-6	:	recombinant human IL-6
rhTNFα	:	recombinant human TNFα
RING	:	really interesting new gene
RIP1	:	receptor interacting protein 1
RLU	:	relative light unit
RNA	:	ribonucleic acid
rpm	:	round per minute
RT	:	reverse transcription
Scr kinase	:	sarcoma kinase
SDS	:	sodium dodecyl sulfate
SEL1L	:	sel-1 (suppressor of lin-12-) like
Ser	:	amino acid serine
SH2 domain	:	Src-homology-2 domain
SHP2	:	SH2 domain-containing tyrosine phosphatase 2
siRNA	:	small interfering RNA
SOCS3	:	suppressor of cytokine signaling 3
SODD	:	silencer of death domain
STAT3	:	signal transducers and activators of transcription 3
Syk	:	spleen tyrosine kinase

TAB1/2/3	:	TAK1-binding protein 1/2/3
TAK1	:	transforming growth factor β-activated kinase 1
TANK	:	TRAF family member-associated NF-κB activator
TBK1	:	IKK related kinases TANK-binding kinase 1
TBS/T	:	Tris-buffered saline / Tween-20
TCR	:	T cell receptor
TEMED	:	Tetramethylethylenediamine
Thr	:	amino acid threonine
TICAM1/2	:	TIR-containing adaptor molecule 1/2
TIMP	:	tissue inhibitor of metalloprotease 1
TIR	:	Toll/interleukin-1 receptor
TLR	:	Toll like receptor
TMB	:	3,3',5,5'-tetramethyl-benzidine
TNFα	:	tumor necrosis factor alpha
TNFR1/2	:	tumor necrosis factor receptor 1/2
Tollip	:	Toll-interacting protein
TRADD	:	TNFR-associated death domain
TRAF2/6	:	TNF receptor associated factor 2/6
TRAM	:	TRIF-related adapter molecule
TRIF	:	TIR domain-containing adapter-inducing IFNβ
Tyr	:	amino acid tyrosine
Ubc13	:	ubiquitin conjugating enzyme 13
Uev1a	:	ubiquitin conjugating enzyme variant 1a
US2	:	unique short 2
US11	:	unique short 11
VIMP	:	VCP (valosin-containing protein)-interacting membrane protein
WB	:	Western blot
wt	:	wild type

PREFACE

IRAK4 (interleukin 1 receptor associated kinase 4) is a critical component of TLR/IL-1R (toll like receptor/interleukin 1 receptor) signaling pathways. IRAK4 is recruited to active receptor complex, which is triggered by ligand engagement, via adapter protein MyD88 (myeloid differentiation factor 88). In receptor complex, IRAK4 becomes active and then activates downstream molecules leading to appropriate response of cells to stimulation [Martin, Wesche – BBA – 2002]. In the other hand, IRAK4 was reported to be involved in T cell receptor signaling and in phosphorylation of NADPH oxidase cytosolic factor $p47^{phox}$ in neutrophils which is completely different from known functions of IRAK4 [Suzuki et. al. – Science – 2006; Pacquelet – Biochemical Journal – 2007]. Thus, it is likely that more not yet defined functions of IRAK4 exist.

In an attempt to seek new partners of IRAK4 using the yeast two-hybrid screening, AUP1 (ancient ubiquitous protein 1) was identified to interact with IRAK4. This interaction was confirmed in HEK293RI cells by co-immunoprecipitation experiments. Moreover, the associations between AUP1 and many components of the IL-1 signaling pathway such as IL-1RI, MyD88, IRAK1, TRAF6 were found. AUP1 was also detected in complex with TNFR1, TRADD, TRAF2 – components of the TNFα pathway – as well as with the specific receptor of IL-6 cytokine IL-6Rα chain. Based on these findings, the aim of this study was to investigate the biological function of AUP1 in the signaling transductions induced by IL-1β, LPS, TNFα and IL-6 in cells in which AUP1 was overexpressed or silenced.

The introduction summarizes current knowledge of AUP1 and some related respects of the present study and puts them into context with IL-1 and cytokine signaling.

1. INTRODUCTION

1.1. Known biological functions of AUP1

Since the identification of AUP1 in 1996, there have been not so many publications reporting biological functions of AUP1.

AUP1 associates with cytoplasmic tail of α_{IIb} subunit but not with β_3 subunit of integrin receptor in UT7/TPO cells, a megakaryocyte-derived cell line. Integrin $\alpha_{IIb}\beta_3$ (GPIIb-IIIa) is one of the receptors on cellular surface of platelets and megakaryocytes. It binds to various adhesive proteins including fibrinogen, von Willebrand factor, vitronectin, and fibronectin. Binding of fibrinogen to $\alpha_{IIb}\beta_3$ leads to platelet aggregation and finally to thrombus formation at the injured vascular sites. $\alpha_{IIb}\beta_3$ on resting platelets does not bind soluble fibrinogen, once platelets are activated, conformation of the extra cellular domains of $\alpha_{IIb}\beta_3$ is altered and its ligand binding affinity is increased. This process of the inside-out signaling is considered to be mediated by modification of the short cytoplasmic tail of α_{IIb} and β_3 subunits. Besides α_{IIb} subunit, AUP1 was shown to bind to cytoplasmic tails of various integrin α subunits such as α_1, α_2, α_5, α_V, α_M but not with β_1, β_2, β_3 subunits. Full-down experiment revealed that AUP1 interacts with cytoplasmic tails of α_{IIb} subunits at the conserved membrane-proximal domain which exerts a negative regulatory function and block $\alpha_{IIb}\beta_3$ in a low affinity state [Kato et.al. – JBC – 2002].

In resting platelets, AUP1 was detected in a complex with $\alpha_{IIb}\beta_3$, Src and Syk (spleen tyrosine kinase) constitutively. *In vitro* binding study revealed that recombinant Syk binds directly to AUP1 and the β_3 cytoplasmic tail. In platelets stimulated with thrombin, activated/phosphorylated Syk does not interact with AUP1. *In vitro* kinase assay of Syk in the presence or absence of AUP1 proved that AUP1 does not directly influence autophosphorylation or tyrosine phosphorylation of Syk by Src, a potent activator of Syk. These results indicate that AUP1 is an adaptor recruiting Syk to the α_{IIb} cytoplasmic tail, and suggest that the α_{IIb} – AUP1 – Syk – β_3 complex formation links α_{IIb} and β_3 cytoplasmic tails to sustain $\alpha_{IIb}\beta_3$ in

an inactive state and Syk dissociates from AUP1 after activation [Kato, Oshimi –
Platelets – 2009].

AUP1 was reported to be a component of a protein complex required for dislocation
of misfolded glycoproteins from endoplasmic reticulum (ER) [Mueller et. al. –
PNAS – 2008]. Misfolded membrane or secretory proteins that fail to pass quality
control in the ER are transported back across the ER membrane into the cytosol, a
process called dislocation or retrotranslocation. In cytosol, misfolded proteins are
degraded by the proteasome in a ubiquitin-dependent manner [Mueller et. al. –
PNAS – 2008]. Two viral proteins encoded by human cytomegalovirus US2 and
US11 which facilitate dislocation of newly synthesized class I MHC (major
histocompatibility complex) heavy chains in infected cells, presumably to overcome
the recognition by cytotoxic T cells, have been used as models to study dislocation
protein complex in mammalian cells [Mueller et. al. – JCB – 2006; Mueller et. al. –
PNAS – 2008]. Derlin-1 protein was identified to be essential for the degradation of
class I MHC molecules catalysed by US11, but not by US2 [Lilley and Ploegh –
Nature – 2004]. Derlin-1 interacts with US11 as well as with VIMP, a novel
membrane protein that recruits the p97 ATPase and its cofactor to dislocation
complex [Ye et. al. – Nature – 2004]. Derlin-1 forms a complex with other member
of Derlin family, Derlin-2, as well as HRD1 and SEL1L. HRD1 is a
nonglycosylated ER membrane protein containing a RING domain that acts as an
active E3 ubiquitin ligase *in vitro*. HRD1 participates in the degradation of ER
proteins and protects cells from ER stress-induced apoptosis. SEL1L is predicted to
be a type I membrane protein with five N-linked glycans [Lilley and Ploegh –
PNAS – 2005]. Because the bulk of the SEL1L protein is predicted to be in ER
lumen, it is possible that SEL1L first plays the role in substrate recognition and
identification of misfolded proteins, and then recruits them to the site of dislocation.
Knockdown of SEL1L expression by shRNA impaired US11-mediated dislocation
of class I MHC heavy chains but not with those mediated by US2. Furthermore,
reduction of SEL1L level also inhibited the degradation of a misfolded ribophorin
fragment (RI332) independently of the presence of viral accessories [Mueller et. al.

– JCB – 2006]. In an attempt to identify more components of misfolded glycoprotein dislocation complex, AUP1 was discovered to associate with SEL1L. Overexpression of the dominant negative form of AUP1, GFP-AUP1, inhibited strongly US11-mediated class I MHC heavy chains dislocation and degradation. Although the function of AUP1 in dislocation and degradation of glycoproteins is obscure, its CUE domain may be involved in recruitment of another ubiquitin-conjugating enzyme [Mueller et. al. – PNAS – 2008].

1.2. Identification, characterization and cloning of novel gene aup1

aup1 gene was identified on mouse chromosome 6 in the progress of positional cloning of mouse mnd2 gene whose mutation results in early onset motor neuron disease. In that work, Jang and coworkers found an unknown 0.7kb clone which could hybridize with genomic DNA from human, cat and dog. They used that fragment to screen mouse cDNA library by hybridization and detected five overlapping cDNAs spanning 1490 nucleotides with an open reading frame of 1230 nucleotides. That gene was cloned and named aup1 (ancient ubiquitous protein 1) [Jang et.al. – Genomics – 1996]. Mouse AUP1 protein, containing 410 amino acid residues, is highly conserved to the *Caenorhabditis elegans* F44b9 protein with 53% amino acid similarity. Human aup1 gene which was identified by EST (Expressed Sequence Tag) database searching locates in chromosome 2p13 and encodes a protein with 86% amino acid sequence identity and 90% conservation with the mouse protein. The gene symbol aup1 reflects the evolutionary conservation of the protein [Jang et.al. – Genomics – 1996].

Karpisheva and coworkers reported the cloning of cDNA of human aup1 gene and its genomic organization. Genomic length of the human aup1 gene is composed of 12 exons and 11 introns spanning 3047bp with a single open reading frame of 1230 bp [Karpisheva et. al. – Tsitologiia – 2002].
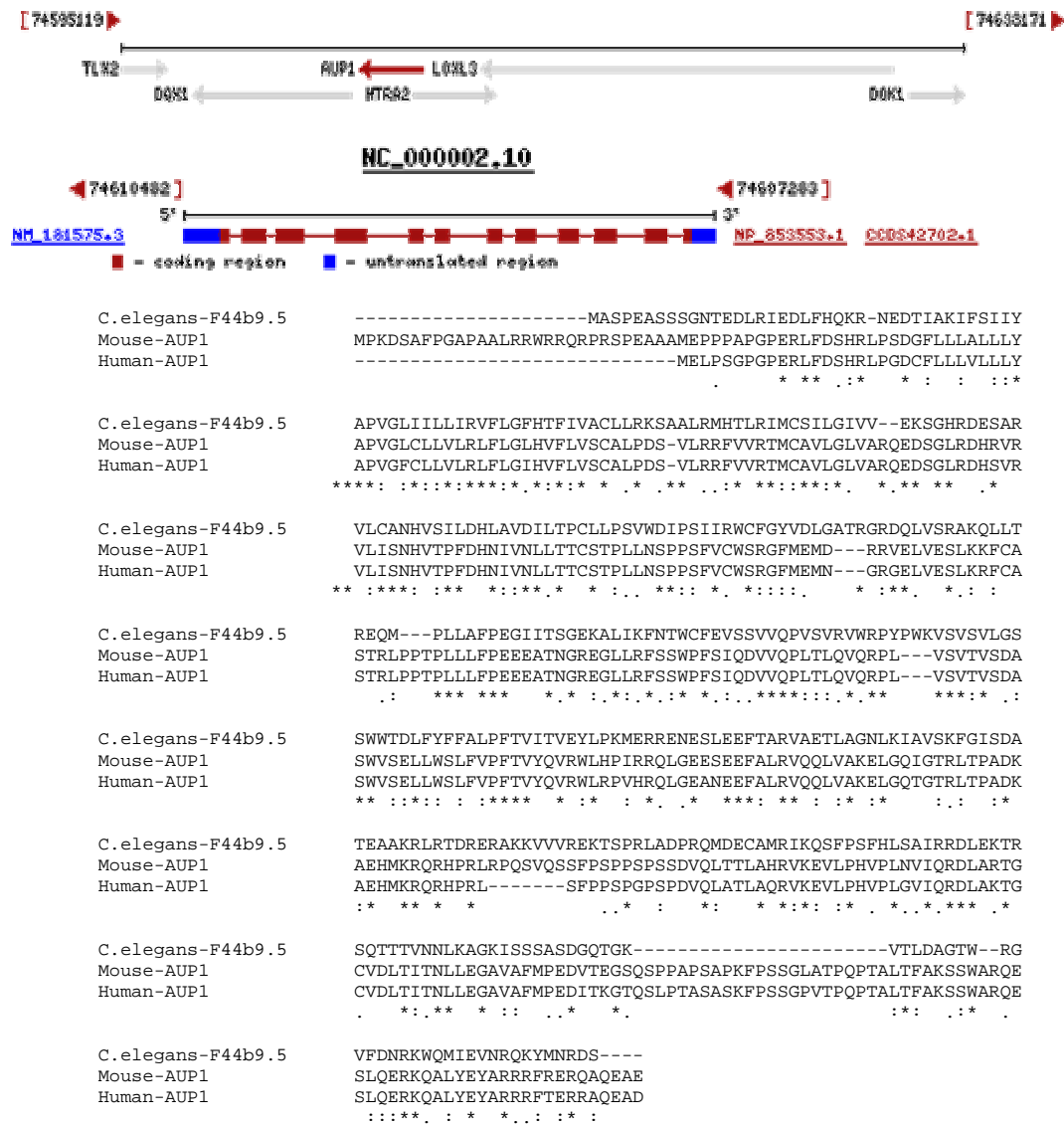
Chromosome: 2; location: 2p13

```
[74595119]                                                    [74638171]

     TLX2              AUP1      LOXL3                         DOK1
         DQX1              HTRA2                           DOK1


                         NC_000002.10

  [74610482]                                   [74607283]
     5'                                              3'
NM_181575.3                                      NP_853553.1  CCDS42702.1
    ■ = coding region    ■ = untranslated region
```

```
C.elegans-F44b9.5   --------------------MASPEASSSGNTEDLRIEDLFHQKR-NEDTIAKIFSIIY
Mouse-AUP1          MPKDSAFPGAPAALRRWRRQRPRSPEAAAMEPPPAPGPERLFDSHRLPSDGFLLLALLLY
Human-AUP1         ---------------------------MELPSGPGPERLFDSHRLPGDCFLLLVLLLY
                                        .    * **  .:*   * :  :   ::*

C.elegans-F44b9.5   APVGLIILLIRVFLGFHTFIVACLLRKSAALRMHTLRIMCSILGIVV--EKSGHRDESAR
Mouse-AUP1          APVGLCLLVLRLFLGLHVFLVSCALPDS-VLRRFVVRTMCAVLGLVARQEDSGLRDHRVR
Human-AUP1          APVGFCLLVLRLFLGIHVFLVSCALPDS-VLRRFVVRTMCAVLGLVARQEDSGLRDHSVR
                    ****: :*::*:***:*.*:*:* * .* .** ..:* **::**:*.  *.** **  .*

C.elegans-F44b9.5   VLCANHVSILDHLAVDILTPCLLPSVWDIPSIIRWCFGYVDLGATRGRDQLVSRAKQLLT
Mouse-AUP1          VLISNHVTPFDHNIVNLLTTCSTPLLNSPPSFVCWSRGFMEMD---RRVELVESLKKFCA
Human-AUP1          VLISNHVTPFDHNIVNLLTTCSTPLLNSPPSFVCWSRGFMEMN---GRGELVESLKRFCA
                    ** :***: :**  *::**.*  * :.. **:: *. *::::.    * :**.  *.: :

C.elegans-F44b9.5   REQM---PLLAFPEGIITSGEKALIKFNTWCFEVSSVVQPVSVRVWRPYPWKVSVSVLGS
Mouse-AUP1          STRLPPTPLLLLFPEEEATNGREGLLRFSSWPFSIQDVVQPLTLQVQRPL---VSVTVSDA
Human-AUP1          STRLPPTPLLLLFPEEEATNGREGLLRFSSWPFSIQDVVQPLTLQVQRPL---VSVTVSDA
                     .:   *** ***   *.* :.*:.*.:* *.:..****::.*.**    ***:* .:

C.elegans-F44b9.5   SWWTDLFYFFALPFTVITVEYLPKMERRENESLEEFTARVAETLAGNLKIAVSKFGISDA
Mouse-AUP1          SWVSELLWSLFVPFTVYQVRWLHPIRRQLGEESEEFALRVQQLVAKELGQIGTRLTPADK
Human-AUP1          SWVSELLWSLFVPFTVYQVRWLRPVHRQLGEANEEFALRVQQLVAKELGQTGTRLTPADK
                    ** ::*:: : :**** * :*  : *. .* ***: ** : :* :*   :.: :*

C.elegans-F44b9.5   TEAAKRLRTDRERAKKVVVREKTSPRLADPRQMDECAMRIKQSFPSFHLSAIRRDLEKTR
Mouse-AUP1          AEHMKRQRHPRLRPQSVQSSFPSPPSPSSDVQLTTLAHRVKEVLPHVPLNVIQRDLARTG
Human-AUP1          AEHMKRQRHPRL-------SFPPSPGPSPDVQLATLAQRVKEVLPHVPLGVIQRDLAKTG
                    :*  ** * *             ..* :   *:   * *:*: :* .  *..*.*** .*

C.elegans-F44b9.5   SQTTTVNNLKAGKISSSASDGQTGK----------------------VTLDAGTW--RG
Mouse-AUP1          CVDLTITNLLEGAVAFMPEDVTEGSQSPPAPSAPKFPSSGLATPQPTALTFAKSSWARQE
Human-AUP1          CVDLTITNLLEGAVAFMPEDITKGTQSLPTASASKFPSSGPVTPQPTALTFAKSSWARQE
                     .   *:.**  * ::  ..*  *.       :*:   .:*   .

C.elegans-F44b9.5   VFDNRKWQMIEVNRQKYMNRDS----
Mouse-AUP1          SLQERKQALYEYARRRFRERQAQEAE
Human-AUP1          SLQERKQALYEYARRRFTERRAQEAD
                     :::**. : *  *.: :* :
```

Figure 1.1: Position and structure of human aup1 gene in genome and amino acid sequence alignment of human AUP1 protein and its counterparts in mouse and *Caenorhabditis elegans*. Asterisks, amino acid identity between mammal and *C.elegans*; double dots, conservative substitutions; single dots, semi-conserved substitutions.
Reference database: www.ncbi.nlm.nih.gov, alignment program: www.ebi.ac.uk/Tools/muscle

1.3. Expression of AUP1 in cells and tissues

The transcript of aup1 gene was detected in many tissues of adult mouse such as brain, fat, intestine, heart, kidney, liver, lung, muscle, ovary, pancreas as well as in mouse embryo [Jang et.al. – Genomics – 1996]. In human tissues, aup1 transcript is also expressed in leukocytes, lung, placenta, small intestine, liver, kidney, spleen, thymus, colon, skeletal muscle, heart and brain. With immunoblot analysis using

rabbit antiserum against a synthetic peptide for hAUP1, the expression of AUP1 protein was determined as duplicate bands of ~40 kDa in many human cell lines (UT7/TPO, CMK, HL60, K562, U937, Jurkat, Raji, HepG2, HEK293, HeLa, MCF7, A547) [Kato et.al. – JBC – 2002].

Because of its evolutionary conservation and ubiquitous expression, AUP1 likely plays basic and essential roles in cellular biology [Jang et.al. – Genomics – 1996; Kato et.al. – JBC – 2002].

1.4. Intracellular localization of AUP1

Observation of intracellular localization of AUP1-RFP (red fluorescent protein) fusion protein revealed that AUP1 distributes evenly throughout the cytoplasm without any colocalization with Golgi apparatus or ER but not in the nucleus [Karpisheva et. al. – Tsitologiia – 2002; Kato et.al. – JBC – 2002]. Although it was reported that N-terminus of mouse AUP1 resembles the signal peptide of secreted proteins, human AUP1 was undetectable in concentrated culture supernatant of UT7/TPO cells. These findings indicated that AUP1 is a cytoplasmic protein [Kato et.al. – JBC – 2002].

However, in another study, immunofluorescence with anti-AUP1 antibody showed a reticular endoplasmic reticulum staining pattern and colocalization with the ER marker PDI. AUP1 readily cosediments with the microsomes in the absence of detergent and is largely resistant to extraction with alkaline sodium carbonate and urea [Mueller et. al. – PNAS – 2008]. These data suggest that AUP1 resides, at least partially, in the ER.

1.5. Structure of AUP1 protein and its putative function domains

A database search for the homologous domain structure revealed that human AUP1 possesses three putative domains: transmembrane domain (residues 23-45), phosphate acyltransferase domain (PlsC, residues 90-193) and coupling of ubiquitin conjugation to the endoplasmic reticulum degradation domain (CUE, residues 296-

337) [Kato et.al. – JBC – 2002; analysis programs: SMART (http://smart.embl-heidelberg.de/); InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/)].

The presence of a putative transmembrane domain in N-terminus suggests that one of functions of AUP1 may carry out in close proximity of plasma or intracellular membrane. It is supported by the observation that AUP1 locates in ER. [Mueller et. al. – PNAS – 2008]

Acyltransferase domain is typical for a superfamily of proteins involved in phospholipid biosynthesis. Characterized enzymes of this superfamily have either glycerolphosphate (GPAT, EC2.3.15), 1-acylglycerolphosphate (AGPAT, EC2.3.1.51), or 2-acylglycerolphosphoethanolamine acyltransferase activity [Neuwald – Current Biology – 1997]. Phospholipid biosynthesis is initated with the acylation of glycerol-3-phosphate by GPAT to form lysophosphatidic acid (LPA), followed by acylation of LPA by AGPAT to form phosphatidic acid (PA) [Kato et.al. – JBC – 2002]. Besides to be the precursor for phospholipid biosynthesis in both eukaryotic and prokaryotic cells, LPA is known as an intercellular signaling molecule that is rapidly produced and released by activated cells, notably platelets, to influence target cells by acting on a specific cell-surface receptor. In addition to acting as an autocrine stimulator of platelet aggregation, LPA stimulates the growth of fibroblasts, vascular smooth muscle cells, endothelial cells and keratinocytes. Furthermore, it promotes cellular tension and cell-surface fibronectin binding, which are important events in wound repair. It was established that LPA binding to its G protein-coupled receptor leads to stimulation of phospholipases C and D, inhibition of adenylyl cyclase, activation of Ras and the downstream Raf/MAP kinase pathway, and tyrosine phosphorylation of focal adhesion proteins in remodeling of the actin cytoskeleton [Neuwald – Current Biology – 1997]. PA, another precursor for phospholipid biosynthesis, acts as an intracellular and an extracellular messenger. PA activates phospholipase C and many protein kinases involved in the signaling transduction of protein kinase C and Raf/MAP kinase pathways [West et.al. – DNA Cell Biology – 1997].

In addition to possessing acyltransferase domain, the amino acid sequence of AUP1 exhibits a homology with CUE domain (coupling of ubiquitin conjugation to the endoplasmic reticulum degradation domain). The CUE domain was discovered through bioinformatics analysis to detect homologues of proteins that regulate the ER-associated degradation pathway [Ponting – Biochemical journal – 2000]. Thirty two proteins encoded in the genome databases carry CUE domains [Shih et.al. – EMBO – 2003]. Function of CUE motifs was determined as monoubiquitin-binding domains and they are capable of promoting the ubiquitination of proteins that contain them [Shih et.al. – EMBO – 2003; Donaldson et.al. – Current Biology – 2003; Hurley et.al. – Biochemical journal – 2006]. Ubiquitinlation regulates the activities of a diverse array of cellular proteins. Polyubiquitination in which chains of Lys48-linked ubiquitin are conjugated to proteins marks target proteins for degradation by the 26S proteasome [Shih et.al. – EMBO – 2003; Hurley et.al. – Biochemical Journal – 2006]. In contrast, monoubiquitination, the addition of a single ubiquitin unit to a protein, is an important cellular regulatory signal. The characterized roles of monoubiquitination include acting as a sorting signal to direct protein traffic in the endocytic pathway, regulating vesicle budding machinery, modifying histones, regulating transcriptional machinery and controlling intranuclear localization. More regulatory roles for monoubiquitin are likely to exist [Shih et.al. – EMBO – 2003]. Previous studies reported that Tollip (Toll-interacting protein) and TAB2 (transforming growth factor β-activated kinase 1 (TAK1)-binding protein 2), two functional proteins in IL-1 signal transduction pathway, possess CUE domain [Kato et.al. – JBC – 2002; Shih et.al. – EMBO – 2003; Kishida et.al. – Genes to Cells – 2005; Tian et.al. – JBC – 2007].

Tollip was determined for the first time to pre-associate with IRAK1 (IL-1R-associated kinase 1) and under IL-1β stimulation, MyD88 and Tollip-IRAK1 are recruited to IL-1RI and IL-1RAcP (interleukin 1 receptor type I and IL-1R acessory protein) complex. Then IRAK1 is phosphorylated/autophosphorylated and releases from receptor complex as well as Tollip. Overexpression and silencing experiments showed that Tollip may act as a silencer of IL-1 signaling [Burns et.al. – Nature –

2000]. Tollip also associates directly with TLR2 and TLR4 (Toll like receptor 2 and 4) and inhibits TLR signaling by suppression of the activity of IRAK. In another hand, Tollip is phosphorylated by IRAK upon stimulation with lipopolysaccharide (LPS) or IL-1 [Zhang, Ghosh – JBC – 2002]. Tollip was reported to bind with phosphatidylinositol-3-phosphate (PtdIns(3)P) and phosphatidylinositol-3,4,5-phosphate (PtdIns(3,4,5)P) *in vitro*. Mutation of lysine 150 residue to glutamic acid of Tollip abolished this binding as well as inhibition activity of Tollip with LPS-induced NF-κB (nuclear factor kappa B) activity. Tollip-mediated inhibition being alleviated by wortmannin, a PI3 (Phosphoinositide 3) kinase inhibitor, implies that PI3 kinase may regulate Tollip function [Li et.al. – Molecular Immunology – 2004]. However, NF-κB as well as MAPK (mitogen activated protein kinase) signaling appeared normal in Tollip-deficient cells stimulated with IL-1β or the TLR4 ligand LPS. In contrast, the production of the proinflammatory cytokines, IL-6 and TNFα was significantly reduced after IL-1β and LPS treatment. Tollip therefore controls the magnitude of inflammatory cytokine production in response to IL-1β and LPS [Didierlaurent et.al. – Molecular and Cellular Biology – 2006]. Moreover, Tollip was shown involving in trafficking of IL-1RI from the cell surface to late endosomes, lysosomes and IL-1RI degradation. In Tollip-deficient cells and cells expressing only mutated Tollip (incapable of binding IL-1RI and ubiquitin), IL-1RI accumulates on late endosomes and is not efficiently degraded [Brissoni et.al. – Current Biology – 2006].

TAB2 is an adapter protein that mediates activation of TAK1 in IL-1 signaling pathway. Overexpression of TAB2 induces JNK (c-Jun N-terminal kinase) and NF-κB activation, whereas a dominant-negative mutant TAB2 impairs their activation by IL-1. IL-1 stimulates translocation of TAB2 from the membrane to the cytosol where it mediates the association of IL-1-dependent activated TRAF6 (tumour necrosis factor receptor associated factor 6) with TAK1 leading to autophosphorylation and activation of TAK1. Activated TAK1, in its turn, activates NF-κB and MAPK [Takaesu et.al. – Molecular Cell – 2000; Martin, Wesche – BBA – 2002]. Study in TAB2-deficient mouse embryo fibroblast revealed that TAB2 is

required for IL-1-induced TRAF6 ubiquitination. TAB2, with CUE domain, binds monoubiquitin in yeast two-hybrid system. Point mutation at critical phenylalanine residue to alanine or aspartic acid of CUE domain or deletion of CUE domain abolished NF-κB -activation mediated by TAB2 overexpression. Thus, CUE domain is likely important for function of TAB2 in IL-1 signaling pathway [Kishida et.al. – Genes to Cells – 2005].

There is no data about the activity of acyltransferase domain as well as CUE domain of AUP1. However, the presence of these domains in AUP1 can provide clues to understand biological functions of AUP1.

