NUB1L and FAT10, two ubiquitin-like proteins involved in protein degradation

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ABBREVIATIONS

AA	amino acid
AAA	ATPase associated with various cellular activities
AIPL1	aryl hydrocarbon receptor-interacting protein-like 1
APC	Anaphase Promoting Complex
APG12	Autophagy protein 12
ATP	adenosine triphosphate
BAG	Bcl-2-associated athanogene
CBC	elongin C-elongin B-Cul2
CHIP	carboxyl-terminus of Hsc70 interacting protein
CP	core particle
CSN	COP9 signalosome
CUE	Cue1-homologous
DEN1	deneddylase 1
DUB	deubiquitylating enzyme
E6-AP	E6-associated protein
EBV	Epstein-Barr virus
ENaC	epithelial amilorid-sensitive sodium channel
ERAD	endoplasmic reticulum-associated degradation
FT-ICR	fourier transform-ion cyclotron resonance
GFP	green fluorescent protein
GST	glutathione *S*-transferase
HCC	hepatocellular carcinoma
HECT	homologous to E6-AP carboxyl terminus
HSC70	heat shock cognate 70
HSP70	heat shock protein 70
HPV	human papillomavirus
HUB1	homologous to ubiquitin 1
IKK	IκB kinase
IκBα	NF-κB inhibitory protein
IFN-γ	interferon-γ
IPTG	isopropyl β-D-1-thiogalactopyranoside
ISG15	Interferon-stimulated gene-15
JAMM	JAB1/MPN/Mov34 metalloenzyme
LCA	Leber´s congenital amaurosis
LCMV	lymphocytic choriomeningitis virus
MALDI	matrix-assisted laser desorption ionization
MCMV	mouse cytomegalovirus
MDa	Mega-Dalton
Mg	magnesium
MHC	major histocompatability complex
MIR	modulator of immune recognition
NMNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NEDD8	Neural precursor cell-Expressed Developmentally down-regulated
NEDP	NEDD8 protease
NEPHGE	non-equilibrium pH gradient gel electrophoresis
NF-κB	nuclear factor-κB

NLS	nuclear localization signal
NPC	nuclear pore complex
Ntn	N-terminal nucleophile
NUB1	NEDD8 Ultimate Buster-1
NUB1L	NUB1long
ODC	ornithine decarboxylase
P_i	orthophosphate ion
PA28	28-kDa proteasome activator
PGPH	peptidylglutamyl peptide hydrolytic
PIAS	Protein inhibitor of activated STATs
PML	promyelocytic leukemia
RanGAP1	Ran-GTPase-activating protein
RING	really interesting new gene
SCF	Skp1-Cullin-F-box protein
SUMO	small ubiquitin like modifier
SP-RING	Siz/PIAS RING
RUB	related to ubiquitin
TCR	T-cell antigen receptor
tet	tetracycline
TNF-α	tumor necrosis factor-α
Tsg101	tumour susceptibility gene 101 protein
UBA	ubiquitin-associated
UBL	ubiquitin like modifier
UBP	ubiquitin specific processing protease
UCH	ubiquitin C-terminal hydrolase
UCRP	ubiquitin cross reacting protein
UDP	ubiquitin domain protein
UEV	Ubiquitin-conjugating enzyme variant
Ulp1	ubiquitin like protease 1
UIM	ubiquitin interacting motif
VHL	von Hippel-Lindau

Amino acids

Alanine	Ala	A
Cysteine	Cys	C
Glycine	Gly	G
Histidine	His	H
Glutamic acid	Glu	E
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Proline	Pro	P
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y

DEUTSCHE ZUSAMMENFASSUNG

FAT10 ist ein Mitglied der Proteinfamilie der ubiquitinähnlichen Proteine. Es kann, wie Ubiquitin, mit seinem carboxy-terminalen Diglycinmotiv kovalent an die Lysinseitenketten anderer, noch unbekanneter Proteine gebunden werden. Durch die Zytokine Tumor necrosis factor-α und Interferon-γ ist es synergistisch induzierbar. Das Gen für FAT10 liegt im Haupthistokompatibilitätskomplex.

In dieser Doktorarbeit zeige ich, dass sowohl freies, als auch an Zielproteine gebundenes FAT10 schnell durch das Proteasom abgebaut wird. Die Halbwertszeit von FAT10 beträgt circa eine Stunde. Wenn man FAT10 an den Amino-Terminus von sehr langlebigen Proteinen anhängt, wird deren Halbwertszeit in einem Maße verringert, der mit dem Effekt einer Fusion von Ubiquitin mit diesen Proteinen zu vergleichen ist.

Die Entdeckung eines mit FAT10 interagierenden Proteins könnte eine mögliche Erklärung für diesen Effekt liefern, indem es eine Verbindung zwischen FAT10 und dem Proteasom herstellt. Bei einem „Yeast two hybrid screen“ konnte NEDD 8 ultimate buster 1 long (NUB1L) als nicht-kovalenter Interaktionspartner von FAT10 identifiziert werden. Diese Interaktion wurde durch Koimmunopräzipitationen und eine *in vitro* Interaktion mit Glutathion-S-transferase-gekoppeltem FAT10 bestätigt.

NUB1L ist genauso wie FAT10 durch Interferone induzierbar, und es wurde berichtet, dass NUB1L mit dem ubiquitinähnlichen Protein NEDD8 wechselwirkt, und dessen Abbau beschleunigt. In meiner Arbeit zeige ich, dass die Bindung von NUB1L an FAT10 deutlich stärker ist, als die an NEDD8, und dass NEDD8 die Bindung von FAT10 an NUB1L nicht behindert. Da SUMO oder Ubiquitin nach Kopplung an andere Proteine nicht mit NUB1L interagieren, kann man davon ausgehen, dass die Bindung von FAT10 oder FAT10 gekoppelten Proteinen an NUB1L spezifisch ist.

FAT10 wird achtmal schneller in Gegenwart von NUB1L abgebaut, als in dessen Abwesenheit. Da *in vitro* gezeigt wurde, dass NUB1 an die Rpn10 Untereinheit des Proteasoms bindet, und in 26S Proteasomenpräperationen enthalten ist, könnte es sein, dass NUB1L FAT10 und FAT10-konjugierte Proteine zum Abbau in die Nachbarschaft des 26S Proteasoms transportiert.

Der über FAT10 vermittelte Proteinabbau scheint ubiquitinunabhängig zu sein, da weder die Deletion aller Lysinreste von FAT10, noch die Expression von FAT10 in Zellen mit defekter Ubiquitinierungsmaschinerie den Abbau verzögert.
Im Gegensatz zur Ubiquitinierung scheint die Modifikation mit FAT10 ein irreversibler Prozess zu sein. Eine Fusion von FAT10 mit GFP wurde nicht gespalten, sondern es wurden beide Proteine abgebaut. Dies deutet darauf hin, dass keine FAT10-dekonjugierenden Enzyme existieren.
In dieser Arbeit zeige ich, dass sich der FAT10-vermittelte Proteinabbau in mehreren Punkten vom ubiquitinvermittelten unterscheidet. Zusammengefasst kann man sagen, dass die Modifikation mit FAT10 ein alternativer, ubiquitinunabhängiger Mechanismus ist, der zum Abbau von Proteinen durch das Proteasom führt. Dieser Weg ist Zytokin-induzierbar und nicht umkehrbar.

SUMMARY

FAT10 is a small ubiquitin-like modifier that is encoded in the major histocompatibility complex and is synergistically inducible by tumor necrosis factor-α and interferon-γ. It is composed of two ubiquitin-like domains and possesses a diglycine motif at its C-terminus, which probably can form isopeptide bonds to so far unidentified target proteins.

In this thesis I show that unconjugated FAT10 and a FAT10 conjugate are rapidly degraded by the proteasome with a similar half-life of approximately one hour. Fusion of FAT10 to the N-terminus of very long-lived proteins reduces their half-lives as potently as achieved by fusion with ubiquitin.

A possible explanation for this is given through the identification of a new interaction partner of FAT10, which links FAT10 to the proteasome. A yeast two hybrid screen identified NEDD8 ultimate buster-1long (NUB1L) as a non-covalent binding partner of FAT10 and this interaction was confirmed by coimmunoprecipitation and glutathione-S-transferase pull down experiments.

NUB1L is like FAT10 an interferon inducible protein, which has been reported to interact with the ubiquitin-like protein NEDD8 and lead to accelerated NEDD8 degradation. Here I show that NUB1L binds to FAT10 much stronger than to NEDD8 and that NEDD8 can not compete with FAT10 for NUB1L binding. The interaction of FAT10 and NUB1L is specific, as GFP fusion proteins containing ubiquitin or SUMO-1 do not bind to NUB1L.

The coexpression of NUB1L enhances the degradation rate of FAT10 eightfold. Since NUB1 was shown to bind to the proteasome subunit RPN10 *in vitro* and to be contained in 26S proteasome preparations it may function as a linker that targets FAT10 as well as FAT10 modified proteins for degradation by the proteasome.

The FAT10 mediated degradation is ubiquitin-independent, since the prevention of ubiquitylation of FAT10 by mutation of all lysines of FAT10 or by expression of FAT10 in ubiquitylation-deficient cells did not affect FAT10 degradation.

In contrast to ubiquitylation the conjugation of FAT10 seems to be irreversible, since FAT10-green fluorescent protein fusion proteins were not cleaved but entirely degraded suggesting a lack of FAT10-specific deconjugating enzymes.

I show in this thesis that the FAT10 mediated degradation has many differences compared with the ubiquitin mediated degradation. In summary it appears that conjugation with FAT10 is an alternative, ubiquitin-independent, targeting mechanism for degradation by the proteasome, which in contrast to polyubiquitylation, is cytokine-inducible and irreversible.

1.) GENERAL INTRODUCTION

UBIQUITIN

Ubiquitin is a conserved 76 amino acid (AA) protein, which is able to form stable chemical bonds with other proteins. Ubiquitin is encoded on several genes, either as an oligomer of ubiquitin or as a fusion with other proteins, particularly with small ribosomal subunits. In both cases a maturation step is needed where monomeric ubiquitin is released through ubiquitin specific proteases, which recognize the C-terminal diglycine motif along with the ubiquitin domain, and which cleave after the diglycine motif. This free C terminal glycine (Gly-76) can form an isopeptide bond with the ε-amino group of lysine residues of a substrate protein. In some cases the conjugation also appears at the N-terminus of the substrate (Bloom et al., 2003; Breitschopf et al., 1998).

This modification of a substrate with ubiquitin, which is termed ubiquitylation, is an ATP dependent process that involves the sequential action of at least three different classes of enzymes (Figure 1). An E1 or ubiquitin activating enzyme, an E2 or ubiquitin conjugating enzyme, an E3 or ubiquitin protein ligase and in some cases an E4 chain elongation factor (Pickart, 2001).

To activate ubiquitin the E1 binds first to MgATP and then to ubiquitin. This leads to the formation of a ubiquitin adenylate that serves as a donor of ubiquitin to a cysteine at the active site of the E1 (Haas and Rose, 1982). The thiol-linked ubiquitin is then transferred to the active site cysteine of an E2.

While there is only one E1 in mammals, there exist several E2s. All E2s share a conserved core domain of about 150 AA that contains an invariant cysteine. Some members of the E2 family have N- or C-terminal extensions that may be involved in specific E2-E3 interactions. In the human genome there can be found 36 E2 domains (von Arnim, 2001).

The E3 ubiquitin protein ligases transfer the activated ubiquitin from the E2 to an ε-amino group of a lysine side chain of a previously bound protein, thereby forming an isopeptide bond. This can be done in two different ways; either the E3 forms a covalent ubiquitin intermediate, or the E3 positions the E2 and the potential substrate

in such a way that the ubiquitin transfer is facilitated, directly from the E2 to the substrate.

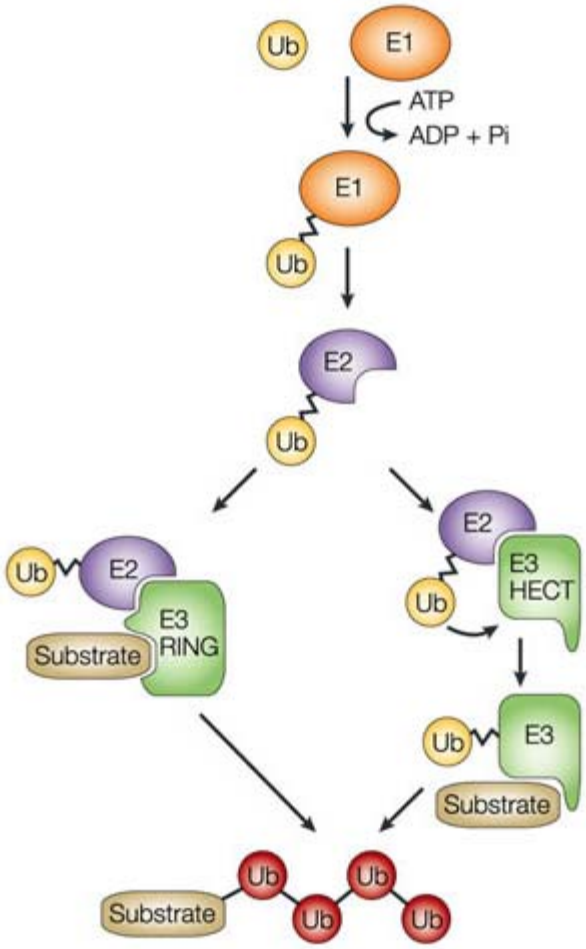

Figure 1. The ubiquitin conjugation pathway. (From (DiFiore et al., 2003))
At least three classes of enzymes are involved in the ubiquitylation of a substrate protein. An E1 or ubiquitin activating enzyme, an E2 or ubiquitin conjugating enzyme, and an E3 or ubiquitin protein ligase. The E3 ubiquitin protein ligases transfers the activated ubiquitin (Ub) from the E2 to the substrate. In HECT-type E3s the E3 forms a covalent ubiquitin intermediate, or in RING-E3-mediated catalysis ubiquitin is transferred directly from the E2 to the substrate, and the RING E3 functions as a adaptor between the two.

The first E3 family is defined by a HECT (homologous to E6-AP carboxyl terminus) domain. The first member of this family, E6-AP (E6-associated protein), was discovered as a protein, that together with the papillomavirus E6 oncoprotein is needed for the rapid ubiquitin dependent degradation of the tumor suppressor p53 (Scheffner et al., 1993). In the following time it was recognized that homology of the

carboxy-terminal half of this protein exists in a number of otherwise unrelated proteins. This conserved region of about 350 AA is located at the carboxy-terminal region of the HECT-domain proteins (Huigbregtse et al., 1995). A conserved cysteine residue within the last 32-34 AA accepts ubiquitin from bound E2, forms a thioester and then transfers it to the substrate.

Another example for a HECT domain E3 is Nedd4, which mediates the ubiquitylation of subunits of ENaC (epithelial amilorid-sensitive sodium channel) (Abriel et al., 1999; Anan et al., 1998).

The second big E3 family is defined by a RING (really interesting new gene) finger domain. The RING finger is a specialized type of Zn-finger of 40 to 60 residues that binds two atoms of zinc, and is probably involved in mediating protein-protein interactions. The RING finger is defined by eight conserved cysteines and histidines that together coordinate two zinc ions in a cross-braced fashion. There are two different variants, the C3HC4-type and a C3H2C3-type. The spacing of the cysteines and histidines is as follows: C-x(2)-C-x(9 to 39)-C-x(1 to 3)-H-x(2 to 3)-C/H-x(2)-C-x(4 to 48)-C-x(2)-C.

There is no evidence that RING E3s can act as catalytic intermediates, but he RING finger motive together with non-RING sequences can bind to E2s and so provide docking sites for the E2s and the substrates, creating an environment where the ubiquitin can be transferred efficient to the target protein.

There are two different groups of RING E3s one type consists of just the RING protein. Single subunit E3s are for example Mdm2 for p53 (Fang et al., 2000) and c-Cbl for activated receptor protein tyrosine kinases (Joazeiro et al., 1999).

In the other group, the RING protein is just a subunit of a multiprotein complex. Multi subunit E3s include the SCF (Skp1-Cullin-F-box protein), the APC (Anaphase Promoting Complex)/Cyclosome, and CBC (elongin C-elongin B-Cul2).