1.6. IL-1 and LPS signaling pathways

The Toll like receptor / Interleukin 1 receptor (TLR/IL-1R) superfamily includes multiple receptors playing crucial role in innate and adaptive immunity. The TLR subfamily consists of 13 members that contain leucine-rich repeat motifs in their extracellular domain. They recognize distinct microbial patterns such as LPS, flagellin, viral double stranded RNA and unmethylated CpG motifs. Members of the IL-1R subfamily are characterized by Ig (immunoglobulin)-like structures in their extracellular domain that bind specifically members of the IL-1 cytokine family, which are involved in multiple immunological and inflammatory processes. In contrast to their distinct extracellular domains, all members of the TLR/IL-1R family (with exception of IL-1RII) are characterized by an intracellular TIR (Toll IL-1 receptor) domain. [Verstrepen et.al.– Cellular and Molecular Life Sciences – 2008]

1.6.1. IL-1 signaling pathway

Upon engagement of IL-1 to IL-1RI, the coreceptor molecule IL-1RAcP (IL-1R accessory protein) comes to and forms the heterodimeric receptor complex by homotypic protein–protein interaction of the TIR domains. Presumably, the conformation of the TIR domains are altered and create scaffolds for MyD88 recruitment. In this model, MyD88 associates with the TIR domains of receptor and

coreceptor possibly as a dimer and thus introduces death domains (DD) into the active receptor complex. By DD-DD interaction, IRAK1 and IRAK4, IL-1R-associated kinase 1 and 4, are recruited to the IL-1R complex via MyD88 [Martin, Wesche – BBA – 2002]. Besides MyD88, there is another molecule involved in recruitment of IRAK1 to receptor complex: Tollip. Tollip associates with unphosphorylated IRAK1 most likely with the N-terminal, DD containing portion of IRAK1 in cytosol prior to cytokine stimulation. Upon IL-1 stimulation, preformed Tollip-IRAK1 complex is recruited to the receptor, which allows IRAK1 to bind MyD88 via its DD. IRAK4 does not bind IRAK1 directly but is recruited to complex by binding with MyD88. By this way, MyD88 mediates a close interaction of the two related IRAK molecules which is essential to allow IRAK4 to phosphorylate IRAK1. Through three TRAF6 interaction consensus motifs at C-terminal, IRAK1 interacts with and recruits TRAF6 to receptor complex. [Martin, Wesche – BBA – 2002; Janssens, Beyaert – Molecular Cell – 2003; Verstrepen et.al.– Cellular and Molecular Life Sciences – 2008]

In the receptor complex, very rapidly, IRAK4 becomes activated by intramolecular autophosphorylation of three residues (Thr342, Thr345 and Ser346) within its activation loop, which is required for optimal kinase activity of IRAK4. Active IRAK4 phosphorylates IRAK1 on Thr209 and Thr387 in its activation loop, leading to full kinase activity of IRAK1. Subsequently, IRAK1 becomes hyperphosphorylated in its ProST region, probably via autophosphorylation. Since MyD88 and Tollip only bind to non-phosphorylated IRAK1, hyper-phosphorylated IRAK1 is then released from MyD88 and the receptor complex, but not from the downstream signaling molecule TRAF6.

However, many publications reported that kinase activity of IRAK1 is dispensable for IL-1 signaling toward NF-κB activation. In contrast, the role of IRAK1 as an adapter is critical for the formation of signaling complex consisting of MyD88, Tollip, IRAK1, IRAK4, TRAF6 which is crucial for IL-1 induced NF-κB activation. [Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008; Gottipati et.al. – Cellular Signaling – 2008]

IRAK1-TRAF6 leaves receptor complex and interacts with a pre-existing TAK1 (transforming growth factor β-activated kinase 1)-TAB1 (TAK1-binding protein)-TAB2 (or TAB3) membrane-bound complex. TRAF6, an E3 ubiquitin ligase, then auto-ubiquitinates, attaching K63-polyubiquitin to itself. In this process, the E2 conjugating complex Ubc13 and Uev1a is involved in. The ubiquitination of TRAF6 is essential for the direct interaction of TRAF6 with TAK1-TAB1-TAB2(3) complex via TAB2. TAB2 and TAB3 both contain nuclear zinc finger motifs that interact with K63-polyubiquitin chains. TAB1 does not recognize poly-ubiquitin chains, but is implicated in the regulation of the kinase activity of TAK1. In this complex, TAK1 is activated somehow. The TRAF6-TAK1-TAB1-TAB2/3 complex then translocates to the cytosol, whereas IRAK1 stays at the membrane and becomes polyubiquitinated and degraded. [O'Neill – Immunological Reviews – 2008; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]

Activated TAK1 then phosphorylates IKKβ (inhibitor of NF-κB (IκB) kinase β) in IKK complex thus activates this complex. In its turn, IKKβ phosphorylates the NF-κB inhibitor IκB, leading to its ubiquitination and subsequent degradation by the proteasome. This allows NF-κB to translocate to the nucleus and bind to specific promotor sequences. Activated TAK1 also phosphorylates MKK3/4/6 (MAPK kinase). Activated MKKs phosphorylate and activate members of the JNK/p38 MAPK family. These also translocate to the nucleus where they can phosphorylate several transcription factors of the basic leucine zipper (bZIP) family, like c-Jun and c-Fos. [Martin, Wesche – BBA – 2002; Janssens, Beyaert – Molecular Cell – 2003; Verstrepen et.al.– Cellular and Molecular Life Sciences – 2008]

In the other hand, IRAK1 also undergoes K63-linked polyubiquination. Pellino-1 and Pellino-3b are proposed important in this process. These proteins are E3 ligases and they interact with IRAK1. Importantly, their activity is greatly enhanced, at least *in vitro*, when phosphorylated by IRAK1 and IRAK4. The role of the kinase activity of IRAK1 and/or IRAK4 might therefore be at least initially to activate Pellinos, such that they can cause K63-linked polyubiquitination of IRAK1. This recruits NEMO to IRAK1, with NEMO binding to polyubiquitin chain. It therefore

appears that there are two mechanisms to activate the IKK complex, one involving recruitment of the complex to IRAK1 and another to TRAF6-TAK1. Both require recognition of K63-linked polyubiquitination, and both appear to be essential. [O'Neill – Immunological Reviews – 2008; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]

Another member of IRAK family, IRAK2, appears to compensate for IRAK1 at least partially in signaling events mediated by the IL-1R and TLR families. IRAK2 can associate with MyD88, TRAF6 and activates NF-κB dependent reporter gene. IRAK2, instead of IRAK1 can also interact with TLR adaptor molecule Mal/TIRAP. Dominant negative IRAK2 can block Mal/TIRAP-induced signaling while dominant negative IRAK1 fails to do so. Thus, IRAK2 may selectively be recruited by Mal/TIRAP to participate in subsequent NF-κB activation. Furthermore, IRAK2 was shown to be involved in TRAF6 ubiquitination and NF-κB activation [Ringwood, Li – Cytokines – 2008]. Since IRAK2 lacks kinase activity, it covers only adapter function of IRAK1 in IL-1R and TLR signaling pathway [Gottipati et.al. – Cellular Signaling – 2008]

The fact that IL-1 and LPS induced NF-κB activation is not abrogated completely in TAK1 deficient cells suggests the existence of TAK1-independent pathways. MEKK3 (mitogen-activated protein kinase kinase kinase 3) is a good candidate because IL-1-induced NF-κB activation is impaired in MEKK3 deficient cells. In addition, IL-1-induced IκBα phosphorylation is only completely abolished when both TAK1 and MEKK3 are impaired. The TAK1-independent, MEKK3-dependent pathway involves IKKγ phosphorylation and IKKα activation. Interestingly, this pathway results in NF-κB activation through IκBα phosphorylation and dissociation from NF-κB, but does not cause IκBα degradation. Furthermore, MEKK3 interacts with IRAK1 and TRAF6 upon IL-1 stimulation. Protein kinase C (PKC) is also likely a candidate for substituting TAK1. Indeed, depletion of atypical PKC or p62, an atypical PKC-interacting protein, inhibits NF-κB, but not JNK activation in response to IL-1 or TRAF6 overexpression. In addition, p62 specifically interacts with TRAF6 upon IL-1 stimulation through binding to polyubiquitin chain of

TRAF6 [Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]. Finally, ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) is another TRAF6-interacting protein that was shown to be involved in LPS- and IL-1-induced signaling. Overexpression of a dominant-negative mutant or RNA interference mediated knock-down of ECSIT expression abolishes LPS- and IL-1-mediated NF-κB activation. The exact function of ECSIT, is however, still unclear. [Martin, Wesche – BBA – 2002; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]
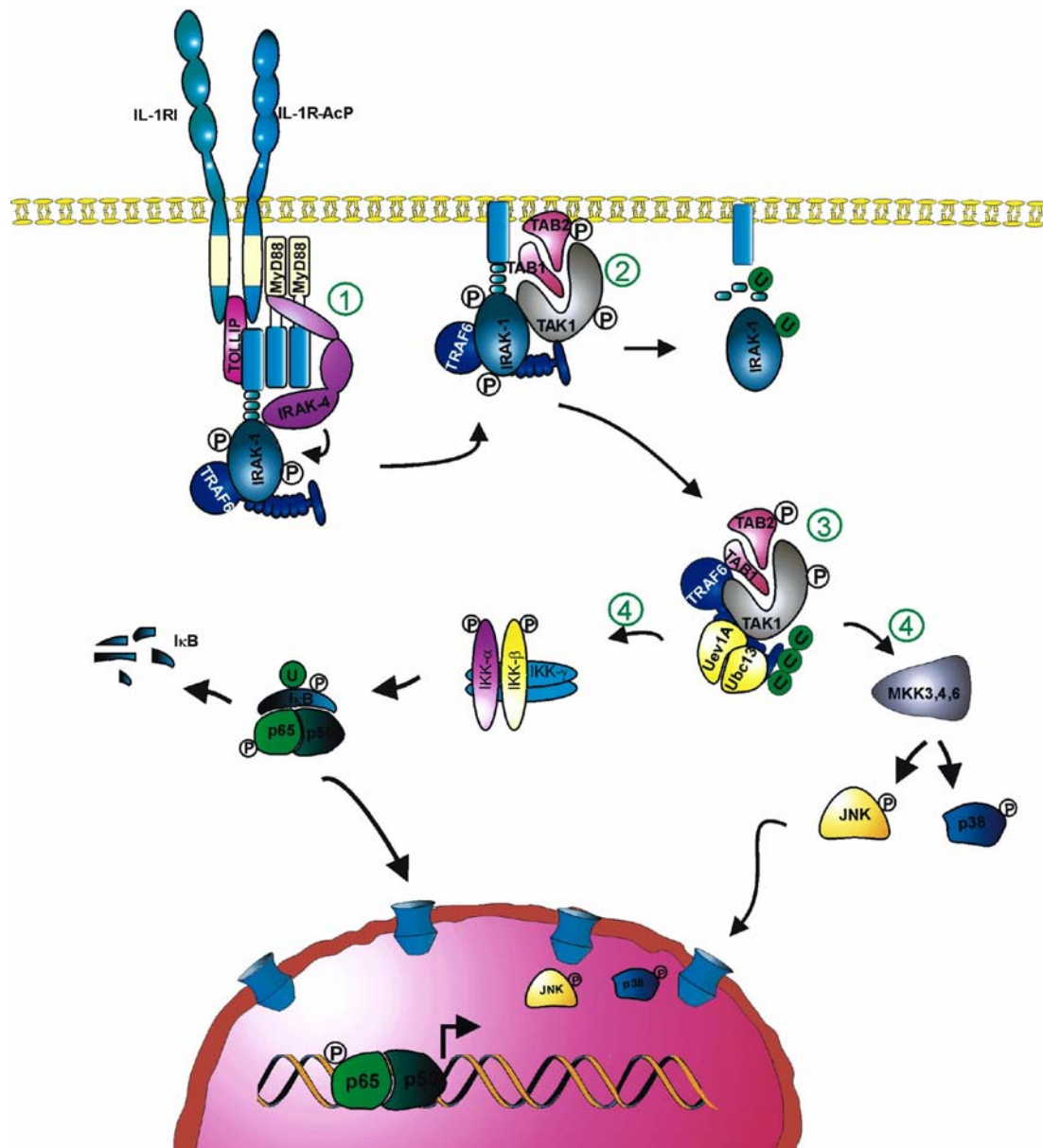

Figure 1.2: Overview of IL-1 signaling pathway
[Janssens, Beyaert – Molecular Cell – 2003]

1.6.2. LPS signaling pathway

In contrast to IL-1 signaling, the introduction of LPS to TLR4 requires additional components. LPS in complex with LPS-binding protein (LBP) binds CD14 on the cell membrane, which transfers LPS to MD2 and TLR4 leading to homologous dimerization of TLR4. MyD88 is then recruited to TLR4 indirectly through TIR domain – TIR domain interaction with another adapter Mal/TIRAP (MyD88 adaptor like / TIR domain-containing adaptor protein). Mal contains a phosphatidylinositol 4,5-bisphosphate (PIP2)-binding domain localizing it to the plasma membrane and a TIR domain in C-terminal. Upon LPS stimulation, Mal becomes tyrosine phosphorylated by Bruton's tyrosine kinase (Btk) which is necessary for NF-κB activation by LPS in a not yet defined mechanism. In a similar way with IL-1R signaling, MyD88 recruits the complex of Tollip-IRAK1, IRAK4 and TRAF6 to LTR4 complex, but in addition, IRAK1 interacts with BCL10 (scaffolding protein B-cell leukemia/lymphoma 10). After the phosphorylation of IRAK1, the complex IRAK1-TRAF6-BCL10 is released from receptor and associates with pre-existing TAK1-TAB1-TAB2/3 membrane bound complex. Pellino2 also participates to the complex via interaction with IRAK1 and BCL10. Then BCL10-Pellino2-TRAF6-TAK1-TAB1-TAB2/3 complex translocates to cytosol while IRAK1 is ubiquitinated and degraded. In cytosol, TRAF6 becomes poly-ubiquitination followed by the activation of TAK1 leading to activation of NF-κB and MAPK JNK/p38. The BCL10-interacting protein MALT1 (mucosa-associated lymphoid tissue 1) also participates to this process and it was shown to be essential in the LPS-signaling cascade towards NF-κB. [Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]

Besides the MyD88-dependent pathway described above, there is another TLR4 signaling pathway termed MyD88-independent/TRIF-dependent pathway. This MyD88-independent signaling pathway involves the recruitment of TRAM (TRIF-related adapter molecule, also known as TICAM2) and TRIF (TIR domain-containing adapter-inducing IFNβ, also known as TICAM1). Upon the engagement of LPS to TLR4, TRAM and then TRIF are recruited to TIR domain of TLR4. In

receptor complex, N-terminal portion of TRIF interacts with and activates IKK related kinase TBK1 (TANK-binding kinase 1) and IKKε resulting in dimerization and phosphorylation of the transcription factor IFN regulatory factor 3 (IRF3), which binds and activates type I IFN promoters in the nucleus. TRIF also initiates signaling toward NF-κB by interaction with, and thus activation, TRAF6. N-terminal portion of TRIF can interact directly with TRAF6 while C-terminal portion of TRIF does so indirectly via RIP1 (receptor interacting protein 1). [Akira, Takeda – Nature – 2004; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008] TLR4 MyD88 dependent pathway and TRIF dependent pathway have recently been shown to signal from two locations. At the plasma membrane, TLR4 first induces Mal/MyD88 signaling. It then appears to traffic to the endosome, where it recruits TRAM/TRIF and initiates TRIF dependent pathway. This capacity for signaling from two locations appears to be unique to TLR4 among the TIR domain-containing receptors. [O'Neill – Immunological Reviews – 2008; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]

1.7. TNFα signaling pathway

Tumor necrosis factor alpha (TNFα) is a multifunctional cytokine playing a key role in apoptosis and cell survival as well as in inflammation and immunity. TNFα acts via two distinct receptors TNFR1 and 2 (TNF receptor 1 and 2). Although the affinity for TNFα of TNFR2 is five times higher than that of TNFR1, the latter initiates the majority of the biological activities of TNFα. TNFR1 (p60) is expressed on all cell types, while TNFR2 (p80) expression is mainly confined to immune cells. The major difference between the two receptors is the death domain (DD) of TNFR1 that is absent in TNFR2. In contrast, TNFR2 contains consensus motifs for TRAF signaling proteins binding which TNFR1 does not have. [van Horssen et.al. – The Oncologist – 2006; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]

TNFα, in homotrimer form, binds to TNFR1 and induces the trimerization of TNFR1. Silencer of death domain (SODD) protein is released and intracellular tails

of TNFR1 becomes docking place to recruit DD containing adapter proteins. First, TRADD (TNFR-associated death domain) binds to TNFR1 by DD-DD interaction and, in its turn, TRADD recruits TRAF2 and RIP1 (receptor interacting protein 1) which rapidly signal NF-κB activation. At later time, the internalization of TNFR1 occurs followed by the dissociation of TRADD-TRAF2-RIP1 complex from receptor. From endosome, TNFR1 associates with another DD containing adapter FADD (Fas-associated death domain) which binds itself to pro-caspase-8 and subsequently activates this caspase. This activation initiates a protease cascade leading to apoptosis involving the mitochondria with caspases as key regulators. [van Horssen et.al. – The Oncologist – 2006; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]. It is unclear how the TNFα-induced life-death pathway is regulated. It is possible that the life pathway is activated first leading to expression of NF-κB-regulated genes such as c-FLIP (cellular FLICE-like inhibitory protein), c-IAP1, cIAP2 (cellular inhibitor of apoptosis protein 1) which inhibit the death pathway. In some cases, when the NF-κB pathway is suppressed, the death pathway is carried out. [Muppidi et. al. – Immunity – 2004]

In receptor complex, TRAF2, an E3 ligase, auto-ubiquitinates attaching K63-link polyubiquitin to itself in the assistance of E2 conjugating complex Ubc13/Uev1A. TRAF2 also ubiquitinates RIP1. Polyubiquitination of RIP1 is not only necessary to recruit TAK1, via binding to TAB2, but is also needed to directly recruit IKKγ. Binding of IKKγ via its NEMO ubiquitin-binding domain to K63-polyubiquitinated RIP1 carries IKK complex to close proximity with TAK1. TAK1 then phosphorylates IKKβ and activates NF-κB activation. Moreover, RIP1 initiates a TAK1 independent/MEKK3 dependent pathway toward NF-κB activation. Upon TNFα stimulation, RIP1 also recruits MEKK3, which phosphorylates IKKβ, leading to activation of the IKK-complex. These two pathways regulate each other's activation and TAK1 is necessary for the regulation of the kinase activity of MEKK3.

Upon TNFα stimulation, TRAF5 interacts with RIP1 but not with TRADD and by this way it also participates in TNFα signaling pathway to activate NF-κB.

Depletion of either TRAF2 or TRAF5 could not abolish NF-κB activation, but it is done when both of them are removed. This implicates that TRAF2 and TRAF5 are redundant in TNFα-induced NF-κB activation. [Chen – Nature – 2005; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]
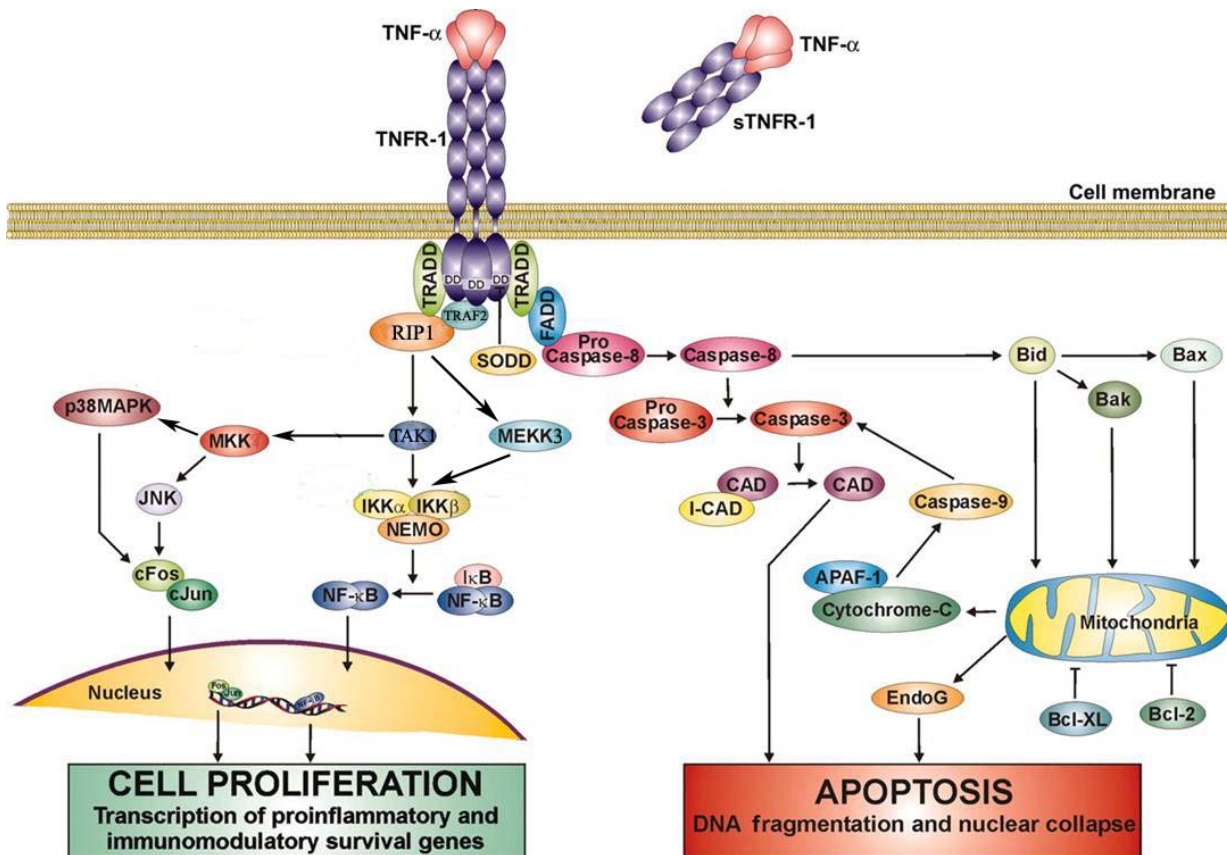

Figure 1.3: Overview of TNFα signaling pathway
[modified from van Horssen et.al. – The Oncologist – 2006]

1.8. IL-6 signaling pathway

IL-6 plays a key role in the maturation of B cells, as well being as a member of three cytokines (IL-6, TNFα and IL-1) that drives the acute inflammatory response. More recent reports have shown how dysregulation of IL-6 signaling contributes to inflammation-associated disease conditions, including obesity and insulin resistance, inflammatory-bowel diseases (IBDs), inflammatory arthritis, sepsis and even cancer. [Naugler, Karin – Trends in Molecular Medicine – 2008]

IL-6 binds specifically to IL-6Rα, non-signaling receptor, leading the recruitment and homodimerization of signaling receptor gp130. Interestingly, in the binding with IL-6, the soluble form of the IL-6Rα lacking the transmembrane and cytoplasmic parts is also capable of forming signal transduction complex. This is one of the rare situations in which a complex of cytokine and soluble receptor can act agonistically instead of antagonistically. [Heinrich et.al. – Biochemical Journal – 2003]

IL-6 signaling is initiated by JAK1 (Janus Kinase 1) [Murray – Journal of Immunology – 2007] that is noncovalently bound to gp130. IL-6-induced gp130 dimerization leads to JAK1 apposition and transphosphorylation on tyrosine residues, releasing their catalytic activity. Active JAK1 then phosphorylates gp130 at tyrosine residues within cytoplasmic domains. Phosphorylated tyrosines of gp130 then provides binding sites for Src-homology-2 (SH2) domain of the STAT3 (signal transducers and activators of transcription) proteins [Murray – Journal of Immunology – 2007] leading to recruitment of STAT3 to gp130. Thus, STAT3 proteins are located in close proximity with JAK1 and therefore be phosphorylated on a single tyrosine residue. Phosphorylated STAT3 form homodimer through reciprocal phosphotyrosine-SH2 interactions and become active and then translocate to nucleus resulting in gene expression program [Levy, Darnell – Nature Reviews – 2002]. This program's primary function is to promote growth and differentiation and prevent apoptosis, and includes the induction of genes encoding Bcl2, Bcl-xL, JunB, cFos, C/EPB, IRF-1 (interferon regulatory factor 1), TIMP (tissue inhibitor of metalloprotease 1), SOCS3 (suppressor of cytokine signaling 3), LPS-binding protein and others. The IL-6–STAT3 signal is regulated negatively by SOCS3, whose gene expression is also induced by STAT3 activation. [Naugler, Karin – Trends in Molecular Medicine – 2008]

The IL-6 signal also activates the MAPK (mitogen-activated protein kinase) pathway, specifically ERK (extracellular signal-related kinase) through the JAK activation of SHP2 (a protein-tyrosine phosphatase). SHP2 then activates Ras, a GTPase leading to activation cascade of MAPK. The importance of this pathway,

however, has not yet to be elucidated fully, especially because most of the effects of IL-6 can be abolished by disrupting the STAT3 pathway. [Naugler, Karin – Trends in Molecular Medicine – 2008]
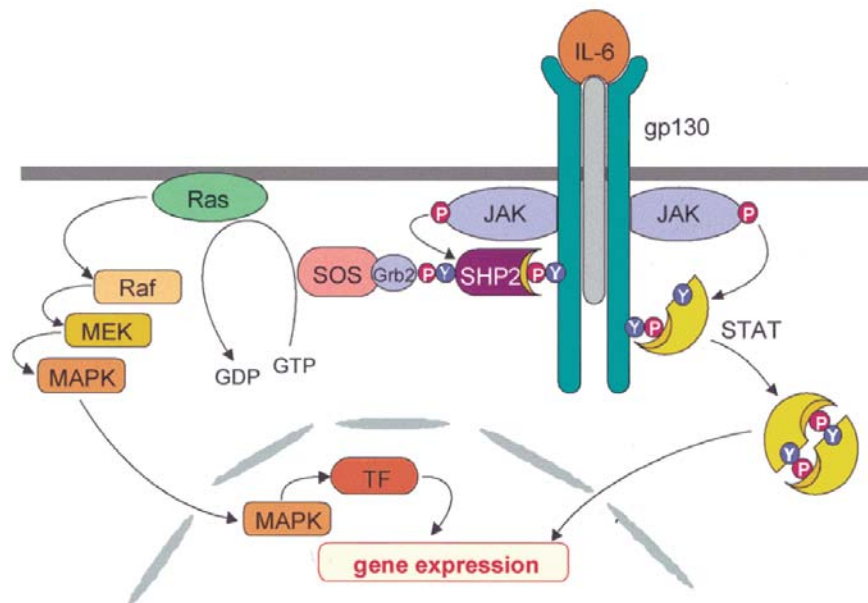
Figure 1.4: Over view of IL-6 signaling pathway

[Heinrich et.al. – Biochemical Journal – 2003]

2. AIM OF THE STUDY

As mentioned in the preface, by the yeast two-hybrid screening AUP1 was identified to interact with IRAK4 and this interaction was also confirmed in mammalian cells. From this point of departure, aim of the present study was to identify the biological function of AUP1 in the IL-1 signaling pathway and it was extended to the LPS-, the TNFα- and the IL-6-activated pathways. Accordingly, the following objectives were addressed.

- The interaction of AUP1 with several functional components of the IL-1 signaling transduction machinery.

- The influence of overexpression of AUP1 on the IL-1β-, LPS-, TNFα-stimulated activation of NF-κB and the IL-6-stimulated activation of STAT3.

- The interaction of AUP1 with the TNFα and IL-6 receptor complexes.

- The influence of silencing of AUP1 on the IL-1β-, TNFα-stimulated activation of NF-κB and the IL-6-stimulated activation of STAT3.

- Investigation of the interference of AUP1 overexpression with the IL-1 and LPS signaling transduction events upstream of the NF-κB activation.