In the SCF E3 ligases (Figure 2) Cul1 functions as a molecular scaffold that links the RING finger containing protein Rbx1/Roc1/Hrt1 with the adaptor subunit Skp1. Skp1also binds to one of many F-box proteins. The F-box proteins are the substrate recognition elements of the SCF E3s. In both yeast and human cells, there are multiple SCF complexes present that differ only in the F-box protein component

(Kipreos and Pagano, 2000). The interaction of a F-box with a certain substrate is often regulated by the phosphorylation status of the target protein.

This structural organisation enables a common core ubiquitin ligase to target numerous substrates in a specific manner for degradation. It is believed that the other multisubunit RING E3s are organized in a similar manner.

Rbx1/Roc1/Hrt1 is also the RING finger subunit for CBC. For the APC it is Apc11. A member of the cullin family (Cul1 for SCF; Cul2 or Cul5 for CBC; and Apc2 for APC) binds the RING finger protein.

For the CBC the role of Skp1 is performed by the elongin C-elongin B dimer. In the case of the APC it was not possible to identify the precise arrangement of the subunits. In the case of the APC, substrate recognition elements/ APC activators include Cdc20 and Cdh1/Hct1 (Fang and Weissman, 2004).

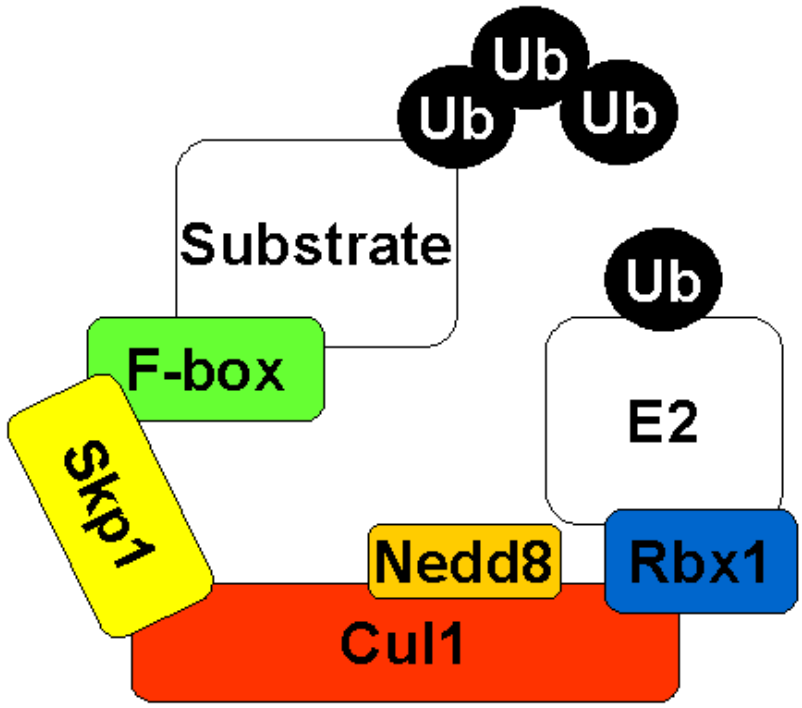

Figure 2. Model of a multisubunit RING finger E3-ligase. Rbx1 contains the RING finger and is involved in the binding of an E2. It binds to the cullin Cul1. The cullin, which can be modified by NEDD8, functions as a molecular scaffold and interacts with the adaptor subunit Skp1. Skp1, in turn binds to one of many F-box proteins. Each F-box-protein appears to be able to bind certain specific substrates.

Two other families of E3s are related to the RING finger, the PHD finger and the U-box.

The PHD finger is a C4HC3 zinc-finger-like motif that is distinct from the C3HC4 type RING finger and possesses an invariant tryptophan before the seventh zinc-binding residue. The herpes virus encoded proteins MIR (modulator of immune recognition) 1 and MIR2 contain PHD domains and use the E3 activity of these proteins to downregulate cell surface molecules that are involved in the immune recognition of infected cells (Coscoy et al., 2001).

The U-box is another modified RING finger, but does not possess the zinc coordinating residues (Hatakeyama et al., 2001). The U box containing E3 CHIP (carboxyl-terminus of Hsc70 interacting protein) was shown to ubiquitylate Hsc70 (Jiang et al., 2001).

For some proteins also the action of a ubiquitin chain elongation factor, an E4 is necessary for more efficient multiubiquitylation (Koegl et al., 1999).

The work of this ubiquitylation cascade can be reversed by the action of deubiquitylating enzymes (DUBs). These enzymes can be divided in two different classes: ubiquitin C-terminal hydrolases (UCHs) and ubiquitin specific processing proteases (UBPs). In general UCHs hydrolyze primarily carboxyl-terminal ester and amide bonds of ubiquitin. They process ubiquitin fusion proteins and cleave ubiquitin precursors to generate active ubiquitin, while UBPs remove ubiquitin from proteins and disassemble polyubiquitin chains (Wilkinson, 2000).

With one exception all DUBs are cysteine proteases. Only the 26S proteasome subunit Rpn11 is a metalloprotease (Verma et al., 2002; Yao and Cohen, 2002).

Among the proteins that can be modified by ubiquitin is also ubiquitin itself, especially when it is already conjugated to a different protein. There are seven lysine residues in ubiquitin and all of them can be found ubiquitylated (Peng et al., 2003) but only for some of the linkages a function is known.

The best-characterized chains are those linked through Lys-48. A chain of four or more Lys-48 linked ubiquitins (it seems that polyubiquitin chains always contain only one type of linkage) works as the principal signal for degradation through the proteasome. The discovery of this role of ubiquitin(-chains) in protein degradation has been awarded in 2004 with the Nobel prize in chemistry (Figure 3). Also Lys-29

linked chains seem to direct their substrate to proteasomal degradation (Johnson et al., 1995). Lys-63 chains seem to be involved in the activation of IκB kinase (IKK) (Deng et al., 2000), in post replicative DNA repair in the RAD6 pathway (Hofmann and Pickart, 1999; Spence et al., 1995), in endocytosis (Galan and HaguenauerTsapis, 1997) and in translational regulation (Spence et al., 2000).

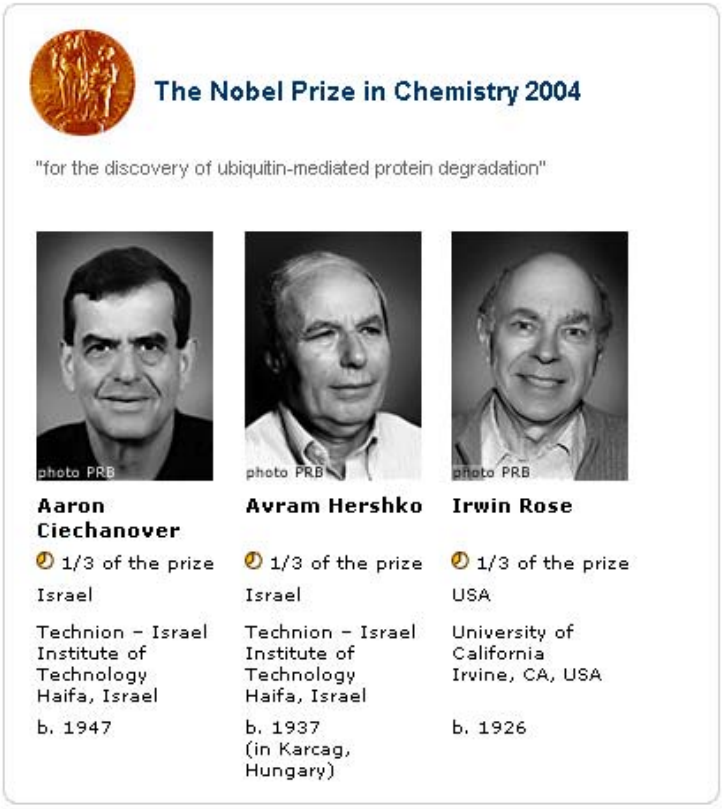

Figure 3. The Royal Swedish Academy of Sciences has decided to award the Nobel Prize in Chemistry for 2004 "for the discovery of ubiquitin-mediated protein degradation" jointly to Aaron Ciechanover, Avram Hershko and Irwin Rose (http://nobelprize.org/chemistry/laureates/2004/index.html)

But also without any polyubiquitin-chain formation the modification of a protein with a single ubiquitin residue (monoubiquitylation) can have effects. Monoubiquitylation is involved in histone regulation, endocytosis and the budding of retroviruses from the plasma membrane (reviewed in (Hicke, 2001))

UBIQUITIN INTERACTING DOMAINS

Ubiquitin associated domain (UBA)

The ubiquitin binding UBA domains are a commonly occurring sequence motif of ~45 amino acid residues that are found in E2s, E3s and other proteins that are linked to ubiquitylation, but they are also present in diverse proteins involved in DNA excision-repair, and cell signalling *via* protein kinases (Hofmann and Bucher, 1996).

The human homologue of yeast Rad23A (HHR23A) is one example of a protein that contains both an internal and a C-terminal UBA domain. The structural similarity between both UBA domains, in spite of their low level of sequence conservation, lead to conclusion that the structural variability of UBA domains in general is likely to be rather small. On the basis of the structural similarities as well as analysis of sequence conservation, it was predicted that a conserved large hydrophobic surface patch is a common protein-interacting surface present in diverse UBA domains. The fact that ubiquitin binds to UBA domains lead to the prediction that the hydrophobic surface patch of UBA domains interacts with the hydrophobic surface on the five-stranded β-sheet of ubiquitin (Mueller and Feigon, 2002).

The specifity of UBA domains with respect to ubiquitin chain length and linkage type is still under debate. For example UBA domains are able to bind to monoubiquitin, but the binding is a lot weaker than the binding to tetraubiquitin (Wilkinson et al., 2001). While one group could show a binding of the UBA domains of Rad23 to Lys-29 and Lys-48 linked polyubiquitin (Rao and Sastry, 2002) another group showed a preference for the Rad23 UBA domains to bind to Lys-48 linked polyubiquitin chains in comparison to Lys-63 or Lys-28 (Raasi and Pickart, 2003). Alternatively different UBA domains might bind to different ubiquitin chains or even to ubiquitin-precursors (Tanaka et al., 2004) or ubiquitin like modifiers like NEDD8 or FAT10 (Tanaka et al., 2003) and Figure 12 in this thesis.

Ubiquitin-interacting motif (UIM)

The UIM was originally identified as the polyubiquitin binding site of the S5a/Rpn10 subunit of the 26S proteasome (Young et al., 1998). It is a stretch of about 20 amino

acids that forms a short α helix that can be embedded into different protein folds and occurs in a wide variety of proteins, including proteins involved in ubiquitylation, ubiquitin metabolism and receptor-mediated endocytosis (Hofmann and Falquet, 2001). UIMs not only functions as a receptor for polyubiquitylated proteins (Young et al., 1998) but also for proteins carrying a ubiquitin-like domain (Walters et al., 2002).

Ubiquitin-conjugating enzyme variant (UEV)

UEV proteins show significant sequence similarity to E2 ubiquitin conjugating enzymes but are unable to catalyze ubiquitin transfer, as they lack the active site cysteine that forms the transient thioester bond with the C-terminus of ubiquitin (Sancho et al., 1998). UEVs have been found in several proteins, including tumour susceptibility gene 101 protein (Tsg101) and Uev1/Mms2. Human Tsg101 has recently been identified as the functional receptor required for budding of the enveloped human immunodeficiency virus (HIV). In performing this function, Tsg101 binds both ubiquitin and a P(S/T)AP tetrapeptide motif located within the viral Gag protein. These interactions are mediated by the N-terminal domain of Tsg101, which contains the UEV domain (Pornillos et al., 2002).

Uev1A/Mms2 forms together with the functional E2 Ubc13 a heterodimer that is involved in the formation of Lys-63 linked polyubiquitin chains (Deng et al., 2000; Hofmann and Pickart, 1999).

Cue1-homologous (CUE)

The CUE domain is structurally related to the UBA domain, and it could be shown that CUE domains are monoubiquitin-binding domains (Shih et al., 2003). The solution structure of a CUE domain of the yeast Cue2 protein in complex with ubiquitin showed that the contact surface encompasses Lys-48, a site of polyubiquitin chain formation. This suggests an occlusion mechanism for inhibiting polyubiquitin chain formation during monoubiquitin signalling (Kang et al., 2003).

Several CUE domain proteins have been shown to interact with ubiquitin-conjugating enzymes (Biederer et al., 1997; Fang et al., 2001) or are involved in endocytotic events and in signal transduction (Donaldson et al., 2003; Shih et al., 2003).

UBIQUITIN-LIKE PROTEINS

Several proteins have been discovered in the last years that are related to ubiquitin or function similarly. These ubiquitin-like proteins can be divided into two groups: Proteins of the first group can, like ubiquitin itself, modify other proteins by covalent formation of an isopeptide-bond with their target. These proteins are called “ubiquitin like modifiers” (UBLs). The other group is only structurally linked with ubiquitin, since all its members possess a ubiquitin homology domain, but not through function, since these proteins cannot become covalently conjugated to target proteins. These proteins are called “ubiquitin domain proteins” (UDPs) (Jentsch and Pyrowolakis, 2000).

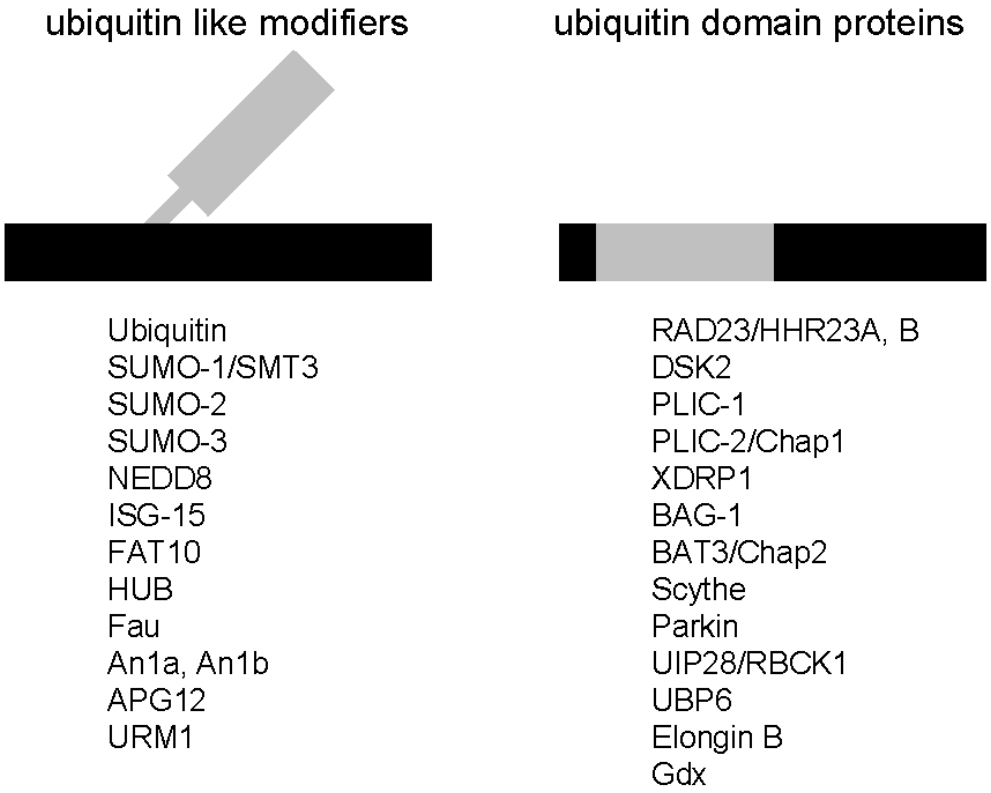

Figure 4. Two different groups of ubiquitin like proteins Proteins of the first group function as modifiers (gray), in a manner analogous to that of ubiquitin, by covalent formation of an isopeptide-bond with their target. These proteins are called “ubiquitin like modifiers”. “Ubiquitin-domain proteins” bear ubiquitin-like domains (gray) but do not form conjugates with other proteins (modified from (Jentsch and Pyrowolakis, 2000)).

Ubiquitin like modifiers

Small ubiquitin like modifier (SUMO)

SUMO is also known under the names Smt3p, Pmt2p, PIC-1, GMP1, Ubl1, DAP1 and sentrin. In mammalian cells three different SUMO related proteins could be detected ubiquitously, called SUMO-1, SUMO-2 and SUMO-3. Recently also a tissue specific SUMO-4 has been identified in human kidney (Bohren et al., 2004). SUMO-2, SUMO-3 and SUMO-4 show much bigger sequence relatedness to each other than to SUMO-1.