3. MATERIALS and METHODS

3.1. Materials

3.1.1. Plasmids
Table 3.1.1: List of plasmids used in the study

Plasmid	Encoded protein	Sequence	Tag	Vector	Source
pMyD88-FL	hMyD88 (NM_002468.4)	Full length Arg2-Pro309	C-terminal FLAG	pRK5C-FLAG	A kind gift from Dr.Wesche, Tularik Inc., California, USA
pFL-hTRAF6	hTRAF6 (NM_145803.1)	Full length Ser2-Val521	N-terminal FLAG	pRK5	A kind gift from Dr. Cao, Tularik Inc.,California,USA
pRK5-hTollip C-FL	hTollip (NM_019009)	Full length Met1-Pro274	C-terminal FLAG	pRK5C-FLAG	Dr. Christian Kollewe
pCMV-Peli2 N-FL	hPellino2 (NM_021255.2)	Full length Phe2-Asp420	N-terminal FLAG	pCMV-tag2B	A kind gift from Dr.Wesche, Tularik Inc., California, USA
pFL-hIRAK4wt	hIRAK4 (AF445802)	Full length Met1-Ser460	N-terminal FLAG	pRK7N-FLAG	A kind gift from Dr.Wesche, Tularik Inc., California, USA
pFL-hIRAK4†	hIRAK4 kinase inactive (KK213/4AA)	Full length Met1-Ser460	N-terminal FLAG	pRK7N-FLAG	A kind gift from Dr.Wesche, Tularik Inc., California, USA
pFL-hIRAK1wt	hIRAK1 (NM_001569)	Full length Ala2-Ser712	N-terminal FLAG	pcDNA3-FL	Dr. Johannes Knop (formerly Hannover Medical School)
pFL-hIRAK1†	hIRAK1 kinase inactive (K239S)	Full length Ala2-Ser712	N-terminal FLAG	pcDNA3-FL	Dr. Johannes Knop (formerly Hannover Medical School)
pFL-hAUP-1	hAUP1 (NM_181575)	Full length Glu2-Asp410	N-terminal FLAG	pRK7N-FLAG	Dr. Christian Kollewe
phAUP1-FL	hAUP1 (NM_181575)	Full length Met1-Asp410	C-terminal FLAG	pRK5C-FLAG	Dr. Christian Kollewe
pMyc-hAUP-1	hAUP1 (NM_181575)	Full length Glu2-Asp410	N-terminal Myc	pRK7 N-Myc	Dr. Christian Kollewe
phTNFR1	hTNFR1 (NM_001065)	Full length Met1-Arg455	N/A	pRK5	A kind gift from Dr.Kieser-Helmholtz Zentrum Muenchen - Germany
pMyc-hTNFR1	hTNFR1 (NM_001065)	Ile22-Arg455 without leader	N-terminal Myc	pMyc-CMV9 mIL-2 leader	DQ. Nguyen
pMyc-hTRADD	hTRADD (NM_003789)	Glu9-Ala312	N-terminal Myc	pRK5N-Myc	A kind gift from Dr.Kieser-Helmholtz Zentrum Muenchen - Germany
pMyc-hTRAF2	hTRAF2 (NM_021138)	Full length Ala2-Leu501	N-terminal Myc	pRK7N-Myc	DQ. Nguyen
pFL-hIL-1RI	hIL-1RI (NM_000877)	Full length Lys2-Gly569	N-terminal FLAG	pFLAG-CMV-1	A kind gift from Dr.Wesche, Tularik Inc., California, USA
phIL6R-HA	hIL-6R (NM_000565.2)	Full length Met1-Arg468	C-terminal HA	P409B	A kind gift from Prof.Grötzinger – Christian-Albrechts Uni – Kiel – Germany
phgp130-FL	hgp130 (NM_002184.2)	Full length Met1-Gln918	C-terminal FLAG	pcDNA3.1 (-) Zeo	A kind gift from Prof.Grötzinger – Christian-Albrechts Uni – Kiel – Germany
p3xNFκB -Luc	NFκB-dependent firefly luciferase	N/A	N/A	pGL3-Basic	N/A
pGL3-α₂M-215Luc	STAT3-dependent firefly luciferase	N/A	N/A	N/A	A kind gift from Prof.Schaper – RWTH – Aachen – Germany

3.1.2. Expression plasmid cloning

- High pure **RNA isolation** kit (Roche, Mannheim, Germany)

- **Reverse transcription**: Oligo d(pT)$_{18}$ (Fermentas #S1326S) 50µM in DEPC H$_2$O; dNTP mixture 10mM for each (Fermentas #R0181); 5x buffer M-MuLV Reverse Transcriptase; RevertAid H Minus M-MuLV Reverse Transcriptase 200U/µl (Fermentas #EP0451)

- **PCR**: 10x buffer Pfu DNA polymerase + MgSO$_4$; Pfu DNA polymerase (Fermentas #EP0501)

- **DNA electrophoresis**: 100bp DNA ladder (Fermentas #SM0321); 10x DNA-loading buffer (5mM Tris-HCl - pH 8.0, 0.0025% Bromphenolblue, 0.0025% Xylen Cyanol, 5% Glycerol); 1x TAE buffer (40mM Tris, 1mM EDTA, 20mM acetic acid); ethidium bromide (#2218.1, Roth, Karlsruhe, Germany) solution 1µg/ml in 1xTAE buffer.

- **DNA purification**: NucleoSpin® Extract II (Macherey-Nagel GmbH & Co. KG, Düren Germany)

- **Restriction endonuclease digestion**: BamHI and 10x buffer BamHI (Fermentas #ER0051), NotI (Fermentas #ER0591), SalI and 10x buffer Orange (Fermentas #ER0641)

- **Ligation**: 10x buffer T4 DNA ligase; T4 DNA ligase 5u/µl (Fermentas #EL0011)

- **Plasmid DNA isolation kits**: JETQUICK Plasmid Spin Kits; Jetstar 2.0 Plasmid purification Midi kit; Jetstar 2.0 Plasmid purification Maxi kit (GENOMED GmbH, Löhne Germany).

3.1.3. Bacterial culture

- *E coli* **strain: XL1-Blue**

- **LB medium:** 171mM NaCl, 10g/L trypton (#8952.3, Roth, Karlsruhe, Germany), 5g/L yeast extract (#2363.3, Roth, Karlsruhe, Germany); pH 7, autoclaved

- **LB agar:** 1.5% bacto-agar (#5210.3, Roth, Karlsruhe, Germany) in LB medium; autoclaved, antibiotic added when needed

- 50x addditives for LB: 625mM $MgCl_2$, 625mM $MgSO_4$, 1M glucose; 0.2μm sterile filtered

- 1000x Ampicillin: 50 mg/ml Ampicillin sodium salt (#K029.1, Roth, Karlsruhe, Germany) in water; 0.2μm sterile filtered

- 1x TSS: 50mM $MgCl_2.6H_2O$, 10% Polyethylenglycol 6000-8000 (#33137, Serva-Feinbiochemica GmbH&Co-Heidelberg), 5% DMSO (#A994.2, Roth, Karlsruhe, Germany) in LB medium; 0.2μm sterile filtered

3.1.4. Mammalian cell culture

- Cell lines:

HEK293RI: human embryonic kidney 293 cell stably expressing hIL-1RI

mouse KeratinoRI-/- : keratinocyte derived from IL-1RI knock-out mouse

HepG2: human hepatocellular carcinoma cell

- Medium: DMEM high glucose (#E15-009), 10% FCS (#A15-151), 2mM L-Glutamin (#M11-004, all PAA, Coelbe, Germany)

- 10x Trypsin/EDTA (#L11-003, PAA, Coelbe, Germany), 1x solution was prepared in DPBS

- 100x Penicillin-Streptomycine (#P11-010, PAA, Coelbe, Germany)

- Dulbecco's phosphate buffered saline (DPBS) (#D5652, Sigma-Alrich, Steinheim, Germany)

- Trypan blue 0.4% (#T8154, Sigma, Steinheim, Germany)

- (4, 5-dimethylthiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (**MTT)** (#M2128, Sigma, Steinheim, Germany) 5mg/ml in PBS

3.1.5. Transfection reagents:

- Polyethylenimine (PEI) (#40,872-7, Aldrich) 1mg/ml in water pH 7 for plasmid transfection.

- DharmaFECT #1 (#T-2001-01, Dharmacon, Thermo Scientific) for siRNA transfection.

3.1.6. Cell lysis and protein quantification

- PBS: 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4, pH 7.4 with HCl, autoclaved if required.

- 10x Cao lysis buffer: 500mM HEPES (acid free), 2.5M NaCl, 200mM β-Glycerophosphat, 50mM Na_2-4-Nitrophenylphosphat.$6H_2O$, 10mM $Na_2EDTA.2H_2O$. pH7.9

- 1x Cao lysis buffer : 10% (v/v) 10x Cao lysis buffer, 10% (w/v) glycerol, 0.5% (w/v) IGEPAL=NP-40 (#I3021, Sigma)

- 1x Cao lysis buffer with additives: 40μl 25x Protease inhibitors complete (#11873580001, Roche, Mannheim, Germany), 10μl DTT 500mM (#6908.2, Roth, Karlsruhe, Germany), 10μl 100x phosphatase inhibitor were added in 1ml 1x Cao lysis buffer.

- 100x Phosphatase inhibitor:100mM NaF, 100mM Na_3VO_4, 500mM β-glycerophosphat, 200mM Na_2MoO_4, 50mM $Na_4P_2O_7$, pH 10

- KE buffer: 4% (v/v) HEPES 0.5M - pH7.9, 8% (v/v) NaCl 5M, 0.2% (v/v) EDTA 0.5M - pH8, 0.2% (v/v) EGTA 0.5M - pH7.6, 1mM DTT, 1mM PMSF (#P7626-5G, Sigma)

- Bradford reagent: Bio-Rad protein assay (#500-0006, Bio Rad Laboratories, Munich, Germany)

- BSA fraction V (#8076.2, Roth, Karlsruhe, Germany)

3.1.7. Immunoprecipitation, kinase assay, SDS-PAGE and Western blot

- Anti-FLAG M2 agarose beads (#A2220, Sigma, Steinheim, Germany), stored in PBS 50%glycerol, 0.02% NaN3, -20°C

- Protein G sepharose beads (#17-0618-02, GE Healthcare, Freiburg, Germany), stored in 20% ethanol, 4°C

- Protein A sepharose beads (#17-0974-01, Amershame), stored in 20% ethanol, 4°C

- 3x Laemmli buffer: 52.7mM Tris pH 6.8, 6% SDS, 30% Glycerol, 15% β-Mercaptoethanol, 0.024% Bromphenolblue

- Table 3.1.2: Polyacrilamide gel (mini gel 7cmx9cm)

Components	Separating gel (5.5ml/gel)				Stacking gel (2ml/gel)
	7%	10%	12.5%	15%	
- H$_2$O	2.2ml	1.74ml	1.28ml	0.83ml	1.22ml
- Tris-HCl 1,5M - pH 8,8	1.38ml	1.38ml	1.38ml	1.38ml	-
- Tris-HCl 1M - pH 6,8	-	-	-	-	0.25ml
- SDS 1%	0.55ml	0.55ml	0.55ml	0.55ml	0.2ml
- AA/Bis-AA 30%/0,8%	1.38ml	1.83ml	2.29ml	2.75ml	0.33ml
- TEMED (#2367.1, Roth)	8μl	8μl	8μl	8μl	5μl
- APS 40%	8μl	8μl	8μl	8μl	5μl

AA/Bis-AA: Acrylamide/Bis-Acrylamide (#3029.1, Roth, Karlsruhe, Germany)

APS: ammonium persulfate (#A6761, Sigma)

- Electrophoresis buffer: 192 mM Glycin, 25 mM Tris Base - pH ~8.5 -, 0.1% SDS.

- Blotbuffer: 192mM Glycin, 25 mM Tris Base - pH ~8.5 -, 10% Methanol.

- TBS/T: 20mM Tris, 137mM NaCl - pH: 7.6 with HCl -, 0.1% Tween-20.

- **PAGE ruler** prestained protein ladder (Fermentas, Germany)

- **PVDF membrane** (741260, Macherey-Nagel GmbH & Co. KG, Düren Germany)

- Table 3.1.3: Primary antibodies and their dilution used in Western blotting

Epitope	Source	Species from	Dilution	Buffer
Flag (bioM2)	Sigma-Aldrich F9291	Mouse	5μg/ml	TBS/T
GAPDH	Santa Cruz sc-25778	Rabbit	1:800	5% MP in TBS/T
Phospho-STAT3-Tyr705	Santa Cruz sc-8059	Mouse	1:100	5% MP in TBS/T
phosphoSTAT3-Ser727	CST #9136S	Mouse	1:1,000	5% MP in TBS/T
STAT3	CST #9132	Rabbit	1:5,000	5% BSA in TBS/T
Phospho-JNK	Becton Dickinson BD #612540	Mouse	1:500	5% MP in TBS/T
JNK	Becton Dickinson BD #610627	Mouse	1:500	5% MP in TBS/T
Phospho-p38	CST # 9211S	Rabbit	1:1,000	5% BSA in TBS/T
p38	CST #9212	Rabbit	1:4,000	5% BSA in TBS/T
Phospho-IκB	CST #9246	Mouse	1:2,000	5% MP in TBS/T
IκB	CST #9242	Rabbit	1:1,000	5% BSA in TBS/T
c-Myc (clone A-14)	Santa Cruz sc-789	Rabbit	1:2,000	1% MP in TBS/T
AUP1	Sigma HPA007674	Mouse	1:10,000	5% MP in TBS/T
IRAK1 (clone 2A9)	Tularik, CA, USA	Mouse	1:10,000	1% MP in TBS/T
hIL-1RI	Rockland 109-401-304	Rabbit	1:2,000	5% MP in TBS/T
HA (clone 12CA5)	Roche 1583816	Mouse	1:2,000	5% MP in TBS/T

- Table 3.1.4: Secondary antibodies / reagents used in Western blotting

Secondary reagent	Source (Concentration)	Dilution	Buffer
Streptavidin-HRP conjugate	Dianova (0.5 mg/ml)	1:10,000	TBS/T
HRP conjugated Mouse anti Rabbit IgG (light chain)	Dianova (0.8 mg/ml)	1:10,000	5% MP in TBS/T
HRP conjugated Goat anti Rabbit IgG (heavy & light chain)	Jackson ImmunoResearch (0.8 mg/ml)	1:10,000	5% MP in TBS/T
HRP conjugated Mouse anti Goat IgG (heavy & light chain)	Jackson ImmunoResearch (0.8 mg/ml)	1:10,000	5% MP in TBS/T
HRP conjugated Rabbit anti Mouse IgG (heavy & light chain)	Jackson ImmunoResearch (0.8 mg/ml)	1:10,000	5% MP in TBS/T

MP: skim Milk Powder; BSA: Bovine Serum Albumin

- ECL (enhanced chemiluminescene) kit (UP99619A, Interchim - Uptima, France).

- Stripping buffer: 25mM glycin – pH 2 with HCl, 1% SDS

- 5x Kinase Buffer: 100mM HEPES (Acid free), 750mM NaCl, 25mM $MgCl_2 \cdot$ 6 H_2O - pH6.5 - , 25mM $MnCl_2 \cdot$ 4 H_2O

3.1.8. Reporter gene assay

- 5x Passive lysis buffer (E1941, Promega)

- Luciferase substrate solution: 436μM D-Luciferin (Acid free – 54568, Biomol), 436μM NaOH (Luciferin in 5 ml water + NaOH solution drop by drop), 20mM Tricin, 2.67mM $MgSO_4 \cdot 7H_2O$, 1.07mM Magnesiumcarbonathydroxid·5H_2O, 33.3mM DTT, 530μM ATP (Disodiumsalt Grade I - A2383, Sigma), 290μM Coenzym A (Trilithiumsalt Dihydrat MP – 100493, Biomedicals).

3.1.9. ELISA (Enzyme-Linked ImmunoSorbent Assay)

- 0.1M sodium carbonate buffer: 8.4g $NaHCO_3$, 3.56g Na_2CO_3/Litter H_2O, pH 9.5

- ELISA substrate buffer: 0.1M Na acetat, 0.1M citric acid - pH 4.9

- ELISA substrate solution: 9ml substrate buffer + 1ml TMB solution (1mg/ml in DMSO) + 15μl H_2O_2 solution (3%)

- 3,3',5,5'-Tetramethyl-Benzidine **(TMB)** (#T2885, Sigma)

- ELISA kits:

     human IL-8 ELISA antibody set (21339088, ImmunoTools)

OptEIA mouseIL-6 set kit (555240, BD Biosciences)

Transciption factor ELISA kit for NFκB-p65 (EK1120, Panomics)

3.1.10. Small-interfering RNA (siRNA) and quantitative PCR

- **ON-TARGET plus huGAPDH control siRNA** (#D-001830-01-05, Dharmacon RNAi Technologies, Thermo scientific)

- **ON-TARGETplus SMARTpool siRNA for hAUP1** (#L-012410-01-0005, Dharmacon RNAi Technologies, Thermo scientific)

- **ON-TARGETplus nontargeting siRNA #1** (#D-001810-01-05, Dharmacon RNAi Technologies, Thermo scientific)

- **siRNA buffer** (20mM KCl, 6mM HEPES, 0.2mM $MgCl_2$ - set pH 7.5 with 2M KOH)

- **2x SYBR Green master mix** (#AB-1166/B, Thermo Scientific)

- **ROX** reference dye (Stratagene)

3.1.11. Cytokines and stimulus

- **rhIL-1β** was a kind gift from Dr. D. Boraschi, Institute for Biomedical Technology, Pisa, Italy).

- **rhTNFα** was a kind gift from BASF AG, Ludwigshafen, Germany.

- **rhIL-6** was purchased from PBH (pharma biotechnologie hannover, 3000 Hannover, Germany)

- **LPS** was purchased from Difco Laboratories, USA.

3.2. Methods

3.2.1. Molecular biology techniques used in the cloning procedure

First of all, mRNA encoding protein of interest was obtained from mammalian cells which express that protein by RNA isolation technique. mRNA was conversed to cDNA by reverse transcription (RT). Then, cDNA was used as template to amplify gene of interest using polymerase chain reaction (PCR). Size of PCR product was verified with agarose gel electrophoresis. Before further steps, PCR product was

purified to eliminate components of PCR reaction and purified PCR product was quantified with a spectrophotometer. Purified PCR product was digested with suitable restriction endonuclease enzymes. Digested product was purified again. In parallel, the backbone vector was also digested with the same restriction endonucleases and purified by agarose gel electrophoresis. The ligation was performed to insert digested purified PCR product of gene of interest into digested vector. Ligation mixture then was transformed to *E coli* and *E coli* colonies taking up plasmid were screened on antibiotic selective agar medium. Survival colonies of *E coli* on selective agar medium were picked up and expanded. Plasmids from *E coli* clones were isolated. The accuracy of recombinant plasmid was verified by digestion of plasmid clones with the same restriction endonucleases used in digestion of PCR product and backbone vector. Gene of interest in one correct clone was finally checked by sequencing.

3.2.1.1. RNA isolation from mammalian cells

Total RNA from mammalian cells was isolated using High pure RNA isolation kit (Roche). $1x10^6$ cells in 200µl PBS were lysed by addition of 400µl Lysis/Binding buffer and vortexed for 15 seconds. The cell lysate was transferred to a High pure filter tube and centrifuged at 8000 x g for 15 seconds. The flowthrough liquid was discarded. 10µl of DNase I solution (18.2U/µl) in 90µl of DNase incubation buffer was applied on the filter and incubated for 15 minutes at room temperature to digest bound DNA. After DNA digestion, 500µl of wash buffer I was added to the filter tube and centrifuged at 8000 x g for 15 seconds. Washing step was repeated with 500µl of wash buffer II. To remove completely wash buffer, 200µl of wash buffer II was applied to filter tube and then was eliminated by centrifuge at 13,000 x g for 2 minutes. RNA bound in the filter was eluted with 50µl of elution buffer and centrifuged at 8000 x g for 1 minute. Isolated RNA was stored at -80°C.

3.2.1.2. Reverse transcription

mRNA in isolated total RNA was conversed to cDNA with reverse transcription procedure. A reverse transcription reaction was set up as following:

- Total RNA: 1µg

- Oligo d(pT)$_{18}$ 50μM: 1μl

- DEPC-treated H$_2$O to 6.5μl

The mixture was incubated at 70°C for 5 minutes. Other components were added to reaction:

- 5x reaction buffer for M-MuLV Reverse Transcriptase: 2μl

- dNTP mixture 10mM for each : 1μl

The reaction was incubated at 37°C for 5 minutes. Finally, 0.5μl of RevertAid H Minus M-MuLV Reverse Transcriptase 200U/μl (Fermentas #EP0451) was added to reaction. Reaction was incubated at 42°C for 60 minutes and then at 70°C for 10 minutes. cDNA was stored at -80°C.

3.2.1.3. Polymerase chain reaction (PCR) for amplification of cloning target genes

Polymerase chain reaction (PCR) for amplification of cloning target genes was established with components listed here:

- Template: 2μl of cDNA synthesized in reverse transcription as described above was used as template in PCR. In other cases, 100pg of expression plasmid of the target protein if it had been cloned was introduced to PCR for template (Table 3.1.1)

- Primers: 2.5μl of corresponding forward primer solution (10μM) and reverse primer solution (10μM) were added to reaction. Primers were designed with an adequately complementary sequence for target gene and a specific extra sequence for restriction endonuclease at 5' end. Sequences of primers were described in Table 3.2.1.

- dNTP mixture 10mM for each: 1μl

- 10x reaction buffer for Pfu DNA polymerase with MgSO$_4$: 5μl

- Pfu DNA polymerase 2.5U/μl (Fermentas #EP0501): 2μl

- H$_2$O was added to 50μl in total.

Table 3.2.1: Sequence of primers used in PCR for cloning target genes

Plasmid	Gene	Template	Forward primer (5'-3')	Reverse primer (5'-3')	Annealing temp.(^{o}C)
pMyc-hTNFR1	hTNFR1	phTNFR1	AAGCGGCCGCA**ATATA CCCCTCAGGGGTTATT**	AAGGATCC**TCATCTGAG AAGACTGGGCGCG**	56
pMyc-hTRAF2	hTRAF2	cDNA – HEK293	AATGTCGACA**GCTGCA GCTAGCGTGACCCC**	AAGCGGCCGC**TTAGAGC CCTGTCAGGT**	56

Bold letters: complementary sequence for target genes

Thermal profile for PCR with Pfu DNA polymerase was performed as following:

- Initial DNA denaturing step: 95°C for 2 minutes

8 cycles
- DNA denaturing step: 95°C for 45 seconds
- Primer annealing step: specific temperature for each pair of primers listed in Table 3.2.1 was established for 30 seconds
- Prolongating step: 72°C for 2 minutes

27 cycles
- DNA denaturing step: 95°C for 45 seconds
- Primer annealing step: specific temperature for each pair of primers listed in Table 3.2.1 was established for 30 seconds
- Prolongating step: 72°C for 2 minutes and 30 seconds

- Finishing step: 72°C for 10 minutes

PCR products were stored at -20°C.

3.2.1.4. Analysis of DNA with agarose gel electrophoresis

PCR products were analysed by agarose gel electrophoresis to confirm whether they were in predicted size.

10µl of PCR product was mixed with 1µl of 10x DNA loading buffer. Samples were loaded to 1% analysis agarose gel in 1x TAE buffer. 100bp DNA ladder (Fermentas #SM0321) was run with samples in the same gel. Electrophoresis was carried out at 100mA, 100V in 1x TAE buffer. Post-electrophoresis agarose gel was stained in ethidium bromide solution (1µg/ml in 1x TAE) for 20 minutes. Stained DNA bands were visualized and recorded using Syngene system.

3.2.1.5. Purification of PCR products and restriction endonuclease digested products

Before further steps, PCR products in correct size shown in agarose electrophoresis were purified to remove PCR reaction components using NucleoSpin® Extract II kit (Macherey-Nagel).

1 volume of PCR product was mixed with 2 volume of NT buffer. Samples were loaded to NucleoSpin Extract II column which had been placed into a collection tube and centrifuged at 11,000 x g for 1 minute. The flowthrough was discarded. DNA-binding silica columns were washed with 600µl of NT3 buffer and were centrifuged at 11,000 x g for 1 minute. The flowthrough was removed and columns were centrifuged one more at 11,000 x g for 2 minutes to get rid of any residue of NT3 buffer. DNA bound in the silica membranes was eluted with 30µl of elution buffer NE, incubated at room temperature for 1 minute and centrifuged at 11,000 x g for 1 minute.

Prior to insertion of target genes to backbone vector by ligation, purified PCR products were digested with appropriate restriction endonucleases. Digested products were purified again to eliminate endonucleases and small DNA fragments which can interfere with subsequent ligation reaction. The purification of digested products was performed with the same procedure applied for PCR products.

In the case of backbone vectors, which were digested with restriction endonucleases as well, digested vectors were dissolved in 0.8% pure agarose gel by electrophoresis followed with ethidium bromide staining as described above. The bands of digested vectors were excised from agarose gel. Gel slices were weighed and solubilized in 200µl of NT buffer for every 100mg of agarose gel. Gel slices were incubated in NT buffer at 50°C and vortexed briefly every 2-3 minutes until they are dissolved completely (5-10 min). Samples were loaded to NucleoSpin® Extract II column and the subsequent steps of purification were performed as those for PCR product purification.

3.2.1.6. Quantification of DNA and RNA with spectrophotometer

RNA and DNA samples were diluted in 200µl of double distilled water with suitable dilution factor (50-100).

200µl of diluted nucleic acid samples was applied for optical density (OD) measurement at wave-lengths of 260nm, 280nm and 320nm using Genesys 6 spectrophotometer (Thermo Spectronic). The spectrophotometer was blanked with 200µl double distiled water prior to measurement of samples.

DNA and RNA concentration of samples was calculated as following formulars:

DNA concentration (ng/µl) = $(OD_{260} - OD_{320})$ x 50 x dilution factor

RNA concentration (ng/µl) = $(OD_{260} - OD_{320})$ x 40 x dilution factor

Quality of nucleic acid samples was considered by the ratio between $(OD_{260} - OD_{320})$ and $(OD_{280} - OD_{320})$. This ratio should be from 1.8 to 2.

3.2.1.7. Restriction endonuclease digestion

A restriction endonuclease digestion reaction was carried out with 4 components including DNA substrate, restriction endonucleases (RE), working buffer for RE and double distiled water. DNA substrate was PCR product of target gene or backbone vector. Amount of DNA substrate was 5µg for one 50µl-reaction. DNA substrate was digested with two RE simultaneously. The combination of RE varied from reaction to reaction depending on cloning strategy of each construct. In most of cases, concentration of each RE was 5u/50µl-reaction. In other cases, concentration of RE was altered corresponding to working buffer system as manufacturer's instruction. Working buffer was chosen for the optimal activity of both REs used in reaction according to manufacturer's instruction. Double distilled water was added to fill up the reaction to 50µl. Reactions were incubated at 37°C for 3 hours.

Criteria of RE digestions performed in this study are listed in Table 3.2.2

Table 3.2.2: Restriction endonucleases and working buffer for cloning

Construct	Retriction endonucleases (activity/50µl reaction)	Working buffer
pMyc-hTNFR1	NotI (10u) and BamHI (5u)	1x BamHI buffer
pMyc-hTRAF2	SalI (5u) and NotI (5u)	1x Orange buffer

After ligation step for insertion of target gene to expression vector, the accuracy of recombinant plasmid was verified by digestion of plasmid clones with the same REs used in digestion of PCR product and backbone vector. 1µg of recombinant plasmid was digested by 1u of each RE in 10µl-reaction with suitable RE working buffer (Table 3.2.2). Digested products were analysed with agarose gel electrophoresis. Accurate recombinant clones would be cleaved into 2 DNA fragments in size of inserted target gene (PCR product) and digested backbone vector.

3.2.1.8. Ligation

To insert target gene to backbone vector, RE digested PCR product and digested backbone vector were mixed in a ligation reaction. Both PCR product and backbone vector were digested with the same REs hence they had compatible sticky ends which can join to each other. The ratio between PCR product and backbone vector was 3:1 in molar. Other components for ligation were 1µl of 10x T4 DNA ligase buffer, 0.4µl of T4 DNA ligase 5u/µl (Fermentas #EL0011) and double distilled water up to 10µl for total reaction. The ligation reaction was incubated at 22°C for 1 hour. The ligation was transformed to *E coli* immediately or was stored at -20°C.

3.2.1.9. Transformation of DNA plasmid to competent XL1-Blue E coli

Before transformation, -80°C-reserved TSS competent XL1-Blue *E coli* was thawed slowly on ice. 1µl of ligation or 100ng of DNA plasmid was added to 50µl of competent XL1-Blue *E coli*. The transformation was mixed by pipetting and was incubated on ice for 30 minutes. Transformation was shocked at 42°C for 45 seconds and then on ice for 2 minutes. 500µl of LB medium with additives (12.5mM $MgCl_2$, 12.5mM $MgSO_4$, 20mM glucose) was added to transformation. Transformation was incubated at 37°C for 1 hour with shaking at 500rpm. 250µl of transformation was plated on 1 dish of appropriate antibiotic selective LB agar and incubated at 37°C for overnight. Survival colonies of XL-1 Blue *E coli* on selective LB agar were picked up and expanded in 3ml of LB medium with antibiotic for plasmid isolation.

3.2.1.10. Plasmid isolation from E.Coli

Plasmid from XL-1 Blue *E coli* cultured overnight in 3ml of antibiotic selective LB medium was isolated with JETQUICK plasmid miniprep spin kit (#400250 – Genomed GmbH) as manufacturer's instruction.

E coli cells were harvested by centrifugation at 5000 x g for 10 minutes. Medium was removed carefully. The bacterial pellet was resuspended with 250µl of solution G1 by vortexing or pipetting. 250µl of solution G2 was added to bacrterial suspension and mixed gently, but thoroughly, by inverting the tube several times. Lysis was incubated at room temperature for 5 min. For neutralization, 350µl of solution G3 was added and mixed gently but thoroughly, by inverting the tube to homogenise suspension. The mixture was centrifuged at room temperature and at 15,000rpm for 10 minutes. The supernatant was loaded to Jetquick spin column and centrifuged at 13,000 x g for 1 minute. The column was washed with 500µl of buffer GX and centrifuged at 13,000 x g for 1 minute. The washing step was repeated with 500µl of buffer G4. After every centrifugation the flowthrough was discarded. To remove completely buffer G4, the column was centrifuged again at 15,000rpm for 1 minute. Bound plasmid in the silica matrix was eluted with 50µl of 70°C-preheated water, incubated for 1 minute and centrifuged at 13,000 x g for 2 minute.

To obtain big yield of plasmid, midiprep or maxiprep was performed using Jetstar2.0 plasmid midiprep kit (#210050 – Genomed GmbH) or Jetstar2.0 plasmid maxiprep kit (#220020 – Genomed GmbH) as manufacturer's instruction. E.Coli in 50µl/200µl (midi/maxi) of overnight culture was pelleted by centrifugation at 6,000 x g for 10 minutes. After complete removal of medium, bacterial pellet was resuspended in 4ml/10ml (midi/maxi) of solution E1. 4ml/10ml of solution E2 (midi/maxi) was added to bacterial suspension to lyse cells. Sample was mixed gently, but thoroughly, by inverting the tube several times and incubated at room temperature for 5 min. For neutralization, 4ml/10ml (midi/maxi) of solution E3 was added and mixed gently but thoroughly, by inverting the tube to homogenise suspension. The mixture was centrifuged at room temperature and at 12,000 x g for

30 minutes. The supernatant was loaded to Jetstar column which had been equilibrated with 10ml/30ml (midi/maxi) of solution E4, lysate was allowed to run by gravity flow. Column was washed with 2 times x 10ml/1 time x 60ml (midi/maxi) of solution E5. Bound plasmid in column was eluted with 5ml/15ml (midi/maxi) of solution E6. Plasmid precipitation was performed by the addition of 3.5ml/10.5ml (midi/maxi) absolute isopropanol and centrifugation at 12,000 x g for 30 minutes at 4°C. Plasmid DNA pellet was washed with 70% ethanol and recentrifuged. Plasmid DNA pellet was air dried and redissolved in suitable volume of 10mM Tris-HCl pH 8. Plasmid solution was stored at -20°C.

3.2.2. Bacterial culture techniques

3.2.2.1. XL1-Blue E coli culture

Plasmid bearing XL1-Blue *E coli* was cultured in LB medium or LB agar plate with addition of appropriate antibiotic (ampicillin) for plasmid maintenance. Liquid culture was incubated at 37°C with shaking at 160rpm for overnight. Agar plate culture was incubated in an incubator at 37°C for overnight.

3.2.2.2. Reservation of plasmid bearing XL1-Blue E coli

750µl of overnight bacterial liquid culture was mixed with 750µl of sterile glycerol 86%. The reservation was frozen and stored at -80°C.

3.2.2.3. Generation of competent XL1-Blue E coli

500µl of fresh overnight XL1-Blue *E coli* culture was transferred to 50ml of LB medium without antibiotic. Culture was incubated at 37°C with shaking at 160rpm. The growth of *E coli* was monitored every hour with optical density (OD) measurement at wave length 600nm. Bacteria were harvested by centrifugation at 4°C and at 5,000 x g for 10 minutes when OD_{600nm} value is from 0.3 to 0.4. After removal of medium, bacterial pellet was resuspended in 5ml of cold, sterile TSS solution (50mM $MgCl_2.6H_2O$, 10% Polyethylenglycol 6000, 5% DMSO in LB medium). 100µl of bacterial suspension was aliquoted to sterile eppendorfs. Aliquotes were frozen directly in liquid nitrogen and stored at -80°C.

3.2.3. Maintenance and culture of mammalian cells

HEK293RI, KeratinoRI-/-, HepG2 cells were cultured in DMEM medium with the addition of 10% FCS and 2mM L-Glutamin at $37^{o}C$, 10% CO_2 in a humidified incubator. Cells were seeded in $75cm^2$-culture flask containing 20ml of culture medium at suitable density and were splitted every 3-4 days. Seeding density for HEK293RI and HepG2 were $2x10^6$ cells/flask and $1x10^6$ cells/flask for KeratinoRI-/-.

For splitting, cells were washed twice with sterile DPBS and treated with 2ml of 1x trypsin/EDTA solution for 2-5 minutes at $37^{o}C$. 8ml of full culture medium was added to terminate activity of trypsin. Cells were resuspended by pipetting. 20µl of cell suspension was stained with 20µl of trypan blue 0.4%. Alive cells which are not stained with trypan blue in suspension was counted using cell counting chamber. Appropriate number of cells was transferred to new culture flask.

For maintainance, cell suspension was centrifuged at 500 x g for 5 minutes to remove culture medium. Cell pellet was resuspended at density of $2x10^6$ cells/ml in FCS with the addition of 10% DMSO. 1ml ($2x10^6$ cells) of reservation was introduced to cryo tube. Cells were frozen at $-80^{o}C$ in an isopropanol-containing box and subsequently in liquid nitrogen.

To thaw cells, frozen cells from liquid nitrogen were thawed immediately at $37^{o}C$. Reservation cells were diluted in 9ml of full culture medium and centrifugated at 500 x g for 5 minutes to remove supernatant. Cell pellet was resuspended in 20ml of full culture medium and cultured as described above.

3.2.4. Transient transfection

HEK293RI, KeratinoRI-/-, and HepG2 cells were transiently transfected with plasmids as indicated in figure legends by using polyethylenimine (PEI) transfection method [Ehrhardt et. al. – Signal Trans – 2006]. A slight optimization of the PEI transfection protocol was performed for each cell line. To optimize the transfection, cells were transfected with EGFP encoding construct and percentage of EGFP expressing cells was monitored by FASC analysis.

In principle, healthy cells were seeded to 94mm-dishes in appropriate density and incubated overnight in culture condition. Plasmids were mixed with PEI (#40,872-7, Aldrich) solution (1mg/ml in water) in DMEM medium free of FCS and glutamin to total volume of 360µl and incubated for 10 minutes at room temperature. The transfection mixture was applied to cells in a 69.4 cm^2 Petri dish in a total volume of 3 ml of culture medium. After 4 hours of incubation in the incubator, 7ml of fresh culture medium was added to the transfected cells in the dish and cells were incubated until assays were performed.

Summary of optimal transfection condition for cell lines:

 * HEK293RI

- Cell density: 3.6x10^6 cells per 69.4 cm^2-culture dish with 10ml of full medium.

- Transfection mixture: 6µl of DNA plasmid solution (1µg/µl), 324µl of medium FCS and glutamin free, 30µl of PEI solution (1mg/ml).

* KeratinoRI-/-

- Cell density: 2.5x10^6 cells per 94mm-culture dish with 10ml of full medium.

- Transfection mixture: 12µl of DNA plasmid solution (1µg/µl), 288µl of medium FCS and glutamin free, 60µl of PEI solution (1mg/ml).

* HepG2

- Cell density: 2x10^6 cells per 94mm-culture dish with 10ml of full medium.

- Transfection mixture: 6µl of DNA plasmid solution (1µg/µl), 306µl of medium FCS and glutamin free, 48µl of PEI solution (1mg/ml).

For other plate formats, all components of transfection were altered according to a corresponding ratio of surface area (Table 3.2.3)

Table 3.2.3: surface area ratio of different plate formats

	94mm-dish (69.4cm^2)	6-well plate (9.6cm^2)	24-well plate (1.82cm^2)	96-well plate (0.28cm^2)
94mm-dish (69.4cm^2)	1	0.14	0.026	0.004
6-well plate (9.6cm^2)	7.23	1	0.189	0.029
24-well plate (1.82cm^2)	38.13	5.27	1	0.154
96-well plate (0.28cm^2)	247.86	34.28	6.5	1

3.2.5. Cell lysis

HEK293RI, KeratinoRI-/-, HepG2 cells transfected with plasmids and stimulated as described in figure legends were lysed as following procedure. Cells in 94-mm culture dish were washed twice with 10ml cold PBS and harvested with a rubber policeman in 500µl of 1x Cao lysis buffer with additives. Cells were incubated for 30 minutes at 4^oC with a gentle rotation. Nuclei were removed from lysate by centrifugation for 10 minutes at 500 x g, 4^oC. Supernatant was harvested and purify with centrifugation for 10 minutes at 13,000rpm, 4^oC to get cytosolic fraction. If nuclear extract is required, nuclear pellet was washed 3 times with 1x Cao lysis buffer without additives, supernatant was discarded by centrifugation for 10 minutes at 500 x g, 4^oC. Then nuclear pellet was sonified in 200µl of KE buffer using a sonificator for 30 seconds, 5x10% intensity. Insoluble fraction was removed from nuclear extract by centrifugation for 20 minutes at 15,000rpm, 4^oC.

3.2.6. Bradford protein quantification method

100µl of cell lysate samples diluted 1:50 or 1:100 in water were pipetted to wells of a flat bottom 96-well plate.

BSA (bovine serum albumin) standard solution was prepared from stock of BSA (2mg/ml) by the addition of 768µl of assay diluent to 32µl of BSA stock to obtain 800µl of BSA solution at concentration of 80µg/ml. Corresponding to sample dilution, assay diluent was 1:50 or 1:100 dilution in water of the lysis buffer used in cell lysate samples. 100µl of standard solutions from 80µg/ml to 0µg/ml (i.e. 80, 60, 40, 30, 20, 10, 5 and 0µg/ml) were applied in duplicate to wells of the assay plate.

Bradford reagent (Bio-Rad protein assay) was diluted 2.5 times in water. 100µl of diluted reagent was added to standard and sample wells. Protein concentration in samples was measured and calculated by an ELISA reader SPECTRAmax® 340 PC384 (OD595nm) using the program for Bradford assay of SOFTmax PRO 4.3 LS software.

3.2.7. Immunoprecipitation and Western blot

HEK293RI, KeratinoRI-/-, HepG2 cells transfected with plasmids and stimulated as described in figure legends were lysed with Cao lysis buffer. 20µl of cell lysate was mixed with 10µl of 3x Laemmli buffer and heated for 10 minutes at 95°C. SDS-PAGE was performed according to the Laemmli procedure [Laemmli – Nature – 1970] using 7.5-15% polyacrylamide gel. Proteins were electrotransferred to PVDF membrane (Macherey-Nagel) at 63mA (for 1 membrane 7x9cm) for 2 hours. Blots were blocked for 1 hour with blocking buffer (5% milk powder in TBS/T). Blocked blots were probed with 10ml solution of appropriate primary antibodies (as listed in Table 3.1.3) for overnight at 4°C on shaker. The blots were washed with TBS/T for 3 times and incubated with 10ml solution of corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies or HRP-conjugated streptavidin (as listed in Table 3.1.4) for 2 hours at room temperature. Blots were washed and subsequently incubated with ECL reagents (Interchim-Uptima) before exposure to chemoluminescence camera (INTAS). Blots were stripped with stripping buffer for 30 minutes, blocked and reprobed with other primary antibodies if needed.

For FLAG immunoprecipitation, 500µl of lysate from cells of 94-mm culture dish was incubated with 10µl of anti-FLAG M2 agarose beads (Sigma) for 18h at 4°C with gentle rotation. Beads were washed for 4 times with 1x Cao lysis buffer without additives and at last with PBS 0.1% IGEPAL. Supernatant was removed by centrifugation for 1 minute at 1000rpm, 4°C. Washed beads were resuspended in 30µl of 3x Laemmli buffer and heated to 95°C for 10 minutes. Immunoprecipitated proteins were analysed with SDS-PAGE and Western blot as described.

With IRAK1 and IL-1RI immunoprecipitation, the same procedure was performed in which 1µl anti-IRAK1 antibody (clone 2A9-Tularik) plus 10µl protein G sepharose (GE Healthcare) and 2µl anti-IL1RI antibody (Rockland 109-401-304) plus 10µl protein A sepharose (Amershame) were used, respectively, instead of anti-FLAG M2 agarose beads.

3.2.8. Forced co-immunoprecipitation and kinase assay

To determine whether AUP1 is phosphorylated by IRAK4 and IRAK1, HEK293RI cells were cotransfected with FLAG-tagged IRAK4 or IRAK1 and FLAG-tagged hAUP1 encoding construct. Cells were lysed and FLAG IP was performed using anti-FLAG M2 agarose beads (Sigma). FLAG-IRAKs and FLAG-AUP1 were forced into close proximity as described previously [Kollewe et.al. – JBC – 2004]. Washed immunoprecipitates were resuspended in a kinase reaction mixture containing 20mM HEPES - pH 6.5, 150mM NaCl, 5mM MgCl2, 5mM MnCl2, 1μM ATP, and 1μCi of [γ-^{32}P]ATP and incubated for 20 min at 30°C. The kinase reaction was stopped by heating at 95°C for 10min in Laemmli buffer. Phosphorylated proteins were separated by SDS-PAGE. The gels were dried, and autoradiographies were performed.

3.2.9. Luciferase reporter gene assay

For studying biological function of AUP1 in signaling transduction by reporter gene assay, HEK293RI, KeratinoRI-/- or HepG2 cells were seeded to wells of 24-well plate at suitable density with DMEM 10%FCS and cultured at 37°C, 10% CO_2. On the next day, cells were cotransfected with p3xNFκB-Luc or pGL3-α_2M-215Luc reporter gene construct and different amounts of pFLAG-hAUP1 construct, DNA concentration was kept constant by addition of empty vector. Transfected cells were either stimulated with rhIL-1β, rhTNFα, LPS, rhIL-6 for 18 hours or were left untreated for control. After stimulation, cells were washed with cold PBS and lysed with 100μl of 1x passive lysis buffer (Promega) for 30 minutes on shaker (300rpm). Cell debris were removed from lysate by centrifugation at 15,000rpm for 1 minute. For luciferase activity measurement, 75μl of luciferase substrate solution was added to 25μl of cell lysates. One second later, light signal was measured for 10 seconds by using a microplate luminometer (MicroLumatePlus LB96V; Berthold Technologies).

For measurement of IL-1β- and TNFα-induced NF-κB activity by reporter gene assay in AUP1-knockdown HEK293RI, 6x10^{4} HEK293RI cells were seeded to

wells of 24-well plate. 24 hours later, 100nM of anti-hAUP1 siRNA or scramble siRNA was transfected to cells. 44 hours post-transfection, cells were transfected with p3xNFκB-Luc reporter gene construct (2μg plasmid /3.6x10^6 cells) by PEI method. Once transfection done (in 4 hours), cells were stimulated either by 100pg/ml rhIL-1β or 10ng/ml rhTNFα for 8 hours or left untreated for control. After stimulation cells were lysed with 100μl of passive lysis buffer for 30 minutes with gentle shaking (300rpm). Cell debris were removed by centrifugation at 15,000rpm for 1 minute. Luciferase activity in lysates was measured as described above.

The same procedure was applied for determination of IL-6-induced activation of α_2M-luciferase reporter gene in AUP1-knockdown HepG2 cells.

3.2.10. Measurement of cytokine production by Enzyme-Linked ImmunoSorbent Assay (ELISA)

HEK293RI or KeratinoRI-/- cells were seeded to wells of 24-well plate at suitable density the day before transfection. Cells were transfected with different amount of pFLAG-hAUP1 construct by PEI method. DNA concentration was kept constant by addition of empty vector. On the next day, transfected HEK293RI and KeratinoRI-/- cells were stimulated by 100pg/ml rhIL-1β, 10ng/ml rhTNFα and 100ng/ml LPS, respectively, for 8 hours. Supernatant was harvested; hIL-8 or mIL-6 in supernatant was measured by ELISA using a set of anti-hIL-8 capture and detection antibodies (Immuno Tools) or OptEIA mIL-6 set kit (BD Biosciences).

ELISA was performed according to manufacturer's instruction. In general, ELISA plate was coated overnight at 4°C with 100μl of capture antibody diluted in 0.1M sodium carbonate buffer pH 9.5 at recommended concentration. Coating buffer was removed and plate was washed with washing buffer (PBS with 0.05% Tween-20). Then, plate was blocked with 200μl of blocking buffer (10%FCS or 5%BSA in PBS for mIL-6 ELISA or hIL-8 ELISA, respectively) for 1 hour at room temperature. After washing step to remove blocking buffer, 100μl of standard and sample solutions were applied to proper wells. Standard was diluted in series of 2 times in DMEM medium 10% FCS. The highest concentration was 300pg/ml and 1000pg/ml

for rhIL-8 (Biosource) and rmIL-6 standard, respectively. Sample supernatant was either diluted 5 times in DMEM 10%FCS for the case of IL-1β-stimulated HEK293RI or undiluted for TNFα-stimulated HEK293RI and LPS-stimulated KeratinoRI-/-.

With hIL-8 ELISA, after 1 hour incubation with standard and samples, plate was washed and 100µl of detection biotin-conjugated antibody diluted in assay diluent (1%BSA, 0.05% Tween-20 in PBS) at recommended concentration was added. After incubation with detection antibody for 1 hour, plate was washed. HRP-conjugated streptavidin solution in assay diluent was applied to wells for 30 minutes.

With mIL-6 ELISA, after 2 hours incubation with standard and samples, plate was washed. 100µl of working dectector including detection biotin-conjugated antibody and HRP-conjugated streptavidin in assay diluent (10% FCS in PBS) was added for 1 hour.

In both cases, after the last washing step to get rid of unbound HRP-conjugated streptavidin, 100µl of substrate solution (90mM Na acetat, 90mM citric acid, 0.1mg/ml TMB, 0.0045% H_2O_2) was introduced to wells. Plate was incubated for 20-30 minutes in dark before the addition of 50µl H_2SO_4 1M to stop reaction. Cytokine concentration was measured using ELISA reader (SPECTRAmax® 340 PC384) and SOFTmax PRO 4.3 LS software.

For measurement of IL-1β/TNFα-induced IL-8 production from AUP1-knockdown HEK293RI cells, $6x10^4$ HEK293RI cells were seeded to wells of 24-well plate. 24 hours later, cells were transfected with 100nM of anti-hAUP1 siRNA or scramble siRNA. 48 hours post-transfection, cells were stimulated either by 100pg/ml rhIL-1β or 10ng/ml rhTNFα for 8 hours or were left untreated for control. Supernantant was harvested and hIL-8 ELISA was performed as described above.

3.2.11. MTT assay

To check whether AUP1 overexpression has an impact on cell viability, $0.5x10^6$ HEK293RI cells or $0.35x10^6$ KeratinoRI-/- cells were plated to wells of 6-well plate

and incubated under culture conditions. On the next day, cells were transfected with pFLAG-hAUP1 construct in increasing amount. DNA concentration was kept constant by addition of empty vector. Transfected cells were trypsinized and reseeded at density of 0.5×10^5 cells per well of 96-well plate. HEK293RI and KeratinoRI-/- cells were stimulated by 100pg/ml rhIL-1β, 100ng/ml LPS, respectively, for overnight or were left unstimulated. Cell viabilities of HEK293RI and KeratinoRI-/- were determined by MTT method.

20μl of MTT solution (5mg/ml in PBS) was added to cells in wells of 96-well plate containing 200μl of culture medium. Cells were incubated with MTT for 4 hours at culture condition. MTT medium was removed carefully from wells and 100μl of isopropanol with 5% formic acid was added. Optical density reflecting mitochondrial activity and thus indirectly quantity of cells in wells was measured with an ELISA reader (SPECTRAmax® 340 PC384) at wave length 570nm.

3.2.12. Detection of DNA-binding activity of NF-κB-p65

3.6×10^6 HEK293RI cells or 2.5×10^6 KeratinoRI-/- cells were seeded to 94mm-culture dishes and incubated overnight in culture condition. 6μg or 12μg of pFLAG-hAUP1 was transiently transfected to HEK293RI or KeratinoRI-/-, respectively. At the same time, instead of pFLAG-hAUP1 plasmid, empty vector was transfected to cells for control. 24 hours later, transfected HEK293RI and KeratinoRI-/- cells were unstimulated or stimulated by 5ng/ml rhIL-1β and 1μg/ml LPS, respectively, for different times from 5 to 180 minutes. After stimulation, cells were lysed in Cao lysis buffer and nuclear extracts were obtained in KE buffer. Activated NF-κB-p65 in nuclear lysates was determined by binding activity to κB-consensus sequence DNA probe using Panomics'TF ELISA kit (EK1120) as manufacturer's instruction. In brief, protein concentration of nuclear extracts was quantified with Bradford method and samples were diluted with nuclear extract dilution buffer to concentration of 2μg protein/μl. 10μl of diluted nuclear extracts were mixed with 40μl binding buffer master mix containing biotin-conjugated κB-consensus sequence DNA probe in wells of 96-well plate. For the binding of activated NF-κB-

p65 to DNA probe, samples were incubated for 30 minutes at room temperature with gently rocking the plate (150rpm). The binding complex was transferred to streptavidin coated assay plate and incubated at room temperature for 1 hour rocking the plate gently (150rpm). Plate was washed with washing buffer to remove unbound molecules. 100µl of a specific anti-p65 antibody solution (1/200 in antibody dilution buffer) was added to wells and incubated for 1 hour. In this step, bound NF-κB-p65 in wells was recognised by anti-p65 antibody. After washing step, 100µl of HRP-conjugated secondary antibody solution (1/200 in antibody dilution buffer) which bind to anti-p65 primary antibody was introduced to wells of assay plate. 1 hour later, plate was washed and 100µl of substrate solution (90mM Na acetat, 90mM citric acid, 0.1mg/ml TMB, 0.0045% H_2O_2) was added to wells. Plate was incubated for 5-15 minutes in dark before the addition of 100µl of stop solution. Optical density reflecting quantity of bound NF-κB-p65 was measured with an ELISA reader (SPECTRAmax® 340 PC384) at wave length 450nm.

3.2.13. Small-interfering RNA (siRNA)–mediated knockdown of AUP1

Small-interfering RNA (siRNA)–mediated knockdown of AUP1 was performed using ON-TARGETplus SMARTpool siRNA for hAUP1 (L-012410-01-0005 – Dharmacon RNAi Technologies – Thermo scientific) and ON-TARGETplus nontargeting siRNA #1 (D-001810-01-05 – Dharmacon RNAi Technologies – Thermo scientific) was used as scramble siRNA control. Anti hAUP1 siRNA pool consists of four siRNAs for target sequences GCACUAUAUGAAUACGCAA;GCAGAUUCGUAGUGCGGAC;GAGCACAU GAAGCGACAAA; CGACCACAACAUAGUCAAU. siRNA transfection was carried out according to manufacturer's instruction with a slight optimization. 24 hours before transfection, $6x10^4$ HEK293RI or HepG2 cells were seeded to wells of 24-well plate in 0.5ml of DMEM medium containing 10% FSC, 2mM L-glutamin. 25µl of anti-hAUP1 siRNA solution or scramble siRNA solution (2µM in siRNA buffer) was mixed with 2µl of transfection reagent DharmaFECT #1 (T-2001-01, Dharmacon, Thermo Scientific) in 73µl of DMEM medium FCS/Glutamin free to

total volume of 100µl. Transfection mixture was incubated for 20 minutes at room temperature. 400µl of full DMEM medium was added to transfection mixture and mixed gently by pipetting. The whole transfection medium (500µl of 100nM siRNA) was applied to each well of 24-well plate which had been removed culture medium. Cells were incubated at $37^{\circ}C$, 10% CO_2 until assays.

For AUP1 mRNA analysis, 24 hours post-transfection, cells were harvested and total RNA from cells was isolated using High pure RNA isolation kit (Roche). cDNA was obtained from 1µg of total RNA by reverse transcription. Amount of AUP1 mRNA in anti-AUP1 siRNA transfectant, scramble siRNA transfectant and mock control were determined and compared using quantitative PCR.

For AUP1 protein analysis, 48 hours post-transfection, cells were harvested and lysed in 35µl of Cao lysis buffer with additives. AUP1 protein in lysates was analysed with SDS-PAGE and Western blotting using anti-AUP1 antibody.

3.2.14. Quantitative PCR (qPCR)

To identify siRNA-mediated AUP1 silencing efficiency in mRNA level, qPCR was performed with RNA samples derived from HEK293RI or HepG2 cells transfected with hAUP1 siRNA pool and scramble siRNA. First of all, mRNA was conversed to cDNA with reverse transciption step using oligo(dT) priming as described before (part 2.2.1.2) and then cDNA products were applied for qPCR. qPCR reaction for quantification of AUP1 mRNA included following components: 6µl of 2x SYBR Green master mix (AB-1166/B, Thermo Scientific), 4µl of 3x primer mixture, 2µl of mixture of template and reference dye ROX. Working concentration of forward primer (hAUP1 201F - GACCATGTGTGCGGTGCTA) and reverse primer (hAUP1 353R - CTATTGAGTAGAGGGGTGCTACA) were 150nM and 600nM, respectively. Thus, 3x primer mixture consisted of 450nM of forward primer and 1800nM of reverse primer. The mixture of template and reference dye ROX contained 0.5ng/µl of total RNA in cDNA form and 0.18µM of ROX. Therefore, 12µl of one qPCR reaction finally consisted of 1ng RNA template in cDNA form

and reference dye ROX at concentration of 0.03μM. Each RNA sample was performed qPCR in triplicate.

For normalization, hGAPDH mRNA – a house keeping gene – in each RNA sample was quantified as an internal control in parallel with AUP1 mRNA quantification. qPCR reaction components for GAPDH were the same to those of AUP1 except the specific primers for GAPDH. Working concentration of GAPDH forward primer (mGAPDH 301F - ACCACCATGGAGAAGGC) and reverse primer (mGAPDH 534R - GGCATGGACTGTGGTCATGA) were 600nM and 150nM, respectively. Notably, this pair of primers mGAPDH 301F and mGAPDH 534R was designed for mouse GAPDH but they also work with human GAPDH.

Thermal profile for qPCR procedure was

95°C for 15 minutes: 1 cycle

95°C for 30 seconds and 60°C for 1 minute: 40 cycles

A single fluorescence reading was obtained at the end of every cycle.

After amplification, the specificity of amplified product was confirmed with melting/dissociation curve.

qPCR experiments were carried out with Stratagene Mx3005P^{TM} system and results were analysed with MxPro software.

Primers for qPCR of hAUP1 and GAPDH were selected from primer database http://pga.mgh.harvard.edu/primerbank/ and http://mouseprimerdepot.nci.nih.gov, respectively, and custom synthesized (Sigma).

The working concentration of primers and templates were defined in initial optimization steps for every target gene. At first the optimal primer concentration were defined by analyzing various concentrations (150, 300 and 600nM) of forward and reverse primers with 10ng of template. Primer concentrations with minimum cycle threshold (ct) and single amplification product (based on dissociation curve) were considered optimal. The second optimization was to determine optimal template amount by analysing the various amounts of template (10ng to 0.156ng/reaction, it was obtained with 1:1 serial dilution from maximum to

minimum) and plotting the standard curve. The optimal template amount was chosen in range which generates a standard curve having 90-110% efficiency.

3.2.15. Statistical analysis

The data was analysed using Excel software, from Microsoft Office 2001. The quantitative PCR data was analyzed using MxPro software (stratagene, USA) provided with the thermal cycler.

4. RESULTS

4.1. AUP1 associates with many components of IL-1 signaling pathway

The original point of departure of the present study was to search for new partners of IRAK4 which seems to exert till unknown functions [Suzuki et. al. – Science – 2006; Pacquelet – Biochemical Journal – 2007]. To approach this question, the yeast two-hybrid system was applied using Matchmaker library construction & screening kits (Clontech). In brief, a cDNA library derived from human placenta tissue was constructed and screened in yeast system for protein-protein interaction in which hIRAK4 was used as the bait protein. Analysis of screening results revealed novel partner proteins of IRAK4 AUP1 being one of them (C. Linke, C. Kollewe & M.U. Martin, unpublished data).

The interaction of AUP1 and IRAK4 was confirmed in HEK293RI cells by co-immunoprecipitation (IP) experiments. In these interaction studies, several others components of the IL-1 signaling pathway were also examined for interaction with AUP1 as depicted in Figure 4.1.