The mechanism involved in the transfer of SUMO to target proteins ("sumoylation") is similar to that of ubiquitin. It requires the E1 heterodimer AOS1/UBA2, the E2 Ubc9 and several E3s (Hochstrasser, 2001; Melchior et al., 2003). At least one of the identified E3s, the nucleoporin RanBP2 has neither a RING nor a HECT domain (Pichler et al., 2002; Pichler et al., 2004), while other SUMO-E3s posses a RING-finger like motif, called Siz/PIAS RING (SP-RING) (Hochstrasser, 2001). A difference to ubiquitylation is that there is a consensus site for sumoylation. Ubc9 recognizes a minimal sumoylation motif ΨKXE (Ψ represents a hydrophobic AA, X represents any AA) present in many known SUMO targets (Bernier-Villamor et al., 2002; Sampson et al., 2001). The SUMO modification occurs at the lysine residue in this sequence.

Since like ubiquitin, also SUMO requires a maturation step, and since like ubiquitylation also sumoylation is a reversible action, there is the need of sumo-specific proteases. All known SUMO-specific proteases belong to the ubiquitin-like protease 1 (Ulp1) cysteine protease family and can either process SUMO to its mature diglycine form, or cleave the isopeptidebond between SUMO and its target proteins, or can do both (Melchior et al., 2003).

SUMO proteins are only about 18% identical in sequence to ubiquitin and also possess short N-terminal extensions. It also seems that the effects of conjugation of SUMO to a target protein are quite different from the effects of ubiquitylation. Parameters that are altered after sumoylation include subcellular localisation, protein partnering, and the DNA binding and/ or transactivation functions of transcription factors (Hilgarth et al., 2004). Sumoylation can also directly interfere with ubiquitylation, when the same lysine residue can be modified by SUMO or ubiquitin.

This is the case for IκBα that is resistant to proteasome mediated degradation after sumoylation on Lys-21 (Desterro et al., 1998).

The first identified substrate of SUMO was RanGAP1, a GTPase-activating protein. While unmodified RanGAP1 is located manly in the cytoplasma SUMO-conjugated forms associate with the cytoplasmic fibers of the nuclear pore complex (NPC).

This process is explained by the formation of a binding interface for RanBP2 through the sumoylation of RanGAP1. Only the SUMO-1 modified form of Ran-GAP1 was found to associate stable with RanBP2 (Mahajan et al., 1997; Matunis et al., 1996).

Other targets of SUMO are promyelocytic leukemia (PML) protein, where only the sumoylated form is located in nuclear bodies (Müller et al., 1998), or the transcription factors c-Jun and p53 (Müller et al., 2000). While SUMO modification plays a negative role for c-Jun, overexpression of SUMO-1 activates the transcriptional activity of p53 (Rodriguez et al., 1999).

Neural precursor cell-expressed developmentally down-regulated (NEDD8)

NEDD8 or RUB (related to ubiquitin) is 60% identical and 80% homologous to ubiquitin (Kumar et al., 1993) and is therefore of all ubiquitin-like proteins the most homologous to ubiquitin. It is conserved from yeast to humans.

Like for ubiquitin and most other UBLs, there is a maturation step necessary, where several amino acids at the C-terminus are cleaved off to gain a free C-terminal glycine, before NEDD8 can be conjugated to other proteins. While UCH-L3 might be involved in maturation of ubiquitin and NEDD8 (Wada et al., 1998) the cysteine protease NEDD8 protease (NEDP1)/ deneddylase 1 (DEN1) is specific for NEDD8 (Mendoza et al., 2003; Wu et al., 2003).

Conjugation of NEDD8 to target proteins involves the heterodimer ULA1/UBA3 as an E1, with ULA1 being related to the N-terminal and UBA3 being related to the C-terminal domain of the ubiquitin activating E1, and UBC12 as E2 (Liakopoulos et al., 1998). The ability of the SCF and the von Hippel-Lindau (VHL) E3 ubiquitin ligase complex subunit Rbx1/Roc1/Hrt1 to promote NEDD8 modification of cullins suggests that it can also act as a NEDD8 E3 (Kamura et al., 1999). Recently it could be shown that Mdm2, another ubiquitin E3, can in addition to ubiquitylation also promote neddylation of p53 (Xirodimas et al., 2004).

Till recently the only known targets for NEDD8 have been the cullin subunits of the multisubunit RING finger containing E3 ubiquitin ligases. The only member of the cullin family that could not be found in a neddylated form is Apc2, a subunit of the APC/Cyclosome.

The neddylation appears to regulate the E3 ubiquitin ligase activity of its targets. The NEDD8 modification facilitates the recruitment of the E2 ubiquitin conjugating enzyme to the SCF complex and mediates the dissociation of CAND1, an inhibitor of the formation of the SCF complex (Parry and Estelle, 2004).

Two new targets for NEDD8 have been discovered just recently, the tumor suppressor protein p53 and the RING finger E3 ubiquitin ligase Mdm2, with Mdm2 being also the E3 for the neddylation of p53. The neddylation of Mdm2 would therefore resemble the autoubiquitylation of many ubiquitin E3s. The effect of the NEDD8

modification of p53 is an inhibition of the transcriptional activity of p53 (Xirodimas et al., 2004).

The NEDD8 modification is a reversible process. The COP9 signalosome (CSN) contains a subunit (CSN5) with a metalloprotease motif called the JAMM motif that is involved in cleavage of NEDD8 from Cul1 (Cope et al., 2002; Lyapina et al., 2001). This JAMM motive is also present in the Rpn11 subunit of the 26S proteasome, which possesses a deubiquitylating activity (Verma et al., 2002). Also DEN1 is able to remove NEDD8 from cullin, but the deneddylation is quite inefficient, especially in comparison with CSN (Wu et al., 2003).

Interferon-stimulated gene-15 (ISG15)

ISG15 was also called ubiquitin cross reacting protein (UCRP) due to its cross reactivity with different anti-ubiquitin antibodies (Haas et al., 1987).

Although ISG15 was the first known member of the UBL family, discovered in 1979 (Farrell et al., 1979), there was very little known about this protein till recently. This in part is due to the fact that there are no homologues known in lower eukaryotes.

Like FAT10 (described below) ISG15 possesses two ubiquitin-like domains in tandem arrangement. Recently the ISG15 activating cascade could be identified; UBEL1 functions as the E1 enzyme for ISG15, and interestingly the influenza B virus NS1 protein inhibits the ISG15 activating step catalysed by this enzyme (Yuan and Krug, 2001). UbcH8, an E2 that also functions in ubiquitin conjugation, serves additionally as an E2 for ISG15 (Kim et al., 2004; Zhao et al., 2004). There also exists an E3 for ISG15 (D.E. Zhang personal communication). Another enzyme identified for the ISG15 system is a deISGylating protease UBP43(USP18) (Malakhov et al., 2002). This enzyme is however not necessary for precursor processing, since UBP43 deficient cells can generate ISGylated proteins upon IFN treatment (Malakhova et al., 2003).

Several components of the ISGylation system, including ISG15 itself, UB1L, Ubc8, and UBP43, are interferon inducible proteins (Kim et al., 2004; Ritchie and Zhang, 2004). ISG15 is inducible through type I interferons (Loeb and Haas, 1992), in contrast to FAT10 that can be induced mainly by the type II interferon IFN-γ.

The first substrate of ISG15 that could be identified is the serine protease inhibitor Serpin 2a (Hamerman et al., 2002). Other ISG15 substrates are phospholipase Cγ1, Jak1 and Erk1. These proteins are involved in signal transduction. The Jak1 kinase substrate Stat1 is also a target of ISG15 (Malakhov et al., 2003). The consequences of ISG15 conjugation and the fate of the conjugates are not known, but it does not appear that ISG15 targets proteins for proteasomal degradation (Malakhov et al., 2003).

In contrast to ubiquitin and to every other UBL, ISG15 can also be detected as an extracellular free protein. Secretion of free ISG15 from human lymphocytes, monocytes and from cell lines of monocyte, T lymphocyte, B lymphocyte, and epithelial origins has been reported after treatment with IFN-β (D'Cunha et al., 1996b;

Knight and Cordova, 1991). Free ISG15 has been found to have immunoregulatory properties and has been described as an interferon-induced cytokine. ISG15 stimulates IFNγ production from $CD3^+$ lymphocytes (Recht et al., 1991) and increases $CD56^+$ nature killer cell proliferation (D'Cunha et al., 1996a).

FAT10

FAT10 or Diubiquitin was discovered quite recently as a ubiquitin-like protein after chromosomal sequencing of the human MHC class I locus. The FAT10 gene is encoded in the MHC class I HLA-F locus of chromosome 6 (Fan et al., 1996).

It encodes a 165 AA protein that has two ubiquitin-like domains, which are separated by a linker of 5 AA. The N-terminal domain is with 29% identity to ubiquitin, and also an initial extension of 6 AA, less well conserved, than the C-terminal domain, which is 36% identical to ubiquitin (Figure 5). The domains are more closely related to ubiquitin than to each other (20% identity), which suggests an evolution towards different functions. Interestingly of the seven lysine-residues in ubiquitin four are conserved in both ubiquitin-like domains of FAT10. These lysine residues correspond with the Lys-27, Lys-33, Lys-48 and Lys-63 of ubiquitin. As mentioned above especially Lys-48 and Lys-63 of ubiquitin are important for polyubiquitin-chain formation. Also the diglycine motif of the very C-terminus of the C-terminal domain is conserved. Atypical of ubiquitin is the appearance of four cysteine residues in FAT10 (Bates et al., 1997).

Fan *et al.* suggested that FAT10 expression is related to the Epstein-Barr virus (EBV) status of the analyzed cell lines, but later it could be shown that EBV^+ status alone is not sufficient for FAT10 expression. Instead, since FAT10 is expressed in mature dendritic cells and mature B cells, it was suggested that FAT10 expression is related to the maturation stats of dendritic cells and B cells (Bates et al., 1997). Two years later our group could show that FAT10 is inducible by the proinflammatory cytokines interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in cells of various tissues in a synergistically manner (Raasi et al., 1999). This result suggests that the mature B cells and mature dendritic cells are not principally different from cells of other tissues with regard to FAT10 expression, but that after a maturation stimulus like for example TNF-α and IFN-γ they express FAT10.

Also other stimuli can alter FAT10 expression. Treatment with *all-trans* retinoic acid, a substance used in treatment of acute promyelocytic leukemia and in the chemoprevention of cancers, could induce FAT10 mRNA 5- to 10-fold. But since FAT10 has no identifiable retinoid response elements in its promoter it is likely that this effect is indirect (Dokmanovic et al., 2002).

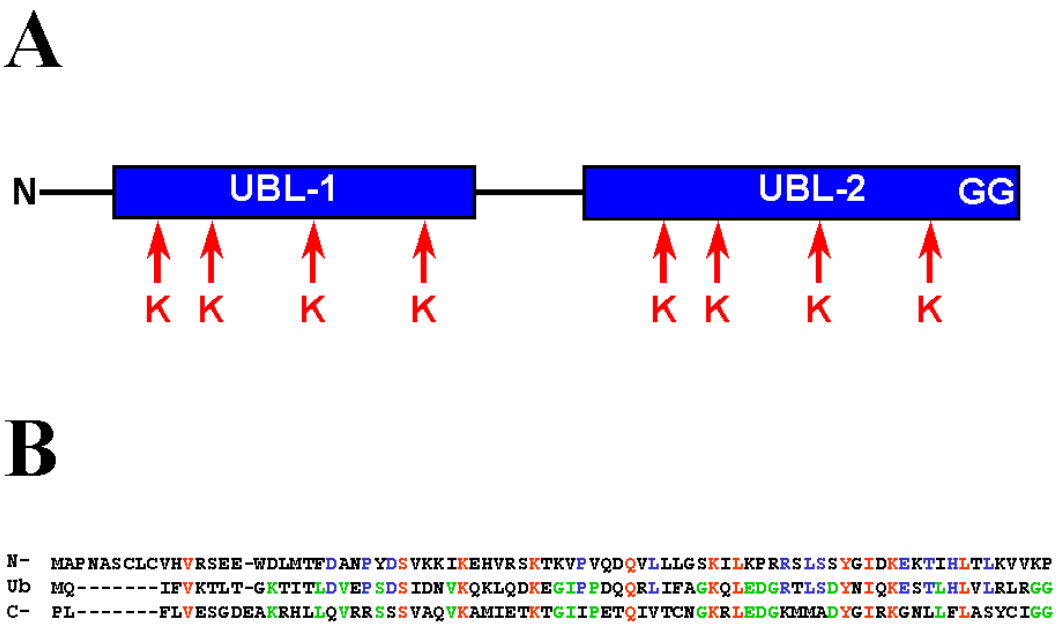

Figure 5. Primary Structure of FAT10 and its sequence similarity to ubiquitin. FAT10 contains two ubiquitin-like domains. The N-terminal domain of human FAT10 is 29% and its C-terminal domain 36% identical to ubiquitin. The C-terminal diglycine-motif is conserved in the C-terminal domain of FAT10. Also the lysine residues that correspond to the ubiquitin lysines 27,48 and 63 are conserved. **(A)** schematic diagram of FAT10 **(B)** sequence comparison of the N and C-terminal halves of FAT10 (N- / C-) with ubiquitin (Ub)

FAT10 mRNA expression was also upregulated after treatment of rats with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a substance that can induce stomach cancers and also an inflammatory response. Interestingly there were differences in the FAT10 induction in two rat strains with dissimilar cancer susceptibilities (Yamashita et al., 2002).

In a study of Lee *et al.* FAT10 was found upregulated in 90% of all examined hepatocellular carcinoma (HCC) patients and was also highly upregulated in other gastrointestinal and gynaecological cancers. Since in this study several other genes involved in an inflammatory or an immunological response were not upregulated, the authors conclude that the elevated FAT10 expression is unlikely to be the result of a general immunological or inflammatory response in cancer (Lee et al., 2003).

Still under debate is the localization of FAT10 in cells. While our group and the group of Sherman Weissman found that FAT10 is mainly localized in the cytosol (Liu et al., 1999; Raasi et al., 2001), Lee *et al.* detected FAT10 primarily in the nucleus (Lee et al., 2003).

The amino acid sequence of FAT10, especially the existence of the C-terminal diglycine motif, suggested a role of FAT10 as a UBL. Our group showed the existence of several forms of FAT10 that have a higher molecular weight in addition to free monomeric FAT10, possibly resembling FAT10 modified proteins. These forms could not be detected when instead of wild type FAT10 a mutant was used where the diglycine motif is deleted. Therefore we concluded, that FAT10 is indeed a UBL and the high molecular weight forms of FAT10 resemble covalent FAT10 conjugates (Raasi et al., 2001). Till today it was not possible to identify these conjugates or the machinery necessary for FAT10 conjugation, so that no E1, E2s or E3s are known.

Since FAT10 is, in contrast to other UBLs and ubiquitin itself, already synthesized in a mature form with a free C-terminal diglycine, there is no need for a processing enzyme.

Liu *et al.* found a noncovalent interaction of FAT10 with the human spindle assembly checkpoint protein MAD2 using several approaches *in vivo* and *in vitro* (Liu et al., 1999) but till now there exist no functional data and no independent confirmation for this interaction.

A functional consequence of FAT10 ectopic overexpression is cell death. Our Lab and another group had problems to constitutively express FAT10 in HeLa cells, since FAT10 expression was not compatible with cell viability and proliferation during a longer period of time (Liu et al., 1999; Raasi et al., 1999). In a mouse fibroblast cell-line it has been possible to create transfectants where FAT10 is expressed under control of a tetracycline (tet) repressible promotor, which allowed the generation and expansion of transfectants in the absence of FAT10 expression. In this system the functions of FAT10 expression could be observed by removing the tet from the growth medium. Also in this cell line induced FAT10 expression led to massive cell death with more than 50% dead cells within 48 hours. Further analyses could show that this cell death is a consequence of caspase dependent apoptosis. It seems that this apoptosis is dependent on the ability of FAT10 to be conjugated to target proteins, since a FAT10 mutant where the C-terminal diglycine motif is deleted did not induce apoptosis (Raasi et al., 2001).