These co-IP experiments demonstrated that AUP1 not only associates with IRAK4 but also with IRAK1. This interaction was seen only in the cases of kinase inactive IRAK4 and IRAK1 mutants but not with enzymatically active wild type forms. This implicates that the kinase activity or phosphorylation state of IRAK molecules may interfere with their interaction with AUP1. In addition to IRAK molecules, AUP1 was found to associate with other components of the IL-1 signaling transduction machinery including MyD88, TRAF6, Tollip. However, AUP1 did not interact with all molecules known to participate in IL-1 signaling, thus no interaction was observed e.g.with Pellino2 (Figure 4.1).
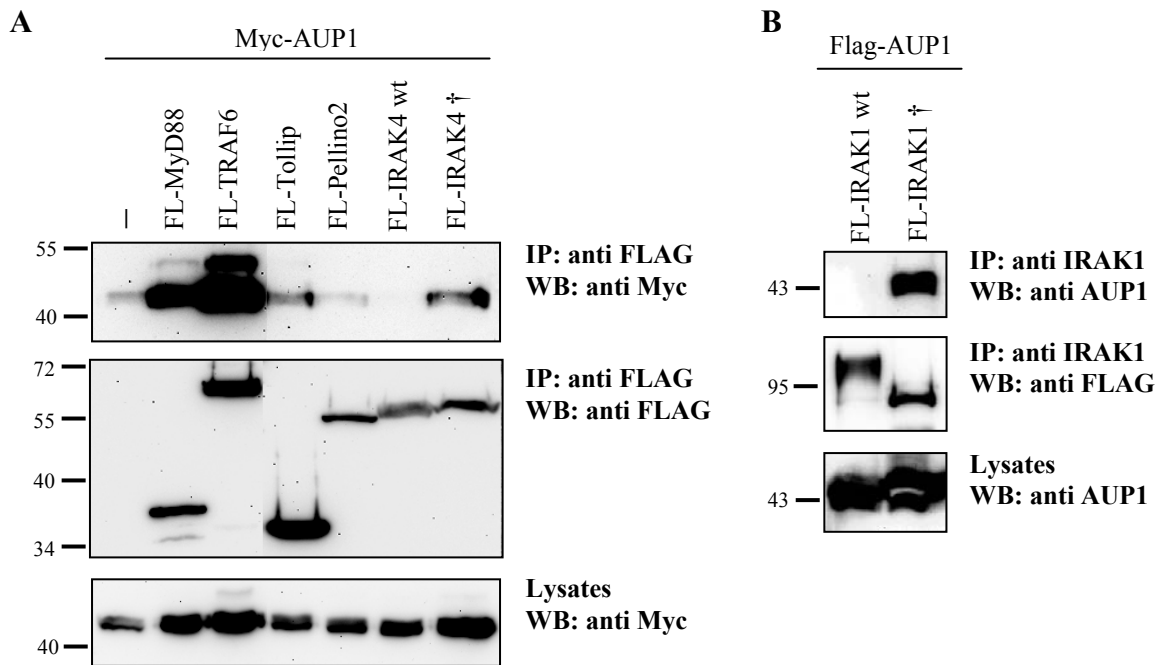
Figure 4.1: AUP1 associates with components of IL-1 signaling pathway as demonstrated by co-immunoprecipitation experiments.
(A) HEK293RI cells were cotransfected with constructs encoding Myc-tagged hAUP1 and either FLAG-tagged hMyD88, hTRAF6, hTollip, hPellino2, IRAK4 wildtype (WT), or IRAK4 kinase inactive (†) or an empty vector for control of unspecific binding. On the next day cells were lysed and FLAG-tagged proteins were immunoprecipitated (IP). Co-immunoprecipitated AUP1 was identified by Western blot (WB) using an anti Myc-epitope specific antibody. Susbsequently, the membrane was stripped and reprobed with an anti FLAG-epitope specific antibody. For control of AUP1 overexpression, cell lysates were separated by SDS-PAGE followed by Western blot using anti Myc-epitope antibody.
(B) HEK293RI cells were cotransfected with plasmids encoding FLAG-hAUP1 and either FLAG-IRAK1 WT or FLAG-IRAK1 kinase inactive. On the next day, an IP was carried out from cell lysates using the anti-IRAK1 antibody clone 2A9 in combination with protein G sepharose. Co-precipitated AUP1 and FLAG-IRAK1 were detected with Western blot using anti-AUP1 antibody and anti FLAG-epitope antibody, respectively. Overexpression of AUP1 in lysates was confirmed with Western blot using anti-AUP1 antibody.
Data shown is one representative experiment out of a series of three with comparable results.

4.2. AUP1 associates constitutively with IL-1RI and dissociates partially from IL-1RI upon IL-1β stimulation

The association of AUP1 with MyD88, Tollip, IRAK, TRAF6 which are components of active IL-1RI complex implicates that AUP1 may interact with IL-1RI as well. To clarify this, IL-1RI was immunoprecipitated, using anti-IL-1RI antibody, from HEK293RI cells which overexpressed AUP1. Before lysis and

immunoprecipitation cells were left untreated or stimulated with IL-1β in order to initiate formation of the signaling IL-1RI complex.

AUP1 was found in association with IL-1RI constitutively in untreated cells, indicating that IL-1RI – AUP1 complexes may pre-exist. Stimulation of cell with IL-1β for 15 minutes resulted in a partial dissociation of AUP1 from IL-1RI (Figure 4.2-upper panel). As the total amount of cellular AUP1 remained constant in the cell in this time frame, the possibility that AUP1 was degraded upon IL-1 stimulation could be excluded (Figure 4.2-lower panel).
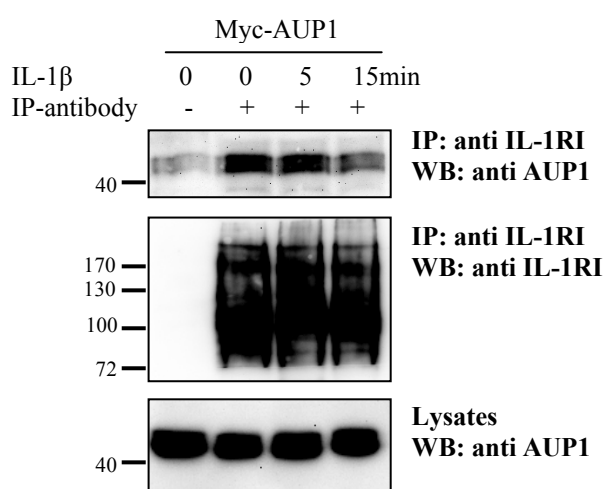
Figure 4.2: AUP1 is constitutively associated with IL-1RI and dissociates partially from the receptor complex upon IL-1β stimulation
HEK293RI cells were cotransfected with constructs encoding Myc-hAUP1 and FLAG-hIL-1RAcP. On the next day, transfected cells were either stimulated with 10ng/ml rhIL-1β for 0, 5, and 15min. Cells were lysed and IL-1RI IP was peformed using anti-hIL-1RI antibody and protein A sepharose. Co-precipitated AUP1 and IL-1RI were detected with Western blot using anti AUP1 and anti IL-1RI antibodies. Overexpression of AUP1 in lysates was confirmed with Western blot.
Data shown is one representative experiment out of a series of three with comparable results.

4.3. AUP1 can be phosphorylated by IRAK4 and IRAK1

The interaction of AUP1 and IRAK4 or IRAK1 was identified only with kinase inactive IRAK mutants but not with wild type forms (Figure 4.1). This suggested that kinase activity of IRAK4 and IRAK1 may play role in the interaction of them with AUP1. It was reported previously that IRAK1 interacts with Tollip and phosphorylates Tollip [Zhang, Ghosh – JBC – 2002]. Thus, it was interesting to know whether AUP1 serves as a substrate for IRAK4 and IRAK1. Since AUP1 was not seen in complex with wild type IRAK4 and IRAK1, forced co-IP assays [Kollewe et.al. – JBC – 2004] were performed using anti-FLAG M2 beads to bring FLAG-tagged AUP1 and FLAG-tagged IRAK4 or IRAK1 into close proximity on the beads. The washed beads were then subjected to an *in vitro* kinase assay.

In these forced co-IP assays IRAK4 and IRAK1 appeared as strongly autophosphorylated bands (Figure 4.3-upper panel). In addition, phosphorylated bands corresponding at the expected size of AUP1 were detected. They were easily seen when AUP1 was phosphorylated by IRAK4 (left panel) and much weaker, when AUP1 was forced together with IRAK1 (right panel). It was irrelevant where the epitope tag was expressed in the AUP1 molecule, both N- and C-terminally tagged AUP1 proteins were accepted as kinase substrates. Regarding the relation of the amounts of protein (shown in Figure 4.3 lower panel) one must conclude that AUP1 is not a very good substrate for IRAK-1. In addition, it cannot be excluded that the phosphorylation of the AUP1 molecules co-immunoprecipitated with IRAK1 was indeed phosphorylated by IRAK4 as IRAK4 also co-precipitates with IRAK1 due to death domain-death domain interactions.
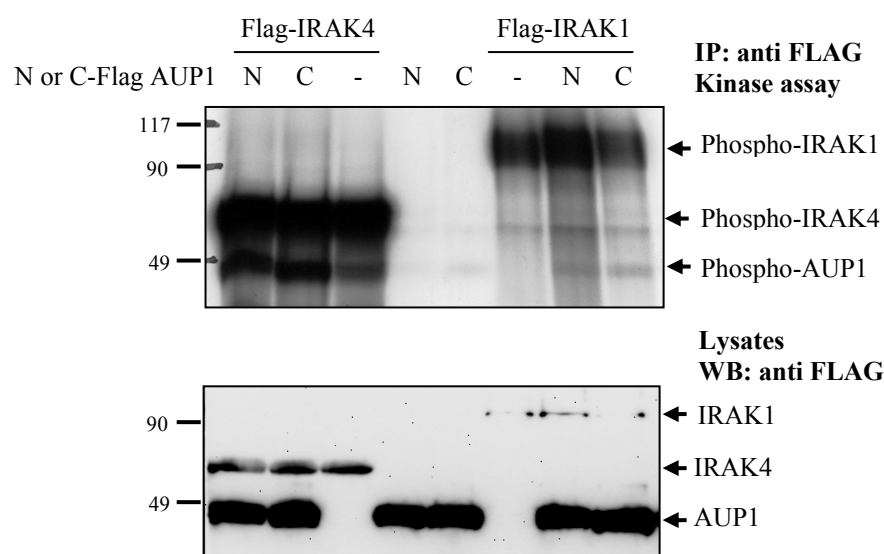
Figure 4.3: AUP1 was phosphorylated by IRAK4 and IRAK1 in forced co-IP assay
HEK293RI cells were cotransfected constructs encoding FLAG-tagged IRAK4 or IRAK1 and C or N-terminal FLAG-tagged hAUP1 as indicated. Cells were lysed and FLAG IP was performed. Beads with bound IRAK and AUP1 molecules were subjected to *in vitro* kinase assay. Proteins were separated by SDS-PAGE followed by auto-radiography. The expression of proteins in cell lysates was verified by Western blot using an anti FLAG-epitope specific antibody.

4.4. AUP1 overexpression impaired IL-1β and TNFα-induced activation of NF-κB in HEK293RI cells

As described above AUP1 associates with IL-1RI constitutively and partially dissociates from the receptor upon IL-1β stimulation. AUP1 also interacts with many components of the IL-1 signaling pathway. In addition, AUP1 can be phosphorylated by IRAK4 and IRAK1 in the receptor complex. Taking into consideration all these findings, it was tempting to speculate that AUP1 may play a regulatory role in the IL-1 signaling pathway.

In order to access the biological function of AUP1 in IL-1-mediated signaling, principally two approaches can be taken. First, it can be investigated how overexpression of AUP1 affects IL-1 signaling and second, it can be ascertained what happens to IL-1 signaling if AUP1 is not available (silencing or knock-down)

To investigate effects of AUP1 overexpression, HEK293RI cells were transfected with increasing amounts of hAUP1 encoding construct together with a luciferase reporter gene (construct encoding for 3xNFκB-Luc) to measure the central transcription factor activated by IL-1. Transiently transfected cells were stimulated with IL-1β and the activation of NF-κB in the IL-1β signaling pathway was estimated by luciferase reporter gene assay. In parallel, transfected cells were also stimulated with TNFα to clarify whether AUP1 was specific to IL-1 signaling or not.

The result showed that overexpression of AUP1 in HEK293RI partially inhibited IL-1β-induced activation of NF-κB in a dose-dependent manner (Figure 4.4 – grey bars). If cells were stimulated with TNFα, a cytokine that utilizes a discrete receptor complex but also addresses the NF-κB-pathway, increasing concentrations of AUP1 also reduced NF-κB activation, demonstrating that this effect was not specific for the IL-1 pathway (Figure 4.4 – black bars).
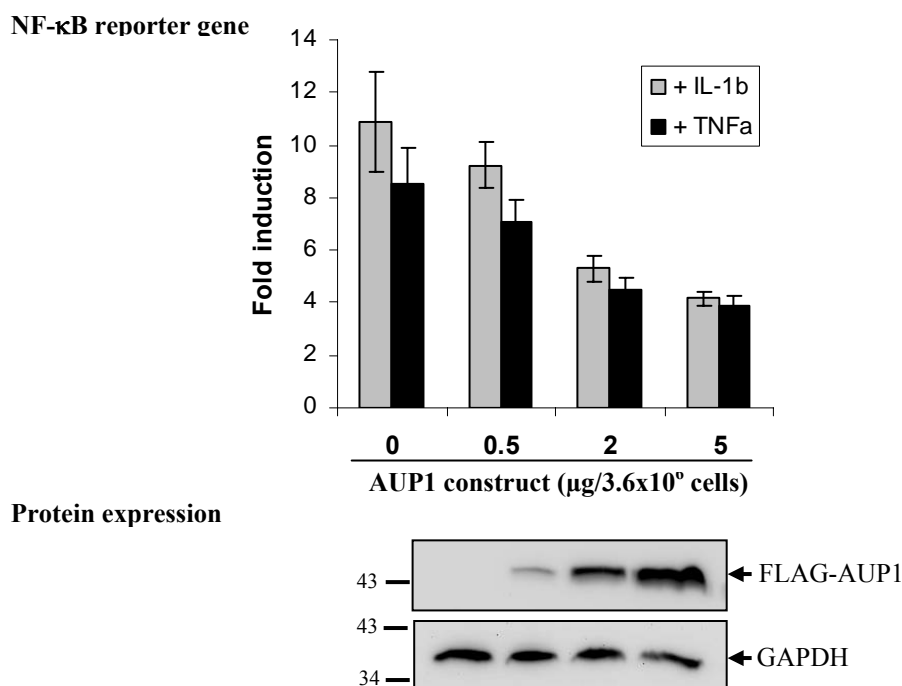
NF-κB reporter gene

Protein expression

Figure 4.4: AUP1 overexpression impaired IL-1β and TNFα-induced activation of NF-κB in HEK293RI

1×10^5 HEK293RI cells/well were seeded in a 24-well plate and cultured overnight. Cells were cotransfected with the 3xNFκB-Luc construct and different concentrations of a construct encoding FLAG-hAUP1 as indicated. Total DNA concentration was kept constant at 6μg DNA/3.6×10^6 cells by addition of empty vector. Transfected cells were either stimulated with 100pg/ml rhIL-1β, 10ng/ml rhTNFα or were left untreated. 18 hours later, cells were lysed and luciferase activity in cell lysates was measured. Depicted is fold induction calculated by dividing relative light unit (RLU) values of stimulated samples by the RLU values of unstimulated controls. Data shown are triplicates of one representative experiment out of a series of three with comparable results. Lower panels: control of protein expression of FLAG-hAUP1 in cell lysates and GAPDH loading control.

4.5. AUP1 overexpression reduced LPS-stimulated activation of NF-κB in KeratinoRI-/- cells

Lipopolysaccharide (LPS) stimulates cells through Toll-like receptor 4 (TLR4), a member of TLR/IL-1 receptor family, which shares many components with the IL-1 signaling pathway to transduce signals and finally activates MAPKs (mitogen activated protein kinases) and the NF-κB transcription factor. [Janssens, Beyaert – Molecular Cell – 2003; Akira, Takeda – Nature – 2004]

Since AUP1 overexpression interfered with IL-1β signaling, it may also interfere with LPS signaling. To clarify this, a keratinocyte cell line derived from an IL-1RI deficient mouse was utilized. This KeratinoRI-/- cell line responds to LPS. In

addition, interfering IL-1 signals, e.g. from endogenously produced IL-1, could by excluded as this cell line lacks the IL-1RI molecule. As mentioned above, NF-κB activation is a hallmark of the signaling pathways initiated by LPS. Therefore, NF-κB-luciferase reporter gene assay was applied to determine LPS-induced activation of NF-κB in KeratinoRI-/- which overexpressed increasing amounts of AUP1. Overexpression of AUP1 in KeratinoRI-/- cells impaired LPS-induced activation of NF-κB in a dose-dependent manner (Figure 4.5).
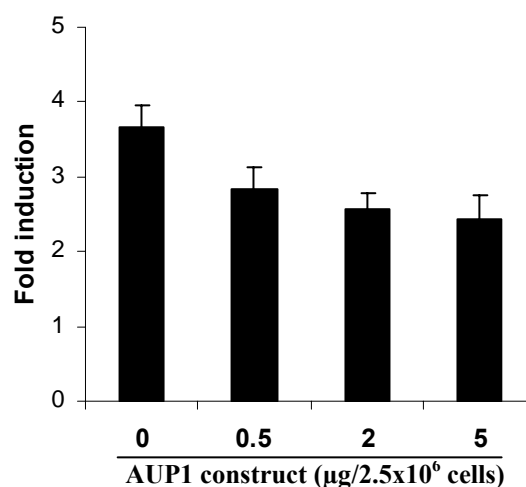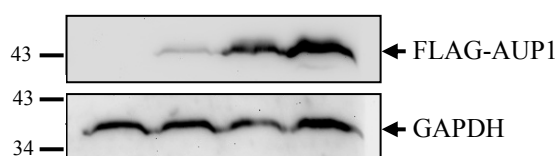
Figure 4.5: AUP1 overexpression reduced LPS-stimulated activation of NF-κB in KeratinoRI-/- cells
$7x10^4$ KeratinoRI-/- cells/well were seeded in a 24-well plate and cultured overnight. Cells were cotransfected with the 3xNFκB-Luc plasmid and different amounts of a construct encoding FLAG-hAUP1 as indicated. Total DNA concentration was kept constant at $12\mu g/2.5x10^6$ cells by addition of empty vector. Transfected cells were either stimulated with 100 ng/ml LPS or were left untreated for control. 18 hours later, cells were lysed and luciferase activity was measured in cell lysates. Depicted is fold induction calculated by dividing relative light unit (RLU) values of stimulated samples by the RLU values of unstimulated controls. Data shown are triplicates of one representative experiment out of a series of three with comparable results. Lower panels: control of protein expression of FLAG-hAUP1 in cell lysates and GAPDH loading control.

4.6. AUP1 overexpression reduced IL-8 production of HEK293RI cells stimulated by IL-1β or TNFα

Stimulation of cells with the pro-inflammatory cytokines IL-1β and TNFα results in the activation of transcription factors which translocate to the nucleus where they bind to specific promotors and turn on transcription of many genes. NF-κB is one of the most important transcription factors activated in inflammation. A large number of ligand-receptor interactions can lead to the activation of NF-κB. Two of the most frequently studied receptor families involved in the recognition of NF-κB-activating signals are the Tumor Necrosis Factor receptor (TNFR) family and the Toll/interleukin-1 receptor (TIR) family. Nuclear accumulation of NF-κB transcription factors causes changes in the expression of target genes involved in innate and adaptive immunity, inflammation, cell survival, hematopoiesis, and lymphoid development [Leeman, Gilmore –Gene – 2008]

The results obtained in the NF-κB reporter gene assays performed in this study suggested that AUP1 overexpression impaired IL-1β, TNFα, and LPS signaling transduction. Thus, reduced NF-κB activation upon AUP1 overexpression should result in diminished secondary cytokine production. In order to confirm this, IL-1β- and TNFα-induced IL-8 production by HEK293RI overexpressing increasing amounts of AUP1 was examined.

Overexpression of AUP1 diminished IL-1β and TNFα-induced IL-8 production by HEK293RI moderately in a dose-dependent manner (Figure 4.6).
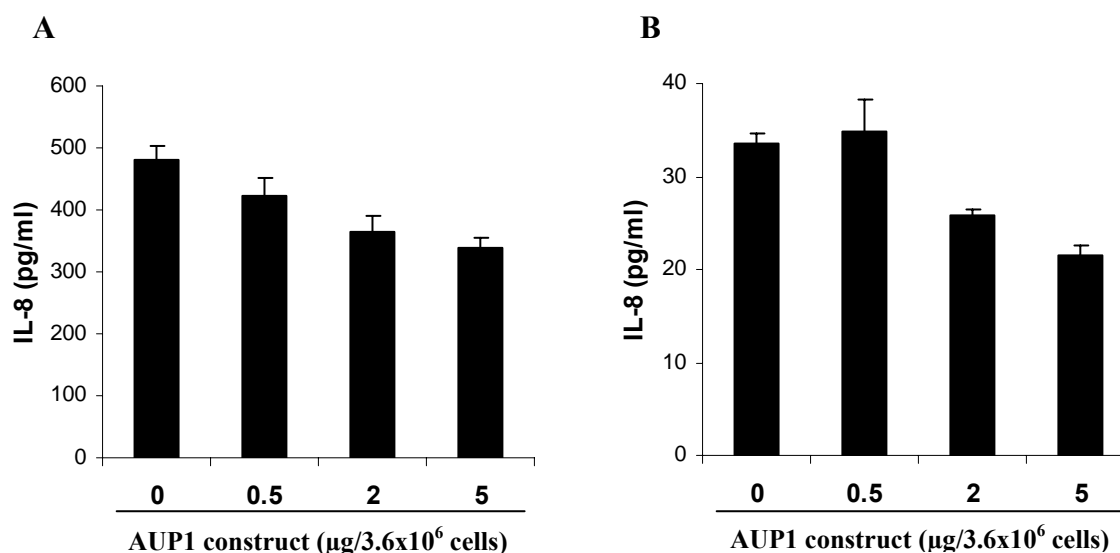
A

B

Figure 4.6: AUP1 overexpression diminished IL-8 production of HEK293RI stimulated with IL-1β and TNFα

$1x10^5$ HEK293RI cells/well seeded and cultured overnight in a 24-well plate were transfected with increasing amounts of a construct encoding FLAG-hAUP1 as indicated. DNA concentration was kept constant at 6μg DNA/3.6x10^6 cells by addition of empty vector. On the next day, transfected cells were either stimulated with 100pg/ml rhIL-1β (**A**) or 10ng/ml rhTNFα (**B**) for 8 hours. Supernatant was harvested and hIL-8 in supernatant was measured by ELISA. Data shown are triplicates of one representative experiment out of a series of three with comparable results.

The regulatory effect of AUP1 in IL-1β and TNFα-induced chemokine production was less pronounced than that observed in NF-κB -luciferase reporter gene assays (Figure 4.4). The possible explanation for this discrepancy is of a technical nature: In a reporter gene assay, the constructs encoding AUP1 and the NF-κB reporter gene are cotransfected so that cells which take up the NFκB-luciferase construct also internalize the AUP1 construct. Under stimulation, all cells induced to produce luciferase are also influenced by AUP1 overexpression. Thus, the background (untransfected cells) is excluded from the read-out, resulting in higher sensitivity of this method compared to the ELISA. In the chemokine production experiment, one hundred percent of cells are stimulated by the cytokine to produce the chemokine IL-8 but maximally 60-70% of the cell population overexpressed AUP1 due to the transfection efficiency. Thus, the background (unstransfected cells) is taken into the account and it reduces the sensitivity of this method. This results in a reduced difference between IL-8 production in control and in cells overexpressing AUP1.

4.7. AUP1 overexpression diminished IL-6 production of KeratinoRI-/- cells stimulated with LPS

As AUP1 overexpression reduced LPS stimulated NF-κB activation, it was investigated whether AUP1 overexpression also affected LPS-induced cytokine productions. Here IL-6 production by LPS-stimulated KeratinoRI-/- cells was investigated.

Consistent with the results obtained with IL-1β and TNFα, overexpression of AUP1 in KeratinoRI-/- reduced LPS-induced IL-6 production in a dose-dependent fashion (Figure 4.7)
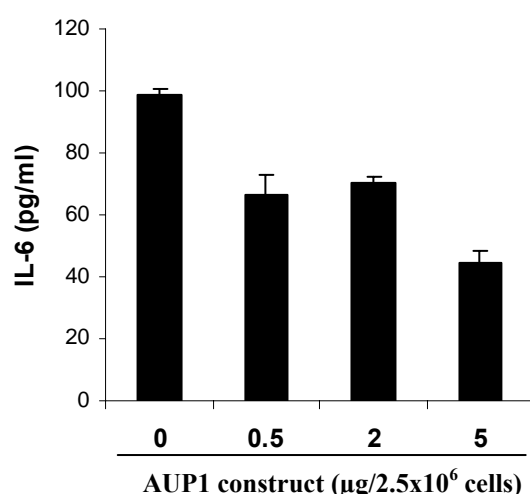
Figure 4.7: AUP1 overexpression diminished IL-6 production of KeratinoRI-/- stimulated with LPS
$7x10^4$ KeratinoRI-/- cells cultured overnight in wells of 24-well plate were transfected with increasing amounts of a construct encoding FLAG-hAUP1 as indicated. DNA concentration was kept constant at $12\mu g/2.5x10^6$ cells by addition of empty vector. On the next day, transfected cells were stimulated with 100ng/ml LPS for 8 hours. Supernatant was harvested and mIL-6 was measured by ELISA. Data shown are triplicates of one representative experiment out of a series of three with comparable results.

4.8. AUP1 overexpression had no effect on the viability of HEK293RI and KeratinoRI-/- cells

The ELISA approach showed that AUP1 overexpression reduced IL-8 and IL-6 production in HEK293RI and KeratinoRI-/- cells stimulated with IL-1β, TNFα or LPS, respectively (Figure 4.6, 4.7). However, one can argue that the observed down-regulatory effect may be the result of enhanced cell death or reduced cellular proliferation due to the increase of AUP1 molecules after overexpression. To

exclude this posibility, MTT assay was performed to estimate the proliferation rate of HEK293RI and KeratinoRI-/- cells transfected with different amounts of a hAUP1 encoding construct.

The results showed that overexpression of AUP1 in the ranges tested had no impact on cell viability and proliferation of HEK293RI and KeratinoRI-/- cells. This was not altered by either stimulation with IL-1β or LPS.
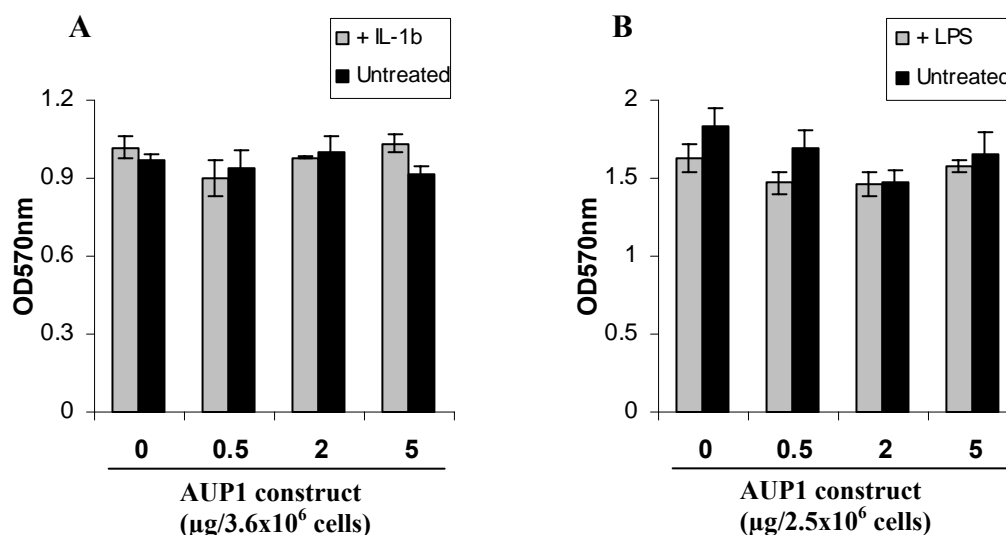

Figure 4.8: AUP1 overexpression did not affect the viabilty of HEK293RI (A) and KeratinoRI-/- (B) cells
$5x10^5$ cells of HEK293RI or $3.5x10^5$ cells of KeratinoRI-/- were plated to wells of 6-well plate and maintained in culture over night. Cells were then transfected with different concentrations of a plasmid encoding FLAG-hAUP1. DNA concentration was kept constant by addition of empty vector. Transfected cells were trypsinized and reseeded at density of $5x10^4$ cells per well of 96-well plate. HEK293RI and KeratinoRI-/- cells were either stimulated with 100pg/ml rhIL-1β or 100ng/ml LPS, respectively or were left unstimulated. 18 hours later, cell viability was determined by the MTT method.

4.9. AUP1 overexpression impaired IL-1β and TNFα-induced activation of NF-κB but did not affect IL-6-induced activation of STAT3 in HepG2 cells

Reporter gene assays showed that overexpression of AUP1 resulted in the reduction of the activation of NF-κB in cells stimulated by IL-1β, LPS as well as TNFα (Figure 4.4, 4.5). One critical question arising from these results is the whether the observed effect of AUP1 is specific for these pro-inflammatory mediators or whether AUP1 generally affects a cellular function irrelevant of cytokine signaling.

Does overexpression of AUP1 globally affect the physiology of cells? To clarify this central question, the influence of AUP1 overexpression on the IL-6 signaling pathway was included in the study. IL-6 signaling is completely different from IL-1, LPS and TNFα pathways. IL-6 cytokine exerts its action via the IL-6 receptor and the signal transducer gp130 leading to the activation of the JAK/STAT (Janus kinase/ signal transducer and activator of transcription) and the MAPK (mitogen-activated protein kinase) cascades [Heinrich et.al. – Biochemical Journal – 2003].