The localization of the FAT10 gene in the MHC class I locus and the finding that FAT10 expression was synergistically inducible with IFN-γ and TNF-α, suggested that FAT10 might be involved in the MHC class I presentation pathway. A role of FAT10 in the immune system is also suggested by the fact that FAT10 is able to inhibit hepatitis B virus expression in a hepatoblastoma cell line (Xiong et al., 2003). However FAT10 induction did not affect cell surface expression of MHC class I molecules or class I restricted antigen presentation (Raasi et al., 2001).

Other ubiquitin like modifiers

The list of UBLs is still growing and for most the functions are still unknown. A list of some UBLs is given in figure 4. Two especially noteworthy examples are:

Autophagy protein 12 (APG12)

APG12 was identified in a screen for yeast mutants that show defects in autophagy. It forms a covalent complex with a particular lysine residue of another protein required for autophagy, APG5. This reaction requires an intact C-terminus of APG12, so that even with APG12 having no sequence similarity to ubiquitin it shows the characteristics of a ubiquitin like modifier (Mizushima et al., 1998).

Homologous to ubiquitin 1 (HUB1)

HUB1 is a ubiquitin like protein which carries a C-terminal dityrosine motif. At the moment it is discussed controversially if HUB1 is a UBL or not (Dittmar et al., 2002; Luders et al., 2003). If HUB1 is indeed a UBL, then also the C-terminal diglycine motif, till now a cardinal feature of the ubiquitin-like family of protein modifiers, is not necessary for modification with a UBL.

Ubiquitin domain proteins

The UDPs constitute a structurally and functionally heterologous group of proteins and they are only similar in the area of their ubiquitin like domain. In contrast to UBLs, UDPs are neither processed nor conjugated to other proteins, and their ubiquitin like domain is an integral part of the protein, like it is the case for most other protein domains.

Some of the UDPs share the ability to interact with the19S regulatory particle of the 26S proteasome. The ability to bind the proteasome is mediated by the ubiquitin like domains of the proteins and seems to be the general function of this domain (HartmannPetersen and Gordon, 2004). Many of the UDPs contain other characterized protein modules in addition to their ubiquitin like domain and are so linked to other cellular functions.

Proteins that carry one or more additional ubiquitin-associated (UBA) domain seem to work as carriers of ubiquitylated substrates. Examples are Rad23/Rhp23 and Dsk2/Dph1. The ability of these proteins to interact with the 26S proteasome via their N-terminal UBL domains (Elsasser et al., 2002; Schauber et al., 1998), while using their C-terminal UBA domain for binding ubiquitin chains, hints that they may function in the transport of ubiquitylated proteins to the 26S proteasome (HartmannPetersen et al., 2003b). It seems that the UBA domains of Rad23 are able to distinguish between certain types of polyubiquitin chains. They bind preferentially to K48 linked polyubiquitin chains (Raasi and Pickart, 2003), and can then deliver their cargo to the proteasome, or protect this chain from disassembly.

UDPs that contain a Bcl-2-associated athanogene (BAG) domain function as chaperone co-factors, since they bind to the ATPase domain of HSP70/HSC70 proteins and modulate their function. The BAG proteins have anti-apoptotic activity and increase the anti-cell death function of BCL-2 induced by various stimuli. Proteins with BAG domains and a ubiquitin like domain are BAG1 or BAG6/Scythe (Doong et al., 2002).

Other UDPs contain RING finger domains or are part of RING finger containing protein complexes. Examples of this group are parkin, which even contains two RING finger domains (Shimura et al., 2000) and elongin B, which is part of the VHL complex, a multi subunit E3. While the binding of parkin to the S5a/Rpn10 subunit

through its ubiquitin like domain could be shown (Sakata et al., 2003), it is at the moment not clear if there is a proteasome interaction of the VHL complex or if such an interaction is mediated by the ubiquitin like domain of elongin B.

Another group of UDPs function as deubiquitylating enzymes, which would add additional deubiquitylation activities in proximity to the proteasome, where already several other deubiquitylating enzymes are located in the 19S particle. Examples are yeast Ubp6 and mammalian Usp14. For Ubp6 it could be shown, that proteasome binding activates its activity 300-fold. Usp14 activity can be enhanced by inhibiting the proteasome (Borodovsky et al., 2001; Leggett et al., 2002).

For all the mentioned examples there exist a functional link to the ubiquitin-proteasome system, because they either bind to ubiquitin, or to proteins that are involved in ubiquitin mediated processes, like this is for example the case for the BAG proteins that can, in addition to chaperons, also bind to the E3 ubiquitin ligase CHIP (Ballinger et al., 1999). But there are also several UDPs where there is no such obvious link to the ubiquitin-proteasome system. For NUB1 (discussed in more detail below) the interaction with the ubiquitin system is at the most indirect, since NUB1 has been reported to interact with NEDD8 and NEDD8 modified proteins (Kamitani et al., 2001). Since, with the exception of p53, all known NEDD8 conjugates are E3 ubiquitin ligases (Mdm2), or parts of ubiquitin ligases (cullins) it could be possible that E3 activity is recruited to the proteasome via this indirect interaction.

There are also UDPs where there is no known function. For example for the fission yeast protein Udp7 there is, besides the ability of this protein to bind to the proteasome (Seeger et al., 2003), no function known.

NEDD8 Ultimate Buster-1

In a yeast two-hybrid screening by using NEDD8 as bait, Kito and colleagues isolated a cDNA fragment encoding a 601 residue protein with a calculated 69.1-kDa molecular mass. It contains two UBA domains in addition to its N-terminal ubiquitin-like domain.

Kito *et al.* found that the amount of NEDD8 was reduced when this protein is coexpressed. Therefore they named the protein NEDD8 Ultimate Buster-1 (NUB1). The decreased level of NEDD8 protein expression is not due to reduced transcription of NEDD8 (Kito et al., 2001) and can be prevented by inhibition of the proteasome (Kamitani et al., 2001). In addition NUB1 overexpression also had a growth inhibitory effect on U2OS cells (Kito et al., 2001).

NUB1 is inducible with interferon-β and interferon-γ. It is mainly localized in the nucleus which is in accordance with a nuclear localization signal (NLS), encoded between AA 414 and 431. The NUB1 mRNA is found specifically expressed in adult human testis, ovary, heart, and skeletal muscle tissues and is developmentally down-regulated in mouse embryos (Kito et al., 2001).

In commercial preparations of 26S but not of 20S proteasomes Kamitani *et al*. could detect cofractionated NUB1. In addition, in an *in vitro* assay, a fusion of the proteasome subunit S5a/Rpn10 with glutathione *S*-transferase (GST) could pull down NEDD8 conjugates only when NUB1 was expressed.

Therefore Kamitani *et al*. suggest that NUB1 has an adaptor function between S5a and NEDD8, and recruits NEDD8 and its conjugates to the proteasome for degradation (Kamitani et al., 2001).

Tanaka *et al.* reported in 2003 the existence of a splicing variant of NUB1 with an insertion of 14 AA and named it NUB1long (NUB1L). These additional amino acids generate a third UBA domain, between the two already existing domains. This additional UBA domain represents a second albeit weak binding site for NEDD8. In NUB1 the only NEDD8-binding site is located at the C-terminus of the protein, in a region outside of the UBA domains, which contains also the half of a PEST domain. The additional binding site for NEDD8 might explain why NUB1L has a slightly greater ability to downregulate NEDD8 than NUB1.

The insertion of the 14 AA is not interfering with the NLS, and NUB1L is, like NUB1, mainly expressed in the nucleus.

Using a different approach than Kito *et al.* (Kito et al., 2001), Tanaka and colleagues detected NUB1 message almost equally in all examined tissues other than the pancreas. In contrast, the NUB1L message could not be detected equally, and was detected to a much lower extent in the prostate, leukocytes, liver, and skeletal muscle compared to other tissues (Tanaka et al., 2003).

In another yeast two hybrid screen published in 2004, Tanaka and colleagues identified the ubiquitin precursor UbC1 as a further noncovalent interaction partner of NUB1. Northern blot analysis showed that the mRNAs of both NUB1 and UbC1 were enriched in the testis, and *in situ* hybridization showed that both mRNAs were strongly expressed in seminiferous tubules of the testis.