It was reported that HEK293 cells express IL-6 receptor, gp130 and respond to IL-6 stimulation [von Laue et. al. – The journal of endocrinology – 2000]. In addition, HEK293 were used to demonstrate the function of AUP1 in IL-1β and TNFα pathway in the present study. Therefore, HEK293 appeared to be the ideal cell line to investigate the function of AUP1 in the IL-6 signaling pathway.

First, the response of HEK293RI to IL-6 stimulation was tested by the phosphorylation of STAT3, the major member of STAT family activated by IL-6 [Heinrich et.al. – Biochemical Journal – 2003]. STAT3 is primarily phosphorylated at Tyr705 residue, and it also requires phosphorylation at Ser727 to achieve maximal activity [Wen et.al. – Cell – 1995; Zhang et.al. – Science – 1995]. HEK293RI cells were either stimulated with rhIL-6 for 15, 30 minutes or left unstimulated for control and the phosphorylation of STAT3 at Tyr705 and Ser727 residues were detected with Western blot using phosphorylation site –specific antibodies. In parallel, human hepatocellular carcinoma HepG2 cells which respond well to IL-6 were used in this experiment as positive control.

In HepG2 STAT3 was phosphorylated strongly at Tyr705 residue after 15 minutes of IL-6 stimulation. The phosphorylation at Ser727 residue also occurred. Although the background in the sample from unstimulated cell was rather high, the induced phosphorylation at Ser727 of STAT3 was obvious (Figure 4.9). In contrast to HepG2 cell, in HEK293RI no phosphorylation of STAT3 at Tyr705 residue could be detected after stimulation of IL-6 for 15 or 30 minutes. In the case of phosphorylation of STAT3 at Ser727, it was undistinguishable between IL-6 stimulation and unstimulation (Figure 4.9). Moreover, IL-8 release into culture

supernatant of HEK293RI stimulated with IL-6 could not by measured by ELISA (data not shown). Thus, in contrast to results from literature, HEK293RI of this laboratory did not seem to react to IL-6 and thus could not be used to test the function of AUP1 with respect to IL-6 responsiveness.
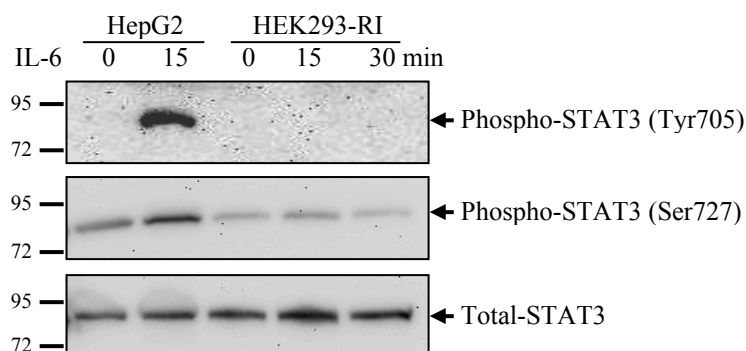

Figure 4.9: HEK293RI cells do not respond to IL-6 stimulation by STAT3 phosphorylation
HEK293RI and HepG2 cells were starved in DMEM medium with 0.1%FCS overnight. Cells were stimulated with 20ng/ml rhIL-6 for the times indicated. Cells were then lysed, lysates were subjected to SDS-PAGE and Western blot using antibodies against phospho-STAT3 (Tyr705), phospho-STAT3 (Ser727) and total STAT3.

For studying possible AUP1 effects on IL-6 signaling HepG2 cells were used. HepG2 cells were transiently transfected with increasing amounts of hAUP1 encoding construct together with pGL3-α_2M-215Luc construct which contains a STAT3-dependent promoter fused to the luciferase encoding sequence (a kind gift of F. Schaper, RWTH, Aachen, Germany [Schaper et.al. – Biochemical Journal – 1998; Schmitz et.al. – JBC – 2000; Lehmann et.al. – JBC – 2003]). The influence of AUP1 overexpression on the IL-6 pathway was measured using luciferase reporter gene assay. To compare the role of AUP1 overexpression in IL-1, TNFα pathway and IL-6 pathway in the same cell line, IL-1β- and TNFα-induced NF-κB activation were also demonstrated in HepG2 overexpressed AUP1.

Overexpression of AUP1 in HepG2 did not alter IL-6-induced activation of STAT3 (Figure 4.10). This result suggested that AUP1 overexpression may not be involved in the IL-6 signaling pathway, demonstrating a relative specificity of AUP1 overexpression on selected signaling pathways. Similarly to the results obtained in HEK292RI cells, overexpression of AUP1 reduced IL-1β- and TNFα-stimulated activation of NF-κB in a dose-dependent fashion in HepG2 cells (Figure 4.10). These results manifested that AUP1 is involved in some signaling pathways

specifically and that overexpression of this protein does not cause a global effect to physiology of cells.
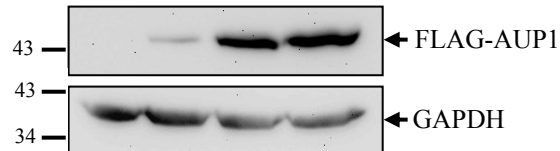
NF-κB reporter
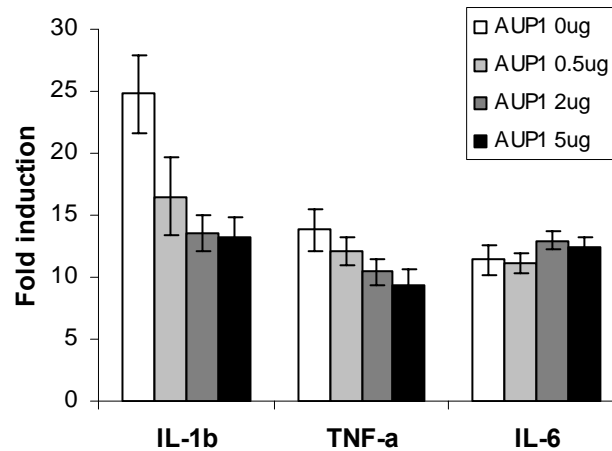
Protein expression

Figure 4.10: AUP1 overexpression impaired IL-1β- and TNFα-induced activation of NF-κB but did not affect IL-6-induced activation of STAT3 in HepG2 cells
5.5×10^4 HepG2 cells/well were cultured in a 24-well plate overnight. Cells were cotransfected with constructs encoding either a 3xNFκB-Luc reporter gene (for IL-1β and TNFα stimulation) or a pGL3-α_2M-215Luc reporter gene (for IL-6 stimulation) and increasing amounts of FLAG-hAUP1 encoding construct as indicated. DNA concentration was kept constant at $6\mu g/2 \times 10^6$ cells by addition of empty vector. Transfected cells were either stimulated with 1ng/ml rhIL-1β, 10ng/ml rhTNFα, 20ng/ml rhIL-6 or were left untreated. Cells were lysed and luciferase activity in cell lysates was measured the next day. Depicted is fold induction calculated by dividing relative light unit (RLU) values of stimulated samples by the RLU values of unstimulated controls. Data shown are triplicates of one representative experiment out of a series of three with comparable results. Lower panels: protein expression of FLAG-hAUP1 in cell lysates and GAPDH for loading control.

4.10. AUP1 associates with TNFR-1, TRADD, and TRAF2

The results given by reporter gene assay (Figure 4.4, 4.10) and ELISA for IL-8 production (Figure 4.6B) suggest that AUP1 may be involved in the TNFα signaling pathway. As AUP1 binds to proteins involved in the IL-1 signaling pathway, it is also possible that AUP1 interacts with components of the TNFα pathway.

To confirm this, the interaction of AUP1 and TNFR1, TRADD and TRAF2 - three molecules of the TNFα-induced survival pathway (see part 1.7) – were investigated

using co-IP experiments. As depicted in Figure 4.11, the association experiment provided evidence that AUP1 interacts with TNFR1, TRADD and TRAF2 at least in the overexpression situation. AUP1 was found to associate with all of three tested molecules but with different intensity. AUP1 associated stronger with TNFR1 than with TRAF2 or TRADD (Figure 4.11 - upper panel), although the expression level of Myc-TNFR1 in cells was extremely low compared to Myc-TRADD and Myc-TRAF2 (Figure 4.11 - lower panel). Hence, the possibility that TRADD and TRAF2 associate with AUP1 indirectly through endogenous TNFR1 can not be excluded.
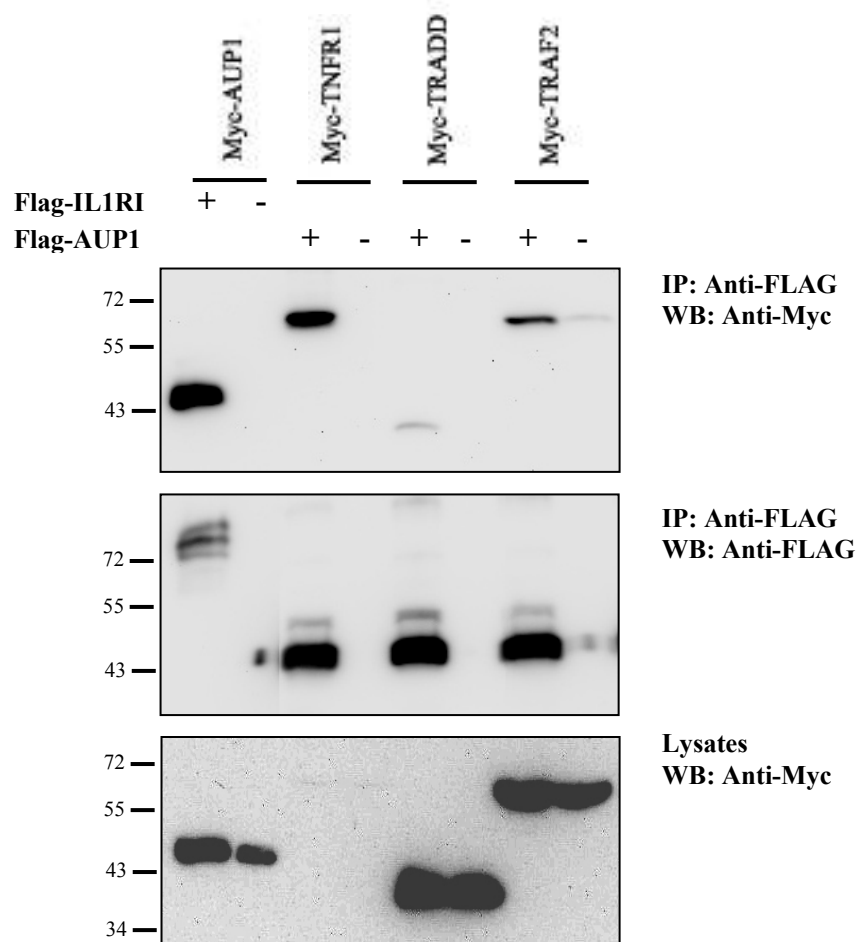

Figure 4.11: AUP1 associates with components of the TNFα signaling pathway
HEK293RI cells were cotransfected with plasmids encoding Myc-hTNFR1, Myc-hTRADD, Myc-hTRAF2 and either FLAG-hAUP or empty vector for unspecific binding control. In parallel, Myc-hAUP1 construct was cotransfected with either FLAG-hIL-1RI or empty vector for control to HEK293RI. On the next day, cells were lysed and cell lysates were incubated with anti-FLAG M2 beads. Co-immunoprecipitated proteins were analysed by Western blot using anti Myc-epitope antibody and then membrane was stripped and reprobed with anti FLAG-epitope antibody. For overexpression control, cell lysates were separated by SDS-PAGE followed by Western blot using anti Myc-epitope antibody. Data shown is one representative experiment out of a series of three with comparable results.

4.11. AUP1 interacts with the IL-6R α-chain (gp80) but not with gp130

In Figure 4.10 it was shown that AUP1 overexpression impairs both the IL-1β and TNFα signaling pathways, but does not interfere with IL-6 signaling in HepG2 cells. And AUP1 interacted with both the IL-1RI complex (Figure 4.1, 4.2) and the TNFR1 complex (Figure 4.11). As AUP1 overexpression did not affect IL-6 signaling, it was investigated whetherAUP1 interacts with IL-6R (gp80) and gp130, the two components of the IL-6 receptor complex. In the IL-6 signaling pathway, IL-6 binds specifically to both receptor subunits, the IL-6 receptor α-chain and gp130, with distinct residues. However, the signal is only transduced by gp130 as soluble IL-6 receptor α-chains in combination with gp130 can initiate IL-6 signaling in cells lacking transmembrane IL-6 receptor α-chains by trans-signaling [Rose-John, Schooltink – Recent Results Cancer Res – 2007]. gp130 which associates with JAKs and becomes tyrosine phosphorylated in response to cytokine stimulation plays the central role in IL-6 signaling transduction [Heinrich et.al. – Biochemical – 2003].

To investigate the association between AUP1 and the IL-6R components, plasmids encoding the candidate proteins were transiently transfected to HEK293RI. Subsequently, co-IP was carried out followed with Western blot analysis.

This association experiment demonstrated that AUP1 interacted with the non-signaling IL-6R α-chain (Figure 4.12-A) but not with the signal transducing receptor subunit gp130 (Figure 4.12-B). Although AUP1 overexpression did not influence IL-6 signaling transduction with respect to STAT3 activation (Figure 4.10), the involvement of AUP1 in IL-6 pathway can not be excluded because of the interaction between AUP1 and IL-6R.
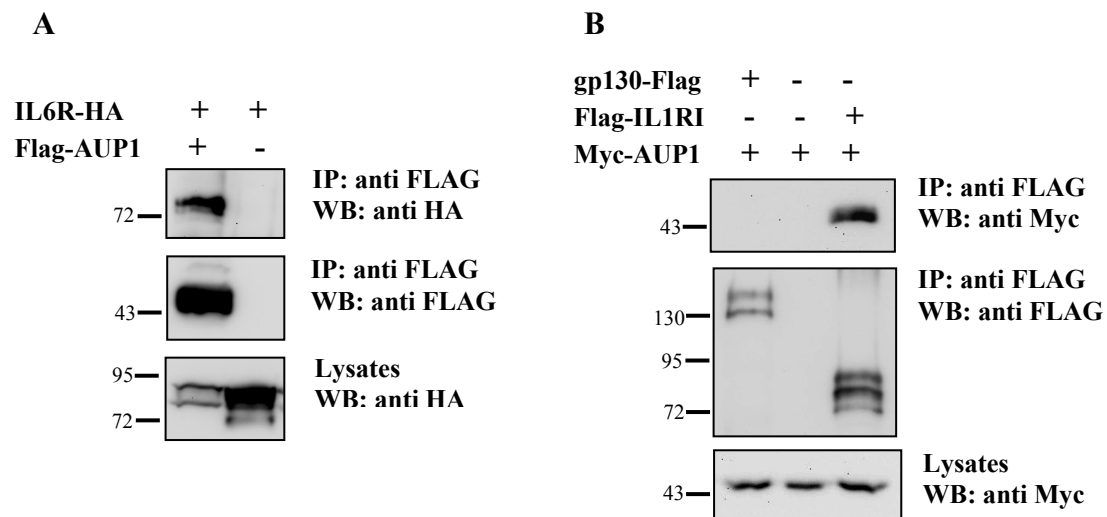
A

IL6R-HA   +   +
Flag-AUP1   +   -

72 —   IP: anti FLAG
            WB: anti HA

43 —   IP: anti FLAG
            WB: anti FLAG

95 —   Lysates
72 —   WB: anti HA

B

gp130-Flag   +   -   -
Flag-IL1RI   -   -   +
Myc-AUP1   +   +   +

43 —   IP: anti FLAG
            WB: anti Myc

130 —   IP: anti FLAG
95 —   WB: anti FLAG
72 —

43 —   Lysates
            WB: anti Myc

Figure 4.12: AUP1 interacts with the non-signaling receptor IL-6Rα chain (A) but not with the signal transducing subunit gp130 (B)
(A) HEK293RI cells were cotransfected with constructs encoding hIL6R-HA and either FLAG-hAUP1 or empty vector for unspecific binding control. On the next day, cells were lysed and cell lysates were incubated with anti-FLAG M2 beads. Co-immunoprecipitated proteins were analysed by Western blot using anti HA-epitope antibody and then membrane was stripped and reprobed with anti FLAG-epitope antibody. The expression of IL6R-HA in lysates was confirmed by SDS-PAGE followed by Western blot using anti HA-epitope antibody.
(B) HEK293RI cells were cotransfected with plasmids encoding Myc-hAUP1 and either hgp130-FLAG or FLAG-hIL1RI or empty vector for unspecific binding control. On the next day, cells were lysed and cell lysates were incubated with anti-FLAG M2 beads. Co-immunoprecipitated proteins were analysed by Western blot using anti Myc-epitope antibody and then membrane was stripped and reprobed with anti FLAG-epitope antibody. The expression of Myc-AUP1 in lysates was confirmed with SDS-PAGE followed by Western blot using anti Myc-epitope antibody.

4.12. Effect of small-interfering RNA (siRNA)–mediated knockdown of hAUP1 in HEK293RI and HepG2 cells

As stated in the introduction to the results section, in order to study biological function of AUP1 removing the protein from cells is as important as overexpressing it. Therefore, silencing of AUP1 expression in HEK293RI and HepG2 cell lines was performed with small-interfering RNA (siRNA) using ON-TARGETplus SMARTpool siRNA for hAUP1 (L-012410-01-0005 – Dharmacon RNAi Technologies – Thermo scientific). Anti hAUP1 siRNA pool consisted of four commercially obtained siRNAs for target sequences (detailed information in materials section). A scrambled siRNA was included as control for the effect of transient transfection of small RNAs in cells. Transfection with siRNA was carried out according to manufacturer's instruction with a slight optimization as described

in part 2.2.13 and legend of Figure 4.13. The AUP1 knock-down efficiency was monitored at mRNA and protein level with RT-qPCR and Western blot.

RT-pPCR experiment showed that siRNA-mediated knock-down efficiency of AUP1 mRNA in HEK293RI and HepG2 reached more than 80% and 70%, respectively. Consistent with the results on mRNA level, AUP1 protein expression in HEK293RI and HepG2 anti-AUP1 siRNA transfectants was reduced significantly as demonstrated by Western blot analysis (Figure 4.13).
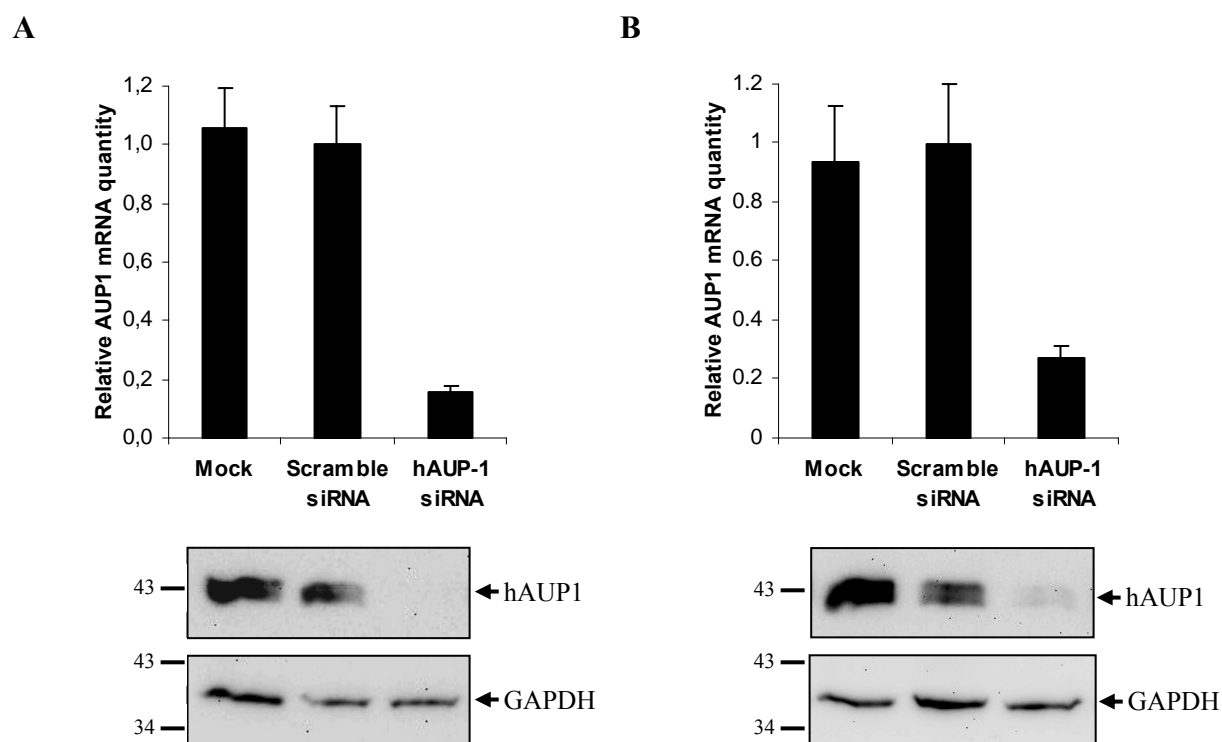

Figure 4.13: Small-interfering RNA (siRNA)–mediated knockdown of hAUP1 in HEK293RI (A) and HepG2 (B) cells
24h before transfection, 6×10^4 cells of HEK293RI or HepG2 /well were seeded in a 24-well plate. Cells were either transfected with 100nM of anti-hAUP1 siRNA, scramble siRNA or treated with transfection reagent only for mock control. 24 hours post-transfection, total RNA from cells was isolated and RT-qPCR was performed to compare quantity of AUP1 mRNA in cells. For AUP1 protein examination, 48 hours post-transfection, cells were lysed and lysates were analysed by SDS-PAGE and Western blot using anti-AUP1 antibody. Membranes were stripped and reprobed with anti-GAPDH antibody for loading control. Data shown is one representative experiment out of a series of three with comparable results.

4.13. Silencing of AUP1 increased IL-1β- and TNFα-induced IL-8 production and activation of NF-κB in HEK293RI cells as well as IL-6-induced activation of STAT3 in HepG2 cells

Overexpression experiments suggested that AUP1 exhibits a partially inhibitory activity in IL-1β, TNFα, LPS signaling pathways (Figure 4.4 – 4.7). To confirm this finding, the influence of siRNA-mediated AUP1 silencing on IL-1β- and TNFα-induced signaling transduction in HEK293RI cells was investigated. Cytokine release in form of IL-8 production and activation of transcription factors in form of NF-κB-luciferase reporter gene assay were measured. In addition, IL-6-induced activation of STAT3 was also examined with reporter gene assay in HepG2 cells in which AUP1 expression had been silenced by use of siRNA..

IL-1β- and TNFα -induced IL-8 production was enhanced in anti-hAUP1 siRNA transfected HEK293RI cells compared to that in control cells receiving scrambled siRNA (Figure 4.14-A). Consistent with this observation, IL-1β and TNFα stimulated NF-κB activation in AUP1 knock-downed cells was higher than in control cells as shown in NF-κB-luciferase reporter gene experiment (Figure 4.14-B). These results, together with previous data in overexpression experiments, provided evidence that AUP1 is a negative regulator of signaling transduction induced by IL-1 (LPS) and TNFα. Silencing of AUP1 in HepG2 resulted in an increase of STAT3 activation upon IL-6 stimulation compared to that in control cells (Figure 4.14-C).

Taken together, AUP1 seems to be a common negative regulator of IL-1β/LPS, TNFα and IL-6 signaling pathways. Moreover, the siRNA-mediated silencing of AUP1 also induced constitutive IL-8 production, and NF-κB as well as STAT3 activation independently of cytokine stimulation (Figure 4.14).
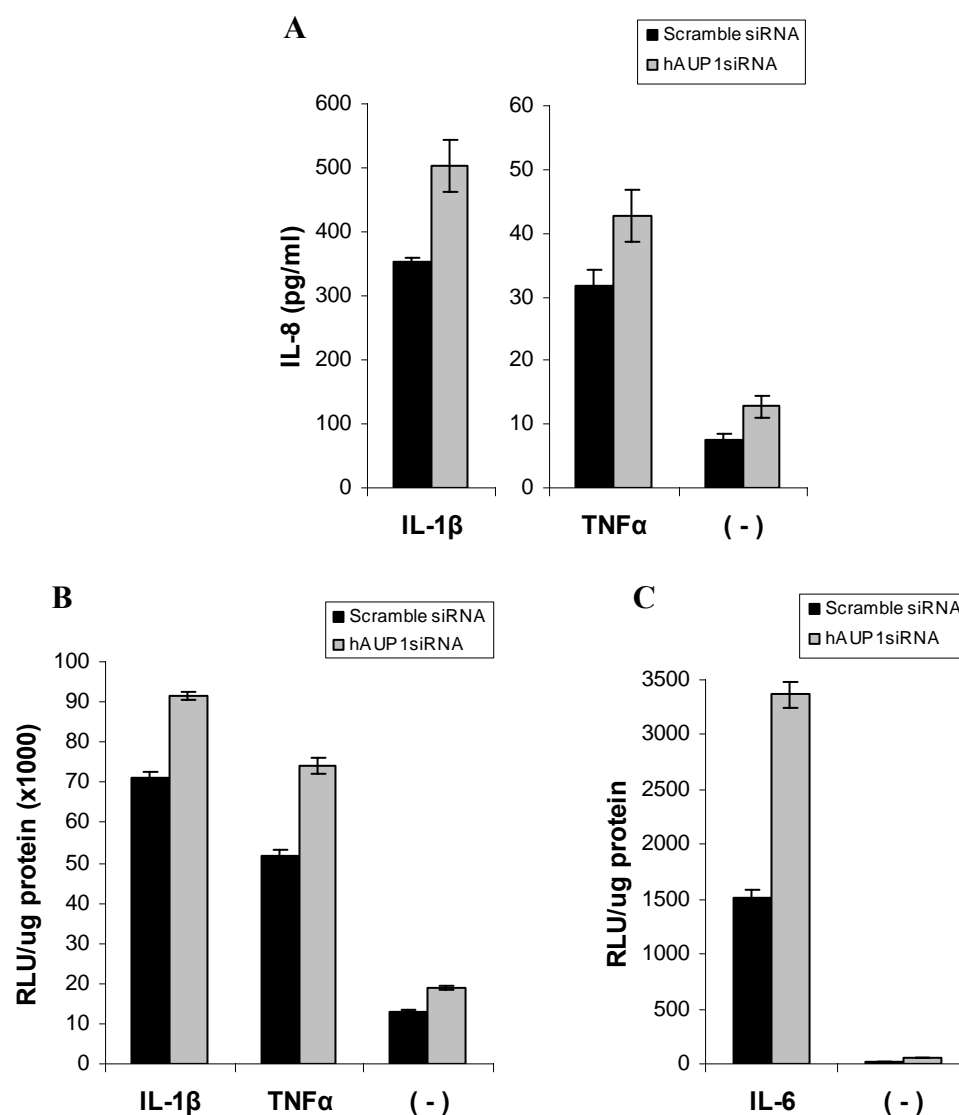
Figure 4.14: Silencing of AUP1 increased IL-1β-, TNFα-induced IL-8 production (A) and activation of NF-κB in HEK293RI cells (B) as well as IL-6-induced activation of STAT3 in HepG2 cells (C)

(A) 48h after transfected with 100nM of anti-hAUP1 siRNA or scramble siRNA, HEK293RI cells were stimulated with either rhIL-1β (100pg/ml) or rhTNFα (10ng/ml) for 8 hours. Supernatant was harvested and hIL-8 in supernatant was quantified with ELISA.

(B) 44h after transfected with 100nM of anti-hAUP1 siRNA or scramble siRNA, HEK293-RI cells were transfected with 3xNFkB-Luc reporter gene construct. Transfected cells were either stimulated with rhIL-1β (100pg/ml), rhTNFα (10ng/ml) for 8 hours or were left untreated for control. Cells were lysed and luciferase activity in cell lysates was measured. RLU was normalized with protein concentration of lysates.

(C) 44h after transfected with 100nM of anti-hAUP1 siRNA or scramble siRNA, HepG2 cells were transfected with pGL3-α_2M-215Luc reporter gene construct. Transfected cells were either stimulated with rhIL-6 (20ng/ml) for 8 hours or were left untreated for control. Cells were lysed and luciferase activity in cell lysates was measured. RLU was normalized with protein concentration of lysates.

4.14. AUP1 overexpression reduced IL-1β-induced phosphorylation of JNK, p38 MAPK and delayed the degradation of IκB in HEK293RI cells

It was reported previously that AUP1 is evolutionarily conserved and ubiquitously expressed, hence it appears to play basic and essential roles in cellular biology [Jang et.al. – Genomics – 1996; Kato et.al. – JBC – 2002]. The data obtained in this study propose that AUP1 is likely a common negative regulator of many signaling pathways. However, it is important to confirm the role of AUP1 to suppress signaling transduction rather than to regulate basic events such as protein synthesis which can result in the reduction of luciferase enzyme and cytokine production as seen in above experiments.

Therefore, the kinetics of some important events in IL-1β-mediated signaling including phosphorylation and degradation of IκB, phosphorylation of JNK, phosphorylation of p38 were investigated in the presence and absence of AUP1 overexpression.

In HEK293RI, AUP1 overexpression did not shift the IL-1β-induced kinetic of JNK phosphorylation compared to those in empty vector control. JNK phosphorylation reached the peak at 30 minute of IL-1β exposure and then decreased. However, in cells overexpressing AUP1, the intensity of phosphorylation of JNK was reduced compared to control (Figure 4.15- phospho JNK). A similar phenomenon occurred with p38 phosphorylation. In the presence of AUP1 overexpression, IL-1β-induced phosphorylation of p38 was decreased significantly compared to that of empty vector control. As with JNK, the kinetic of p38 phosphorylation was not altered by overexpression of AUP1. The phosphorylation of p38 reached the peak after 30 minutes stimulation with IL-1β in both cases with and without AUP1 overexpression (Figure 4.15- phospho p38). IκB is the inhibitor of NF-κB, the phosphorylation and the degradation of IκB is essential for the activation of NF-κB. In cells overexpressing AUP1, the IL-1β-induced degradation of IκB was not reduced but delayed compared to those in control cells. IκB was degraded rapidly after 5 minute stimulation of IL-1β in control cells while it was slower in AUP1 expressing cells. However, after longer stimulation (15 minute), IκB degradation

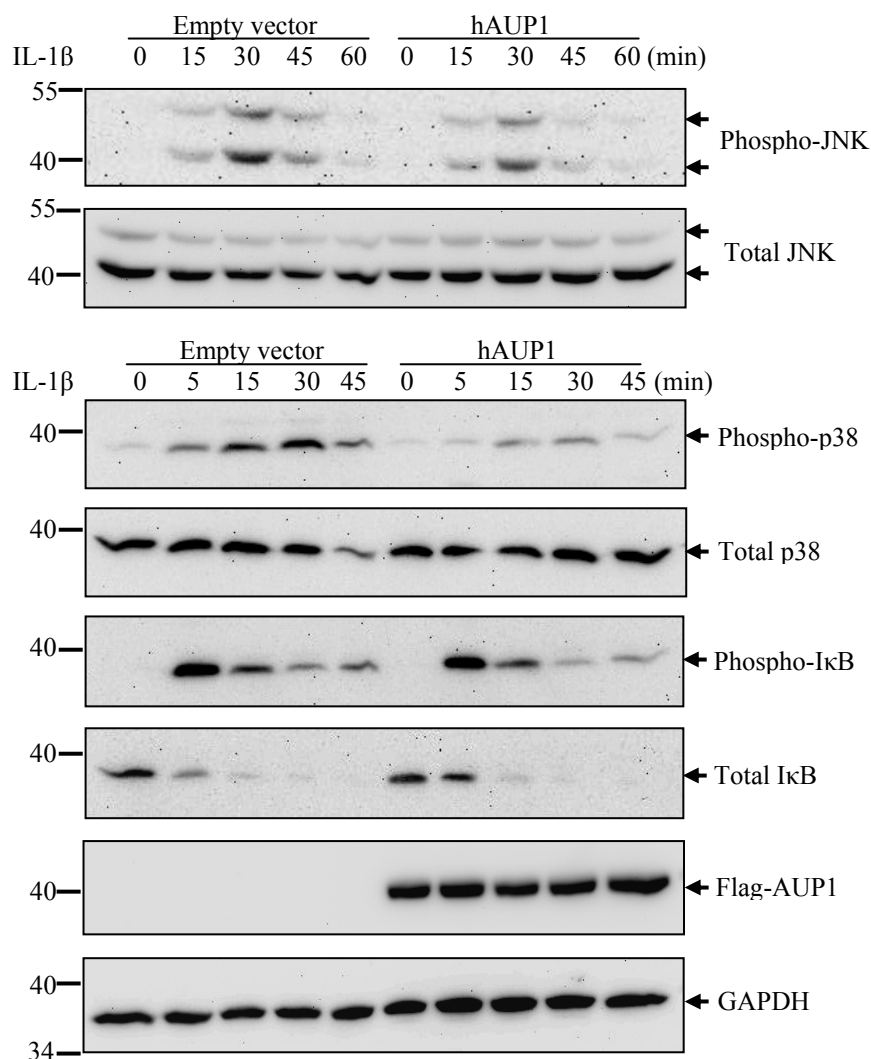was the same in cells with and without AUP1 overexpression (total IκB - Figure 4.15).

Figure 4.15: AUP1 overexpression reduced IL-1β-induced phosphorylation of JNK, p38 MAPK and delayed degradation of IκB in HEK293RI
HEK293RI cells were transfected either with FLAG-hAUP1 encoding plasmid (6μg/3,6x10^6 cells) or empty vector. On the next day, transfected cells were stimulated by 5ng/ml rhIL-1β for the indicated times. Cells were lysed, lysates were subjected to SDS-PAGE and Western blot using antibodies against phospho-JNK, total-JNK, phospho-p38, total-p38, phospho-IκB, total IκB. The expression of FLAG-hAUP1 was confirmed with Western blot using anti FLAG antibody and anti GAPDH antibody was used for loading control. Data shown is one representative experiment out of a series of three with comparable results.

4.15. AUP1 overexpression reduced the degradation of IκB and delayed the phosphorylation of p38 MAPK also in KeratinoRI-/- cells stimulated with LPS

With the same purpose as discussed in part 4.14, the kinetics of LPS-mediated phosphorylation and degradation of IκB, phosphorylation of JNK, phosphorylation of p38 were investigated in the presence and absence of AUP1 overexpression.

In KeratinoRI-/- cells, AUP1 overexpression did not shift the LPS-induced kinetic of IκB degradation compared to those of empty vector control. In both cases, IκB was degraded the most at 60 minute of LPS stimulation and then new IκB was synthesized in feedback mechanism of the signaling pathway. Nevertheless, in AUP1 overexpressed cells, the LPS-mediated degradation of IκB was reduced, especially at time points 30min and 60min, compared to those of control cells (Figure 4.16- total IκB). In contrast, there was no difference in LPS-induced phosphorylation of IκB between AUP1 transfectant and empty vector transfectant (Figure 4.16- phospho-IκB). The remaining phospho-IκB was the result of the balance between two simultaneous processes phosphorylation and degradation. Thus, it is unable to comment upon the relation between LPS-induced phosphorylation of IκB and AUP1 overexpression. In the presence of AUP1 overexpression, LPS-induced phosphorylation of p38 was delayed compared to those of empty vector control. In control cells, the peak of p38 phosphorylation was achieved at 120min after LPS stimulation while in AUP1 overexpressed cells, it occurred at 180min (Figure 4.16- phospho-p38). However, the phosphorylation of JNK was not modified by the overexpression of AUP1 in KeratinoRI-/- cells stimulated with LPS (Figure 4.16- phopho-JNK).
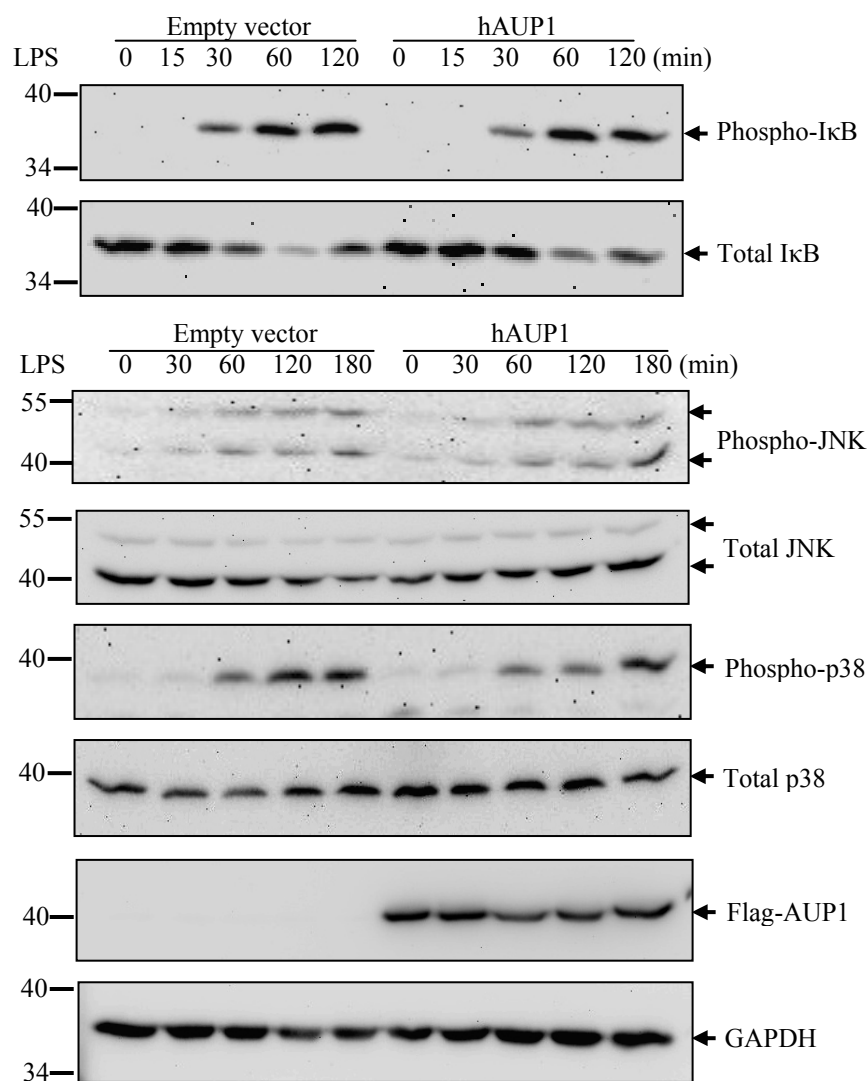
Figure 4.16: AUP1 overexpression reduced the degradation of IκB and delayed the phosphorylation of p38 MAPK in KeratinoRI-/- stimulated with LPS
KeratinoRI-/- cells were transfected either with FLAG-hAUP1 encoding plasmid ($12\mu g/2,5x10^6$ cells) or empty vector. On the next day, transfected cells were stimulated by $1\mu g/ml$ LPS for the indicated times. Cells were lysed, lysates were subjected to SDS-PAGE and Western blot using antibodies against phospho-IκB, total-IκB, phospho-JNK, total-JNK, phospho-p38, total-p38. The expression of FLAG-hAUP1 was confirmed with Western blot using anti FLAG antibody and anti GAPDH antibody was used for loading control. Data shown is one representative experiment out of a series of four with comparable results.

The results shown in Figure 4.15 and 4.16 indicated that AUP1 overexpression impairs IL-1β- and LPS-mediated signaling pathways. AUP1 perhaps interferes with IL-1 and LPS signaling pathways at the level of the activator of IκB: the NEMO-IKK complex and at the activator of the JNK - p38 MAPK: the MKKs or upstream of them.

4.16. AUP1 overexpression reduced IL-1β- and LPS-induced DNA-binding activity of NF-κB-p65 in HEK293RI and KeratinoRI-/- cells

In order to provide more evidence for the direct interference of AUP1 overexpression with signaling pathways, the IL-1β- and LPS-mediated DNA-binding activation of NF-κB-p65 subunit were determined in the presence or absence of AUP1 overexpression.

Once IL-1β or LPS engage to their receptors, they initiate signaling cascades with many events through many mediate proteins. An important outcome of this complicated process is the phosphorylation and degradation of NF-κB inhibitor IκB resulting in the translocation of NF-κB to nucleus [Michael U.Martin, Holge Wesche – BBA – 2002; Foo Y.Liew et. al. – Nature – 2005]. NF-κB mainly exists the heterodimer consisting of p65 (RelA) and p50 in cells. In the nucleus, activated NF-κB p65-p50 binds to promoters and stimulates transcription of genes containing consensus sequence κB 5'-GGGPuNNPyPyCC-3' (Pu: purines, Py: pirimidines) [Okamoto et. al. – Current Pharmaceutical Design – 2007].

IL-1β- and LPS-activated NF-κB-p65 in nuclear extracts of HEK293RI and KeratinoRI-/- cells with or without overexpression of AUP1 were measured using Panomics'TF ELISA kit according to the manufacturer's instruction.

In HEK293RI cells transfected with the empty vector, the DNA-binding activity of NF-κB-p65 increased significantly following the time course of IL-1β stimulation (Figure 4.17A-black square line). However, in AUP1 overexpression, the kinetic of IL-1β-induced DNA-binding activity of NF-κB-p65 was reduced (Figure 4.17A-white round line) compared to the control. In KeratinoRI-/- cells, AUP1 overexpression also impaired LPS-induced DNA-binding activity of NF-κB-p65 compared to the empty vector control (Figure 4.17B). These results support for the regulatory function of AUP1 in IL-1β and LPS pathways.
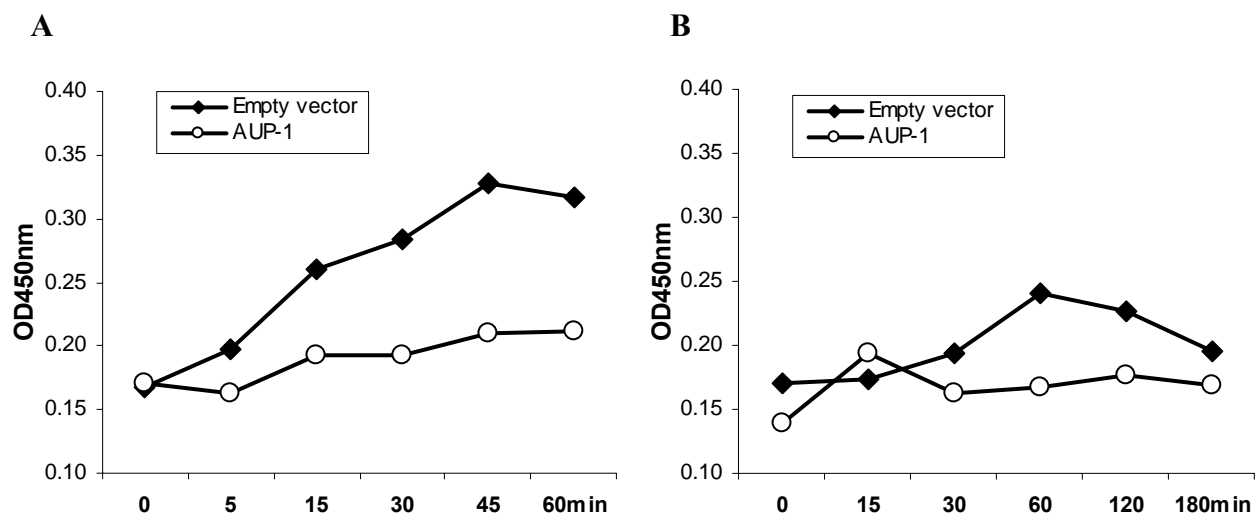
A

B

Figure 4.17: AUP1 overexpression reduced IL-1β- and LPS-induced DNA-binding activity of NF-κB-p65 in HEK293RI and KeratinoRI-/- cells

HEK293RI (A) and KeratinoRI-/- (B) cells transfected with FLAG-hAUP1 encoding construct were stimulated with 5ng/ml rhIL-1β and 1μg/ml LPS, respectively, for the indicated times. Empty vector transfectants were used as control. After stimulation, cells were lysed and nuclear lysates were isolated. Activated NF-κB-p65 in nuclear lysates was determined by binding activity to κB-consensus sequence DNA probe using Panomics'TF ELISA kit as manufacturer's instruction. Depicted are OD450nm values which reflect relative amount of activated NF-κB-p65.

5. DISCUSSION

IRAK4 plays a critical role in TLR/IL-1R signaling pathways. Upon challenges of TLR ligands, IRAK4 deficient mice exhibited severe impairment of NF-κB activation and cytokine production [Suzuki et. al. – Nature – 2002]. The kinase activity of IRAK4 was proved necessary for IL-1/LPS-induced NF-κB activation [Li et. al. – PNAS – 2002]. Recently, a study in IRAK4 kinase inactive knock-in cells showed that IRAK4 kinase activity is required for TLR-mediated JNK and p38 signaling but not for NF-κB and IRF3 activation [Koziczak-Holbro et. al. – Eur J Immunol – 2008]. IRAK4 was proposed to be involved critically in TCR (T cell receptor) – induced T cell proliferation through NF-κB activation. IRAK4, therefore, may be a connection between innate and adaptive immunity [Suzuki et. al. – Science – 2006]. In addition, IRAK4 can phosphorylate NADPH oxidase cytosolic factor p47^{phox} in neutrophils at Thr_{133} and Thr_{356} locating in the consensus sequence (D/E)XQTX(K/R) which is similar to the consensus sequence in the kinase activation loop ($QT_{383}VR$) of IRAK1, the well-known subtrate of IRAK4 [Pacquelet – Biochemical Journal – 2007]. This observation implicates that IRAK4 may phosphorylate structurally related proteins with different roles in distinct cellular events. These diversified data on IRAK4 suggest that more not yet defined functions of IRAK4 may exist. Thus, seeking new partners of IRAK4 is helpful to elucidate the signaling network of IRAK4.

The yeast two-hybrid screening was performed to define partners of IRAK4 from proteins encoded by a cDNA library derived from human placenta tissue (C.Linke & M.U.Martin unpublished). Analysis of screening results revealed novel partner proteins of IRAK4 AUP1 (ancient ubiquitous protein 1) being one of them (C.Kollewe & M.U.Martin unpublished).

AUP1, identified and cloned in 1996, is a protein which is highly conserved in evoultion. The sequence similarity of mouse AUP1 protein with the *Caenorhabditis elegans* or human counterpart molecules is 53% or 90%, respectively. This degree of evolutionary conservation is comparable to that of essential proteins such as pyruvate kinase and ribosomal protein L3. In addition, AUP1 is expressed in nearly

all tissues of mouse and human [Jang et.al. – Genomics – 1996; Kato et.al. – JBC – 2002]. Therefore, it is highly likely that AUP1 plays a basic and essential role in the biology of cell. However, although the molecule is known for some years now, the biological functions of AUP1 are still obscure. AUP1 was proposed to associate with the cytoplasmic tail of the α_{IIb} subunit of the integrin receptor and with Syk protein which binds to the β_3 subunit in megakaryocyte-derived UT7/TPO cells. By this way, AUP1 links the cytoplasmic tails of α_{IIb} and β_3 subunits and sustains the $\alpha_{IIb}\beta_3$ complex in an inactive state [Kato et.al. – JBC – 2002; Kato, Oshimi – Platelets – 2009]. On the other hand, AUP1 was reported to be involved in the dislocation and degradation of glycoproteins in endoplasmic reticulum [Mueller et. al. – PNAS – 2008]. To date, no information is available on the participation of AUP1 in cytokine signaling.

5.1. AUP1 interacts with IRAK4 and IRAK1 in mammalian cells

It is well known that the search for protein interaction with the yeast two-hybrid system is proned to yield false positive results. Therefore, it was necessary to demonstrate the interaction between AUP1 and IRAK4 found in the yeast two-hybrid screening also in mammalian cells. The interaction of AUP1 with IRAK4 was confirmed in HEK293RI cells. If both candidate proteins were overexpressed they could be co-immunprecipitated. As IRAK4 is only one out of four members of serine-threonine protein kinases found to be involved in IL-1 signaling, IRAK1 was also examined for the interaction with AUP1. Interestingly, AUP1 was found to associate with IRAK4 and IRAK1 molecules, but only if kinase inactive mutants were employed for co-immunoprecipitation experiments. The interaction of AUP1 with wild type IRAK4 and IRAK1 which are both kinase active, could not be detected in co-immunoprecipitation experiments suggesting that it was very transient and dependent on the phosphorylation state of IRAK molecules. The phosphorylation state of IRAK4 and IRAK1 strongly influence the interaction with other partners. Phosphorylated IRAK4 and IRAK1 dissociate from the adapter protein MyD88 [Wesche et. al. – Immunity – 1997; Gottipati et. al. – Cellular

Signalling – 2008]. It was previously shown that, under overexpression conditions wild type IRAK4 and IRAK1 become constitutively active and then auto-phosphorylate independent of cytokine stimulation of cells [Li et. al. – PNAS – 2002; Neumann et. al. – Journal of Leukocyte Biology – 2008]. Obviously, kinase inactive IRAK4 and IRAK1 mutants are unable to phosphorylate themselves in an overexpression situation and thus the interaction with AUP1 was sustained as shown in the co-immunoprecipitation studies. Taken together, it is possible that the phosphorylation of IRAK4 and IRAK1 interferes with their interaction with AUP1. This observation is similar to one made with Tollip, a molecule that was described to be a silencer for IRAK1. This molecule can be detected in complex with kinase inactive IRAK1 but not with the wild type molecule due to its sensitivity to phosphates introduced in IRAK1 after activation of its kinase activity [Burns et.al. – Nature – 2000]. Besides of auto-phosphorylation, active IRAK4 and IRAK1 phosphorylate other proteins such as Tollip and Pellino2 [Zhang, Ghosh – JBC – 2002; Strelow et. al. – FEBS Lett – 2003].

5.2. AUP1 is phosphorylated by IRAK4 and possibly IRAK1

Therefore, it was necessary to investigate whether AUP1 is a substrate for IRAK4 and IRAK1. Since wild type IRAK4 and IRAK1 do not interact with AUP1 or the transient interaction is so short lived that it could not be detected in co-immunoprecipitation experiments, forced co-immunoprecipitation assays were performed followed by *in vitro* kinase assays. This experimental approach was originally developed to force together two molecules which normally would only interact briefly. Both molecules, kinase and putative substrate are expressed with the same epitope tag and an antibody is used to immunoprecipitate the two together. Statistically kinase and substrate will be forced into close proximity by the bivalent antibody. If this was performed on a bead, an in vitro kinase assay can easily be perfomed in the presence of radioactive ATP. The results obtained with such experiments showed that AUP1 can be phosphorylated by IRAK4. A much weaker signal was also observed with IRAK1, suggesting that AUP1 was also a substrate

for IRAK1 under these conditions. However, one has to keep in mind that the overepxression was done in HEK293RI cells possessing endogenous IRAK4 and IRAK1. As all IRAK family members readily form heterodimers due to their death domains which are excellent homotypic protein interaction domains, it cannot be excluded that together with overexpressed IRAK1 carrying the tag necessary for forced co-immunoprecipitation endogenous IRAK4 molecules were unwantingly co-precipitated. These molecules would be activated by overexpressed IRAK1 and could then phosphorylate AUP1 on the bead. Thus, at present it cannot be excluded that in fact only IRAK4 accepts AUP1 as substrate. This observation may indicate the involvement of AUP1 in the TLR/IL-1R signaling pathways. However, it was proposed that members of the IRAK family phosphorylate structurally related proteins which play roles in very distinct cellular events [Pacquelet – Biochemical Journal – 2007]. A database search for the homologous domain structure revealed that hAUP1 contains 5 phosphorylation sites for Protein kinase C, 5 phosphorylation sites for Casein kinase II and 1 phosphorylation site for cAMP- and cGMP- dependent protein kinases which are all serine/threonine kinases [http://us.expasy.org/tools/scanprosite/]. Thus, AUP1 is likely susceptive to be phosphorylated by serine/threonine kinases in general. Thus, the phosphorylation of AUP1 in forced close proximity to IRAK4 may be an *in vitro* situation which would need proof *in vivo*.

5.3. AUP1 interacts with many, but not all components in IL-1 signaling complex

In order to ascertain the involvement of AUP1 in the IL-1 signaling pathway, the interaction of AUP1 with several functional components of the IL-1 signaling transduction machinery was investigated including upstream molecules such as IL-1RI, MyD88 and Tollip and downstream components such as TRAF6 and Pellino2. The co-immunoprecipitation results revealed that AUP1 associates constitutively with quiescent IL-1RI and dissociates partially from the receptor complex after a brief IL-1β stimulation. AUP1 also associated with other components of the IL-1RI

complex such as IL-1RAcP (data not shown), MyD88, Tollip, and especially with TRAF6 – the direct downstream signaling transducer of IRAK1. As reported previously, the overexpression of TRAF6 is sufficient to activate downstream events toward activation of NF-κB [Frobose et. al. – Molecular Endocrinology – 2006]. Auto-ubiquitination of TRAF6, an E3 ubiquitin ligase, is critical for the IL-1-dependent signaling pathway [Cao et. al. – Nature – 1996; Lamothe et. al. – JBC – 2007]. The ubiquitination of TRAF6 is essential for the direct interaction of TRAF6 with TAK1-TAB1-TAB2/TAB3 complex via TAB2/TAB3. TAB2/TAB3 binds to the K63-polyubiquitin chain of TRAF6 and by that way links TRAF6 to TAK1 [Tekaesu et. al. – Mol Cell – 2000; Ishitani et. al. – EMBO – 2003]. In addition, TAB2 was reported to facilitate TRAF6 ubiquitination and thereby to mediate IL-1-induced cellular events. The N-terminal region of TAB2 contains an ubiquitin binding domain, the CUE domain, which is important for TAB2-dependent activation of NF-κB. However, the CUE domain is dispensable for the TRAF6 binding of TAB2 [Kishida et.al. – Genes to Cells – 2005]. AUP1 also possesses a CUE domain and it associates strongly with TRAF6, thus AUP1 may play an active role in the TRAF6 function.

As AUP1 interacted with so many molecules in IL-1 signaling it was necessary to question its selectivity. In that respect it is interesting to note that AUP1 does not interact with all molecules known to participate in IL-1 signaling, thus no interaction was observed e.g. with Pellino2.

5.4. Overexpression of AUP1 impairs the IL-1 and LPS signaling transductions but the regulatory effect is not specific for these pathways

All the above findings suggested that AUP1 may play a regulatory role in the IL-1 signaling pathway. In order to elucidate the biological function of AUP1 in IL-1-mediated signaling, the influence of AUP1 overexpression on IL-1β- and LPS-induced signaling transduction was investigated in HEK293RI and KeratinoRI-/- cells, respectively. Cells transfected with different amounts of a hAUP1 encoding plasmid were stimulated by IL-1β or LPS and signaling transduction was measured

by NF-κB -dependent firefly luciferase expression, IL-8, and IL-6 production. Results suggested that AUP1 overexpression partially inhibited IL-1β and LPS-induced signaling pathways in a dose-dependent manner.

The overexpression of AUP1 in the ranges tested had no impact on viability and proliferation of transfected HEK293RI and KeratinoRI-/- cells as shown in MTT assays.

Because of AUP1's evolutionary conservation and ubiquitous expression [Jang et.al. – Genomics – 1996; Kato et.al. – JBC – 2002], it was necessary to verify whether the observed regulatory effect of AUP1 overexpression was specific for IL-1β and LPS signaling. TNFα is a pro-inflammatory cytokine with a biological effector profile overlapping that of IL-1. However, TNF utilizes a discrete receptor system and related but distinct adaptor molecules to activate NF-κB inducing kinase and finally IκB kinases. Thus, TNFα and IL-1 signaling converge at the activation of NF-κB. Therefore, TNFα-activated signaling transduction was investigated in HEK293RI cells overexpressing increasing amounts of hAUP1 by NF-κB -dependent firefly luciferase expression and IL-8 production. Similarly to the results obtained after IL-1β and LPS stimulation, overexpression of AUP1 in HEK293RI cells was also impaired significantly after TNFα stimulated activation of NF-κB and IL-8 production in a dose-dependent manner. Thus, it must be concluded that the negative regulatory effect of AUP1 overexpression is not specific for the IL-1/LPS signaling pathway.

Although IL-1/LPS pathway and TNFα pathway utilize distinct signaling transduction machinery to exert their functions, all of them lead to the activation of NF-κB transcription factor and p38/JNK MAPK in which the activation of NF-κB is the hallmark of the inflammatory response [Martin, Wesche – BBA – 2002; Verstrepen et. al. – Cellular and Molecular Life Sciences – 2008]. Therefore, the involvement of AUP1 in the NF-κB was studied using co-immunoprecipitation experiments to verify whether AUP1 interacts with NF-κB p65 and p50 subunits.

However, there was no evidence for the physical association of AUP1 and the NF-κB transcription factor.

AUP1 associates with components of the IL-1 signaling pathway and seems to be involved in the regulation of this pathway. This observation together with the fact that AUP1 plays a similar role in TNFα pathway led to the presumption that AUP1 may interact with components of the TNFα pathway. Accordingly, AUP1 was examined for the interaction with three components of the TNFα-induced survival pathway including TNFR1, TRADD and TRAF2 by co-immunoprecipitation experiments. AUP1 was found to interact with all of three tested molecules of the TNFα signaling pathway. However, only TNFR1 interacts strongly with AUP1. TRADD and TRAF2 possibly associate with AUP1 indirectly through endogenous TNFR1 in HEK293RI cells.

5.5. AUP1 overexpression does not affect STAT-mediated IL-6 signaling but AUP1 interacts with an IL-6R component

From these results, the critical question arises whether AUP1 is only involved in signaling pathways of pro-inflammatory mediators such as IL-1β, LPS and TNF-α, which all centrally relay on NF-κB, or whether AUP1 plays a more generalized role in cellular functions. To clarify this question, the influence of AUP1 overexpression on another signaling pathway which is completely different from IL-1, LPS and TNFα pathways was examined. The IL-6 pathway appeared to be a good candidate. IL-6 cytokine exerts its action via the IL-6 receptor and signal transducer gp130 leading to the activation of Janus kinases and the phosphorylation and activation of the transcription factor STAT3. STAT3, in turn, once phosphorylated, dimerizes, translocates to the nucleus, and activates the transcription of its regulated genes [Levy, Darnell – Nature Reviews – 2002; Heinrich et. al. – Biochemical Journal – 2003; Murray – Journal of Immunology – 2007]. Human hepatocarcinoma HepG2 cells, which respond well to IL-6, were transiently transfected with different amounts of a hAUP1 encoding construct. Transfected cells were stimulated with rhIL-6 and the signaling performance was evaluated by STAT3-dependent firefly

luciferase expression. The result revealed that overexpression of AUP1 did not alter the STAT3-driven luciferase activity in HepG2 cells stimulated by IL-6, while, similarly to the results observed in HEK293RI cells, overexpression of AUP1 reduced IL-1β and TNFα-stimulated activation of NF-κB in a dose-dependent fashion in HepG2 cells. These results support the working hypothesis that AUP1 is involved in some signaling pathways specifically and that overexpression of this protein does not cause a global effect to physiology of cells.

Surprisingly, when association of AUP1 with IL-6Rα and gp130 – the two components of the IL-6 receptor complex – was investigated, AUP1 was found to associate with one of the two molecules. AUP1 interacted with the non-signaling IL-6Rα chain but not with gp130 – the critical signaling transducer of IL-6 pathway [Rose-John, Schooltink – Recent Results Cancer Res – 2007]. This finding implicated that more work was required to clarify the involvement of AUP1 in the IL-6 signaling pathway.

5.6. The biological function of AUP1: A common negative regulator of many signaling pathways

The overexpression of AUP1 reduced IL-1β-, LPS-, TNFα-stimulated NF-κB activation and cytokine production, whereas it did not affect to IL-6-induced STAT3 activation. In order to further address the biological function of AUP1, besides the investigation of how AUP1 overexpression affects the interested signaling pathways, the influence of AUP1 removal on signaling pathways was investigated. Thus, AUP1 expression in HEK293RI and HepG2 cells was knock-downed by means of siRNA and the responses of cells to IL-1β, TNFα and IL-6 were investigated. IL-1β- and TNFα -induced IL-8 production as well as NF-κB activation were enhanced in AUP1 knock-downed HEK293RI cells compared to those in control cells. These results, together with the previous data obtained in overexpression experiments, provide evidence that AUP1 is an negative regulator of signaling transduction induced by IL-1 (LPS) and TNFα. Since AUP1 associates with IL-6Rα chain, the involvement of AUP1 in the IL-6 pathway could not be

excluded. Here, the involvement of AUP1 with the IL-6 pathway is revealed by the increase of IL-6-induced STAT3 activation in AUP1-silenced HepG2 cells compared to that in control cells. Therefore, AUP1 may also function as a negative regulator of the IL-6 signaling pathway.

Taken together, AUP1 seems to be a common partial inhibitor of all investigated signaling pathways including IL-1β, LPS, TNFα, and IL-6. Moreover, AUP1 possibly regulates many other signaling pathways. The general negative modulator function of AUP1 in the response of cells to stimuli was also manifested by the enhancement of stimulation-independent basal IL-8 production, NF-κB and STAT3 activation in AUP1-silenced cells. The basal NF-κB and STAT3 activation are possibly caused by growth factors in culture medium. The increase of basal NF-κB and STAT3 activation in AUP1-silenced cells implicates that cells respond to growth factors better in the removal of AUP1 or, in another word, AUP1 may be a partial inhibitor of growth factor signaling.

5.7. The biological function of AUP1: Overexpression interferes with IL-1β and LPS signaling transduction upstream of IκB and JNK/p38 MAPK

In the present study, NF-κB - or STAT3-dependent luciferase reporter gene assay and IL-8, IL-6 production were applied to study the biological functions of AUP1 in IL-1β, LPS, TNFα and IL-6 signaling pathways. Although these read-outs reflect the response of cells to stimuli, the underlying mechanism of them is protein synthesis of luciferase enzyme or chemokine/ cytokine. Hence, the partially inhibitory activity of AUP1 in IL-1β, LPS, TNFα or IL-6 signaling pathways suggested by overexpression and silencing of AUP1 experiments is possibly the result of either the down-regulation of signaling transduction or the protein biosynthesis impairment. To distinguish the possible aspects of AUP1 function, kinetics of some important events in IL-1β- and LPS-mediated signaling pathways were investigated in cells with and without overexpression of AUP1. The studied

events included the phosphorylation and degradation of IκB, phosphorylation of JNK, phosphorylation of p38 which are upstream of NF-κB activation and are not directly concerned with the protein synthesis process. Overexpression of AUP1 in HEK293RI cells resulted in the reduction of IL-1β-induced phosphorylation of p38/JNK MAPK and the delay of IκB degradation. Consistent with this, in KeratinoRI-/- cells stimulated by LPS, AUP1 overexpression caused the reduction of IκB degradation and the delay of p38 phosphorylation compared to those of control cells. Furthermore, the IL-1β- and LPS-induced DNA-binding activity of NF-κB-p65 was also reduced in AUP1-overexpressed cells compared to that in control cells. Taken together, it must be concluded that AUP1 overexpression impairs IL-1β and LPS signaling transduction and that the interference happens at the level of the activator of IκB: NEMO-IKK complex and the activator of p38/JNK MAPK: MKKs or up-stream of them.

5.8. Perspective of the present study

The results obtained in the present study shed more light on the biological functions of AUP1. AUP1 exhibits functional properties of a common negative regulator for the response of cells to outside stimuli such as IL-1β, LPS, TNFα, IL-6. It is presently unknown whether AUP1 interacts with TLR4, but it is clear that AUP1 associates with IL-1RI, TNFR1 and IL-6R. Analysis of the influence of AUP1 overexpression in IL-1β and LPS signaling transductions revealed that AUP1 interferes with these signaling pathways upstream of the NEMO-IKK complex and the p38/JNK MAPK. The fact that IL-1β, TNFα and IL-6 – induced pathways are completely distinct, the only thing in common with respect to AUP1 is that all of the three receptors IL-1RI, TNFR1 and IL-6Rα associate with AUP1. This raises the question whether AUP1 is involved in expression and/or performance of these receptors on the cell surface.

Although it is presently not possible to pinpoint the underlying mechanism of AUP1 action in these signaling transduction pathways, this work suggests that AUP1

down-regulates the response of cells to outside stimuli possibly via the regulation of the receptors on the cell surface. There are two hypotheses to explain how AUP1 interferes with the cell surface receptor functions. First, AUP1 may reduce the amount of the receptors expressed on the cell surface by either interfering with the trafficking of receptor molecules from the endoplasmic reticulum to the cell surface or by enhancing the dislocation/degradation of the receptors from endoplasmic reticulum membrane during their synthesis. As reported previously, overexpression of the dominant negative form of AUP1, GFP-AUP1, inhibited strongly class I MHC heavy chains dislocation and degradation [Mueller et. al. – PNAS – 2008], indicating that AUP1 may accelerate the dislocation/degradation of receptor components from the endoplasmic reticulum membrane. Obviously, signaling will be indeed impaired when the amount of the corresponding cell surface receptors is decreased. Second, AUP1 may associate constitutively with components of the receptor complexes and obstruct the recruitment to the receptor complexes of downstream molecules in the signal transduction machinery. Thus, AUP1 may sustain the receptor complexes in inactive states or may attenuate the signaling transductions. A similar model for the function of AUP1 was proposed in the case of the $\alpha_{IIb}\beta_3$ integrin receptor complex in platelet cells, where AUP1 associates with the cytoplasmic tail of α_{IIb} subunit thus mantains the $\alpha_{IIb}\beta_3$ complex in an inactive state [Kato et.al. – JBC – 2002; Kato, Oshimi – Platelets – 2009]. Therefore, the subsequent investigations will aim to elucidate the influence of the overexpression and removal of AUP1 on the quantity of cytokine receptors such as IL-1RI, TNFR1 and IL-6Rα expressed on the cell surface. On the other hand, it may be necessary to study the effect of AUP1 overexpression and silencing on the formation of these active receptor complexes upon cytokine stimulation.

6. SUMMARY

In the progress of seeking for new partners of IRAK4 - a critical kinase of the IL-1R/TLR signaling pathways - using the yeast two-hybrid screening, AUP1 (Ancient Ubiquitous Protein 1) was identified to interact with IRAK4. AUP1 is a protein which is highly conserved in evolution and is ubiquitously expressed in tissues of mouse and human. However, the functions of AUP1 in cell biology have not been clearly defined yet.

The interaction of AUP1 was confirmed for the interaction with IRAK4 and IRAK1 in HEK293RI cells. Interestingly, AUP1 was only found to interact with kinase inactive IRAK4 and IRAK1 but not with the wild type molecules, indicating that the kinase activity of IRAK molecules interferes with their association with AUP1. In addition, AUP1 can be phosphorylated by IRAK4 and possibly IRAK1 as shown in the forced co-immunoprecipitation and *in vitro* kinase assays. Besides IRAK4 and IRAK1, AUP1 interacts with other components in the IL-1 signaling machinery such as IL-1RI, MyD88, Tollip, TRAF6, suggesting the involvement of AUP1 in the IL-1 pathway.

Indeed, overexpression of AUP1 in HEK293RI and KeratinoRI-/- cells reduces IL-1β- and LPS-induced activation of NF-κB as well as chemokine/cytokine production, respectively. However, the regulatory effect of AUP1 overexpression is not specific for IL-1 or LPS signaling as TNFα signaling is also impaired by AUP1 overexpression. AUP1 was also found to associate with TNFR1, TRADD and TRAF2 which are components of TNFα pathway. Interestingly, although overexpression of AUP1 does not affect STAT-mediated IL-6 signaling, AUP1 interacts with the IL-6Rα chain but not with the signaling transducer gp130. In contrast, silencing of AUP1 via siRNA enhances the IL-1β-, TNFα-stimulated activation of NF-κB as well as IL-6-stimulated activation of STAT3, indicating that AUP1 is a common negative regulator of the cytokine signaling pathways investigated.

As overexpression of AUP1 interferes with IL-1β/LPS signaling transduction upstream of IκB and the JNK/p38 MAPK and AUP1 was found to associate with all

three of the cytokine receptors IL-1RI, TNFR1 and IL-6Rα, the role of AUP1 may be to down-regulate the response of cells to outside stimuli via the impairment of the expression and/or performance of these receptors on the cell surface.

7. ZUSAMMENFASSUNG

Im Rahmen der Suche nach neuen Wechselwirkungspartnern von IRAK4 –einer kritischen Kinase des IL-1R/TLR Signalwegs- wurde mit dem *"yeast two hybrid"*-System AUP1 (Ancient Ubiquitious Protein 1) als Partner von IRAK4 identifiziert. AUP1 ist ein Protein, das in der Evolution stark konserviert ist und ubiquitär in Geweben der Maus und des Menschen exprimiert wird. Die Funktionen von AUP-1 in der Biologie der Zelle sind bis heute weitgehend ungeklärt.

Die direkte Proteinwechselwirkung von AUP1 mit IRAK4 und IRAK1 wurde in der Zelllinie HEK293RI nachgewiesen. Interessanterweise konnte AUP1 nur mit den Kinase-inaktiven Varianten von IRAK4 und IRAK1 immunpräzipitiert werden, jedoch nicht mit den Wildtyp-Molekülen, was darauf hindeutet, dass die Kinaseaktivität der IRAK-Moleküle mit der Assoziation von AUP1 interferiert. Zusätzlich konnte durch forcierte Koimmunpräzipitationen mit anschließendem *in vitro* Kinase Assay gezeigt werden, dass AUP1 durch IRAK4, und möglicherweise auch durch IRAK1, phosphoryliert werden kann. Neben IRAK4 und IRAK1 interagiert AUP1 mit weiteren Komponenten der IL-1 Signaltransduktions-maschinerie wie z.B. IL-RI, MyD88, Tollip, TRAF6, was nahe legt, dass AUP1 im IL-1 Signalweg eine Rolle spielt.

Überexpression von AUP1 in HEK293RI und KeratinoRI-/- Zellen verringerte die IL-1β- oder LPS-induzierte Aktivierung von NF-κB genauso wie die Chemokin/ Zytokinproduktion. Dieser negativ regulatorische Effekt der Überexpression von AUP1 ist jedoch nicht spezifisch für die IL-1 oder die LPS Signaltransduktion, da die durch TNFα ausgelösten Signale ebenfalls durch AUP1 Überexpression beeinflusst wurden. AUP1 assoziierte auch mit TNFR1, TRADD und TRAF2; alle drei Moleküle sind Bestandteile des TNFα-Signalwegs. Die Überexpression von AUP1 beeinflusste die STAT-vermittelte IL-6 Signaltransduktion zwar nicht,

dennoch konnte gezeigt werden, dass AUP1 an die IL-6 Rezeptor α-Kette bindet, jedoch nicht an der signaltransduzierende gp130 Untereinheit. Im Gegensatz zur Überexpression, verstärkte das Ausschalten von AUP1 mittels siRNA-Technik die IL-1β- und TNFα-stimulierte Aktivierung von NF-κB ebenso wie die IL-6-stimulierte Aktivierung von STAT3. Dies weist darauf hin, dass AUP1 ein generalisierter negativer Regulator der hier untersuchten Zytokinsignalwege ist. Da die Wirkung der Überexpression von AUP1 im IL-1β/LPS Signalweg oberhalb von IκB und dem JNK/p38 MAPK – Element einzuordnen war und zudem AUP1 an den drei untersuchten Zytokinrezeptoren IL-1RI, TNFR1 and IL-6Rα nachgewiesen werden konnte, liegt es nahe zu vermuten, dass die Rolle von AUP1 darin liegt, die Fähigkeit einer Zelle zu dämpfen, auf Stimuli von außerhalb der Zelle zu antworten, indem die Expression oder Funktionsfähigkeit dieser Rezeptoren auf der Zelloberfläche negativ reguliert wird.

(D. Q. N. was assisted in translating the English summary into the German Zusammenfassung by Prof. M. U. Martin.)

8. REFERENCES

Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004 Jul;4(7):499-511. Review.

Brissoni B, Agostini L, Kropf M, Martinon F, Swoboda V, Lippens S, Everett H, Aebi N, Janssens S, Meylan E, Felberbaum-Corti M, Hirling H, Gruenberg J, Tschopp J, Burns K. Intracellular trafficking of interleukin-1 receptor I requires Tollip. Curr Biol. 2006 Nov 21;16(22):2265-70.

Burns K, Clatworthy J, Martin L, Martinon F, Plumpton C, Maschera B, Lewis A, Ray K, Tschopp J, Volpe F. Tollip, a new component of the IL-1RI pathway, links IRAK to the IL-1 receptor. Nat Cell Biol. 2000 Jun;2(6):346-51.

Cao Z, Xiong J, Takeuchi M, Kurama T, Goeddel DV. TRAF6 is a signal transducer for interleukin-1. Nature. 1996 Oct 3;383(6599):443-6

Chen ZJ. Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol. 2005 Aug;7(8):758-65. Review.

Didierlaurent A, Brissoni B, Velin D, Aebi N, Tardivel A, Käslin E, Sirard JC, Angelov G, Tschopp J, Burns K. Tollip regulates proinflammatory responses to interleukin-1 and lipopolysaccharide. Mol Cell Biol. 2006 Feb;26(3):735-42.

Donaldson KM, Yin H, Gekakis N, Supek F, Joazeiro CA. Ubiquitin signals protein trafficking via interaction with a novel ubiquitin binding domain in the membrane fusion regulator, Vps9p. Curr Biol. 2003 Feb 4;13(3):258-62.

Ehrhardt,C., Schmolke,M., Matzke,A., Knoblauch,A., Will,C., Wixler,V. and Ludwig,S. Polyethylenimine, a cost-effective transfection reagent. Signal Transduct. 2006, 6, 179–184.

Frobøse H, Rønn SG, Heding PE, Mendoza H, Cohen P, Mandrup-Poulsen T, Billestrup N. Suppressor of cytokine Signaling-3 inhibits interleukin-1 signaling by targeting the TRAF-6/TAK1 complex. Mol Endocrinol. 2006 Jul;20(7):1587-96.

Gottipati S, Rao NL, Fung-Leung WP. IRAK1: a critical signaling mediator of innate immunity. Cell Signal. 2008 Feb;20(2):269-76.

Heinrich PC, Behrmann I, Haan S, Hermanns HM, Müller-Newen G, Schaper F. Principles of interleukin (IL)-6-type cytokine signalling and its regulation. Biochem J. 2003 Aug 15;374(Pt 1):1-20.

Hsu H, Xiong J, Goeddel DV. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. Cell. 1995 May 19;81(4):495-504.

Hurley JH, Lee S, Prag G. Ubiquitin-binding domains. Biochem J. 2006 Nov 1;399(3):361-72.

Ishitani T, Takaesu G, Ninomiya-Tsuji J, Shibuya H, Gaynor RB, Matsumoto K. Role of the TAB2-related protein TAB3 in IL-1 and TNF signaling. EMBO J. 2003 Dec 1;22(23):6277-88.

Jang W, Weber JS, Bashir R, Bushby K, Meisler MH. Aup1, a novel gene on mouse chromosome 6 and human chromosome 2p13. Genomics. 1996 Sep 1;36(2):366-8.

Janssens S, Beyaert R. Functional diversity and regulation of different interleukin-1 receptor-associated kinase (IRAK) family members. Mol Cell. 2003 Feb;11(2):293-302. Review.

Karpisheva KV, Touber B, Kisliakova TV, Dobner T. A new human cellular protein AUP1. II. cDNA cloning, genomic organization of Aup1 gene ans preliminary characterization of human AUP1 protein. Tsitologiia. 2002;44(9):839-45. Russian.

Kato A, Kawamata N, Tamayose K, Egashira M, Miura R, Fujimura T, Murayama K, Oshimi K. Ancient ubiquitous protein 1 binds to the conserved membrane-proximal sequence of the cytoplasmic tail of the integrin alpha subunits that plays a crucial role in the inside-out signaling of alpha IIbbeta 3. J Biol Chem. 2002 Aug 9;277(32):28934-41.

Kato A, Oshimi K. Ancient ubiquitous protein 1 and Syk link cytoplasmic tails of the integrin alpha(IIb)beta(3). Platelets. 2009 Mar;20(2):105-10.

Kieser A, Kaiser C, Hammerschmidt W. LMP1 signal transduction differs substantially from TNF receptor 1 signaling in the molecular functions of TRADD and TRAF2. EMBO J. 1999 May 4;18(9):2511-21

Kishida S, Sanjo H, Akira S, Matsumoto K, Ninomiya-Tsuji J. TAK1-binding protein 2 facilitates ubiquitination of TRAF6 and assembly of TRAF6 with IKK in the IL-1 signaling pathway. Genes Cells. 2005 May;10(5):447-54.

Kollewe C, Mackensen AC, Neumann D, Knop J, Cao P, Li S, Wesche H, Martin MU. Sequential autophosphorylation steps in the interleukin-1 receptor-associated kinase-1 regulate its availability as an adapter in interleukin-1 signaling. J Biol Chem. 2004 Feb 13;279(7):5227-36.

Koziczak-Holbro M, Glück A, Tschopp C, Mathison JC, Gram H. IRAK-4 kinase activity-dependent and -independent regulation of lipopolysaccharide-inducible genes. Eur J Immunol. 2008 Mar;38(3):788-96.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970 Aug 15;227(5259):680-5.

Lamothe B, Besse A, Campos AD, Webster WK, Wu H, Darnay BG. Site-specific Lys-63-linked tumor necrosis factor receptor-associated factor 6 auto-ubiquitination is a critical determinant of I kappa B kinase activation. J Biol Chem. 2007 Feb 9;282(6):4102-12.

Leeman JR, Gilmore TD. Alternative splicing in the NF-kappaB signaling pathway. Gene. 2008 Nov 1;423(2):97-107. Review.

Lehmann U, Schmitz J, Weissenbach M, Sobota RM, Hortner M, Friederichs K, Behrmann I, Tsiaris W, Sasaki A, Schneider-Mergener J, Yoshimura A, Neel BG, Heinrich PC, Schaper F. SHP2 and SOCS3 contribute to Tyr-759-dependent attenuation of interleukin-6 signaling through gp130. J Biol Chem. 2003 Jan 3;278(1):661-71

Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol. 2002 Sep;3(9):651-62.

Li S, Strelow A, Fontana EJ, Wesche H. IRAK-4: a novel member of the IRAK family with the properties of an IRAK-kinase. Proc Natl Acad Sci U S A. 2002 Apr 16;99(8):5567-72.

Li T, Hu J, Li L. Characterization of Tollip protein upon Lipopolysaccharide challenge. Mol Immunol. 2004 May;41(1):85-92.

Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. Nat Rev Immunol. 2005 Jun;5(6):446-58. Review.

Lilley BN, Ploegh HL. A membrane protein required for dislocation of misfolded proteins from the ER. Nature. 2004 Jun 24;429(6994):834-40.

Lilley BN, Ploegh HL. Multiprotein complexes that link dislocation, ubiquitination, and extraction of misfolded proteins from the endoplasmic reticulum membrane. Proc Natl Acad Sci U S A. 2005 Oct 4;102(40):14296-301.

Martin MU, Wesche H. Summary and comparison of the signaling mechanisms of the Toll/interleukin-1 receptor family. Biochim Biophys Acta. 2002 Nov 11;1592(3):265-80.

Mueller B, Klemm EJ, Spooner E, Claessen JH, Ploegh HL. SEL1L nucleates a protein complex required for dislocation of misfolded glycoproteins. Proc Natl Acad Sci U S A. 2008 Aug 26;105(34):12325-30.

Mueller B, Lilley BN, Ploegh HL. SEL1L, the homologue of yeast Hrd3p, is involved in protein dislocation from the mammalian ER. J Cell Biol. 2006 Oct 23;175(2):261-70.

Muppidi JR, Tschopp J, Siegel RM. Life and death decisions: secondary complexes and lipid rafts in TNF receptor family signal transduction. Immunity. 2004 Oct;21(4):461-5. Review.

Murray PJ. The JAK-STAT signaling pathway: input and output integration. J Immunol. 2007 Mar 1;178(5):2623-9.

Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med. 2008 Mar;14(3):109-19.

Neumann D, Kollewe C, Pich A, Cao P, Resch K, Martin MU. Threonine 66 in the death domain of IRAK-1 is critical for interaction with signaling molecules but is not a target site for autophosphorylation. J Leukoc Biol. 2008 Sep;84(3):807-13.

Neuwald AF. Barth syndrome may be due to an acyltransferase deficiency. Curr Biol. 1997 Aug 1;7(8):R465-6.

Okamoto T, Sanda T, Asamitsu K. NF-kappa B signaling and carcinogenesis. Curr Pharm Des. 2007;13(5):447-62.

O'Neill LA. The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress. Immunol Rev. 2008 Dec;226:10-8.

Pacquelet S, Johnson JL, Ellis BA, Brzezinska AA, Lane WS, Munafo DB, Catz SD.
Cross-talk between IRAK-4 and the NADPH oxidase. Biochem J. 2007 May 1;403(3):451-61.

Ponting CP. Proteins of the endoplasmic-reticulum-associated degradation pathway: domain detection and function prediction. Biochem J. 2000 Oct 15;351 Pt 2:527-35.

Ringwood L, Li L. The involvement of the interleukin-1 receptor-associated kinases (IRAKs) in cellular signaling networks controlling inflammation. Cytokine. 2008 Apr;42(1):1-7.

Rose-John S, Schooltink H. Cytokines are a therapeutic target for the prevention of inflammation-induced cancers. Recent Results Cancer Res. 2007;174:57-66.

Schaper F, Gendo C, Eck M, Schmitz J, Grimm C, Anhuf D, Kerr IM, Heinrich PC. Activation of the protein tyrosine phosphatase SHP2 via the

interleukin-6 signal transducing receptor protein gp130 requires tyrosine kinase Jak1 and limits acute-phase protein expression. Biochem J. 1998 Nov 1;335 (Pt 3):557-65

Schmitz J, Weissenbach M, Haan S, Heinrich PC, Schaper F. SOCS3 exerts its inhibitory function on interleukin-6 signal transduction through the SHP2 recruitment site of gp130. J Biol Chem. 2000 Apr 28;275(17):12848-56.

Schneider F, Neugebauer J, Griese J, Liefold N, Kutz H, Briseño C, Kieser A. The viral oncoprotein LMP1 exploits TRADD for signaling by masking its apoptotic activity. PLoS Biology. 2008 Jan;6(1):e8

Shih SC, Prag G, Francis SA, Sutanto MA, Hurley JH, Hicke L. A ubiquitin-binding motif required for intramolecular monoubiquitylation, the CUE domain. EMBO J. 2003 Mar 17;22(6):1273-81.

Strelow A, Kollewe C, Wesche H. Characterization of Pellino2, a substrate of IRAK1 and IRAK4. FEBS Lett. 2003 Jul 17;547(1-3):157-61.

Suzuki N, Suzuki S, Duncan GS, Millar DG, Wada T, Mirtsos C, Takada H, Wakeham A, Itie A, Li S, Penninger JM, Wesche H, Ohashi PS, Mak TW, Yeh WC. Severe impairment of interleukin-1 and Toll-like receptor signalling in mice lacking IRAK-4. Nature. 2002 Apr 18;416(6882):750-6.

Suzuki N, Suzuki S, Millar DG, Unno M, Hara H, Calzascia T, Yamasaki S, Yokosuka T, Chen NJ, Elford AR, Suzuki J, Takeuchi A, Mirtsos C, Bouchard D, Ohashi PS, Yeh WC, Saito T. A critical role for the innate immune signaling molecule IRAK-4 in T cell activation. Science. 2006 Mar 31;311(5769):1927-32.

Takaesu G, Kishida S, Hiyama A, Yamaguchi K, Shibuya H, Irie K, Ninomiya-Tsuji J, Matsumoto K. TAB2, a novel adaptor protein, mediates activation of TAK1 MAPKKK by linking TAK1 to TRAF6 in the IL-1 signal transduction pathway. Mol Cell. 2000 Apr;5(4):649-58.

Tian Y, Zhang Y, Zhong B, Wang YY, Diao FC, Wang RP, Zhang M, Chen DY, Zhai ZH, Shu HB. RBCK1 negatively regulates tumor necrosis factor- and interleukin-1-triggered NF-kappaB activation by targeting TAB2/3 for degradation. J Biol Chem. 2007 Jun 8;282(23):16776-82.

van Horssen R, Ten Hagen TL, Eggermont AM. TNF-alpha in cancer treatment: molecular insights, antitumor effects, and clinical utility. Oncologist. 2006 Apr;11(4):397-408.

Verstrepen L, Bekaert T, Chau TL, Tavernier J, Chariot A, Beyaert R. TLR-4, IL-1R and TNF-R signaling to NF-kappaB: variations on a common theme. Cell Mol Life Sci. 2008 Oct;65(19):2964-78. Review.

von Laue S, Finidori J, Maamra M, Shen XY, Justice S, Dobson PR, Ross RJ. Stimulation of endogenous GH and interleukin-6 receptors selectively activates different Jaks and Stats, with a Stat5 specific synergistic effect of dexamethasone. J Endocrinol. 2000 May;165(2):301-11.

Wen Z, Zhong Z, Darnell JE Jr. Maximal activation of transcription by Stat1 and Stat3 requires both tyrosine and serine phosphorylation. Cell. 1995 Jul 28;82(2):241-50.

Wesche H, Henzel WJ, Shillinglaw W, Li S, Cao Z. MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. Immunity. 1997 Dec;7(6):837-47.

West J, Tompkins CK, Balantac N, Nudelman E, Meengs B, White T, Bursten S, Coleman J, Kumar A, Singer JW, Leung DW. Cloning and expression of two human lysophosphatidic acid acyltransferase cDNAs that enhance cytokine-induced signaling responses in cells. DNA Cell Biol. 1997 Jun;16(6):691-701.

Ye Y, Shibata Y, Yun C, Ron D, Rapoport TA. A membrane protein complex mediates retro-translocation from the ER lumen into the cytosol. Nature. 2004 Jun 24;429(6994):841-7.

Zhang G, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by Tollip. J Biol Chem. 2002 Mar 1;277(9):7059-65.

Zhang X, Blenis J, Li HC, Schindler C, Chen-Kiang S. Requirement of serine phosphorylation for formation of STAT-promoter complexes. Science. 1995 Mar 31;267(5206):1990-4.

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