UbC1 is composed of nine tandem repeats of a ubiquitin unit connected through α-peptide bonds. The NUB1-UbC1 interaction is mediated by the UBA1 domains of NUB1 and NUB1L. NUB1 also is able to interact with ubiquitin dimers and trimers if they are linked by α-peptide bonds. When ubiquitin is linked via isopeptide bonds, it was not possible to find an interaction.

It seems that one or more unidentified proteins coprecipitating with NUB1 are able to hydrolyze UbC1, but NUB1 itself has no ubiquitin C-terminal hydrolase activity (Tanaka et al., 2004).

Aryl hydrocarbon receptor-interacting protein-like 1 (AIPL1) was found to interact with NUB1 during a yeast two hybrid screen, when a bovine retinal cDNA library was used (Akey et al., 2002). AIPL1 is present only in the rod photoreceptors of the adult human retina, where NUB1 is also expressed (Akey et al., 2002; Van Der Spuy et al., 2003). The interaction could also be found by co-immunoprecipitation studies of endogenous NUB1 and AIPL1 in Y79 retinoblastoma cells. Interestingly the size of NUB1 in the retina is only about 50kD (Akey et al., 2002).

Mutations in the AIPL1 gene have been found in patients with Leber´s congenital amaurosis (LCA), a genetically heterogeneous, autosomal recessive retinal degenerative disease responsible for about 5% of all inherited retinopathies. The NUB1-binding site on AIPL1 is located between amino acid residues 181 and 330 in AIPL1 and many LCA-associated mutations of AIPL1 have been found at this site. It

was however not possible to find a clear connection between the LCA-associated mutations in this region, and their ability to bind to NUB1, since only some of the mutants lost their NUB1 binding ability. The AIPL1 binding site of NUB1 is located at the C-terminus and in part overlaps with the proposed NEDD8 binding site, but not with the PEST domain (Kanaya et al., 2004).

It was observed recently that co-transfection of the mainly cytoplasmatic localized AIPL1 with the mainly nuclear NUB1, fused to green fluorescent protein (GFP), resulted in a shift of GFP-NUB1 subcellular distribution towards the cytoplasm. In the same study it was observed that AIPL1 was able to act in a chaperone-like fashion and efficiently suppressed inclusion formation by NUB1 fragments (van der Spuy and Cheetham, 2004).

PROTEASOMAL DEGRADATION

The 20S proteasome

The 26S proteasome, or multicatalytic protease complex is the central enzyme of protein degradation in both the nucleus and the cytosol. The proteolytic core complex of the proteasome system is the 20S proteasome or core particle (CP), a cylinder-shaped complex formed out of four stacked rings, each composed out of seven subunits. The 20S proteasomes of prokaryotes and eukaryotes differ in complexity. The proteasomes of prokaryotes consist of 14 copies of 2 subunits, whereas the proteasomes of eukaryotes are composed out of 2 copies of 14 different subunits. Despite this difference, the overall architecture of these complexes is conserved: the two outer rings are composed out of α subunits, homoheptamers in prokaryotes or heteroheptamers (α1-α7) in eukaryotes, and the two inner rings out of seven β subunits, again homo- or heteroheptamers (β1-β7) in pro- and eukaryotes, respectively (Voges et al., 1999). Of the seven different β-subunits in eukaryotes only three bear catalytically active sites. The active sites face toward the lumen of the proteasome, so that the only way for substrates to reach them is through the gated channels that are formed by the α-rings. The proteolytically active β-subunits are N-terminal nucleophile (Ntn)-hydrolases. Common to this family is the ability to hydrolyze amide bonds, but only the proteasomal β-subunits are able to hydrolyze peptide bonds. All Ntn hydrolases are synthesized as inactive precursors and are converted to an active form by an autocatalytical internal cleavage, which exposes a new N-terminus. In the case of the proteasomal β-subunits this is a threonine, which serves as the N-terminal nucleophile (Heinemeyer et al., 2004). Together the three catalytically active subunits are able to cleave C-terminal of virtually all amino acids, but they show a preference for hydrophobic, basic and acidic residues. These three preferred peptidolytic activities have been classified according to the position N-terminal of the cleaved bond. The chymotrypsin-like site cleaves peptide bonds after hydrophobic residues, and the trypsin-like site cuts after basic residues. The third site cuts preferentially after acidic residues and its activity is called caspase-like, peptidylglutamyl peptide hydrolytic (PGPH) or post-glutamyl splitting. In yeast, it

was possible to assign the chymotrypsin-like activity to β5/Pre2, the trypsin-like activity to β2/Pup1 and the acidic activity to β1/Pre3 (Groll et al., 1997).

In mammals there exist three additional, non-essential subunits, β1i (LMP2), β2i (MECL1) and β5i (LMP7) that can replace the constitutive components β1, β2 and β5 during proteasome neosynthesis after induction by IFN-γ. Proteasomes with these inducible subunits are called immunoproteasomes and show an altered cleavage pattern, which can lead to an enhancement of antigen presentation (Groettrup et al., 2001a; Groettrup et al., 2001b). The LMP subunits are encoded by a region of the MHC where also the FAT10 encoding sequence is localized.

Substrates have to access the active sites through a narrow opening (α-annulus) that in the unliganded 20S CP is normally blocked through the amino-terminal sequences of the α-subunits. Therefore the unliganded CP can hydrolyze only short or unstructured polypeptides and, at least *in vitro*, some proteins with hydrophobic or misfolded patches. The peptide products have to leave the proteasome through the same opening.

Proteins are degraded in a processive manner by the 20S proteasome without the release of degradation intermediates. The size distribution of released peptides ranges from 4 to 25 residues, with an average length of 7 to 9 AA.

The 11S regulator

Degradation of small peptides by the proteasome is strongly enhanced by the 11S regulator (also called 28-kDa proteasome activator (PA28), PA26 and REG) in an ATP independent manner. PA28α/β consists of two different subunits α and β, which are inducible by IFN-γ. They form probably a heptamer or hexamer with alternating α and β subunits. PA28α/β exits as a free oligomer or can bind to one or both ends of the 20S CP. Structural analysis of how the 11S regulator of *Trypanosoma brucei* PA26 interacts with the *Saccharomyces cerevisiae* 20S CP might give an explanation of how PA28 works. By binding to the α-endplates of the 20S proteasomes, PA26 induces a conformational change in the α-subunits that opens the normally blocked gate. This would facilitate proteasome access of peptides that do not need to be unfolded to enter the proteasome. What might be more relevant *in vivo* is that the opening of the α-gate might permit the exit of larger peptide products (Whitby et al.,

2000). This would be important for antigen processing, since 75% of the average peptide products of 20S and 26S *in vitro* are too small to serve as ligands for MHC class I molecules. A role of PA28α/β in antigen processing is also supported by the fact that PA28α/β genes are only present in organisms with an adaptive immune system and that the proteins are inducible by IFN-γ.

The Ki antigen or PA28γ is a nuclear protein that can form homo-oligomers and can also activate the proteasome. PA28 -/- mice display a slight reduction in $CD8^+$ T cell numbers and do not effectively clear a pulmonary fungal infection (Barton et al., 2004). But PA28γ it is also present in organisms without an adaptive immune system.

The 26S proteasome

The 26S proteasome is implicated in the degradation of abnormal and damaged proteins, but also of correctly folded proteins that need to be controlled tightly like cell-cycle regulators, oncogenes, and tumor suppressors. It is also involved in processing of antigens and the activation or degradation of transcription factors. Misfolded or damaged secretory proteins are also degraded by the 26S proteasome in the cytosol. These secretory proteins, which are degraded via the so-called endoplasmic reticulum-associated degradation (ERAD) pathway, undergo a retrograde transport from the ER to the cytosol.

The 26S proteasome in eukaryotes is a roughly 2.5MDa complex made out of at least 32 different subunits arranged in two subcomplexes. Proteolysis takes place in the 20S CP (mentioned above), but to degrade polyubiquitylated substrates attachment of a 19S regulatory particle (RP, or PA700) to the surface of the α-ring is required.

There are at least two different nomenclatures used to designate the 19S RP subunits, the mammalian “S”(subunit)- and the *S.cerevisiae* “Rp”(regulatory particle)-nomenclature. The Rp nomenclature differentiates between Rp triphosphatases (Rpt) subunits and Rp non-ATPase (Rpn) subunits.

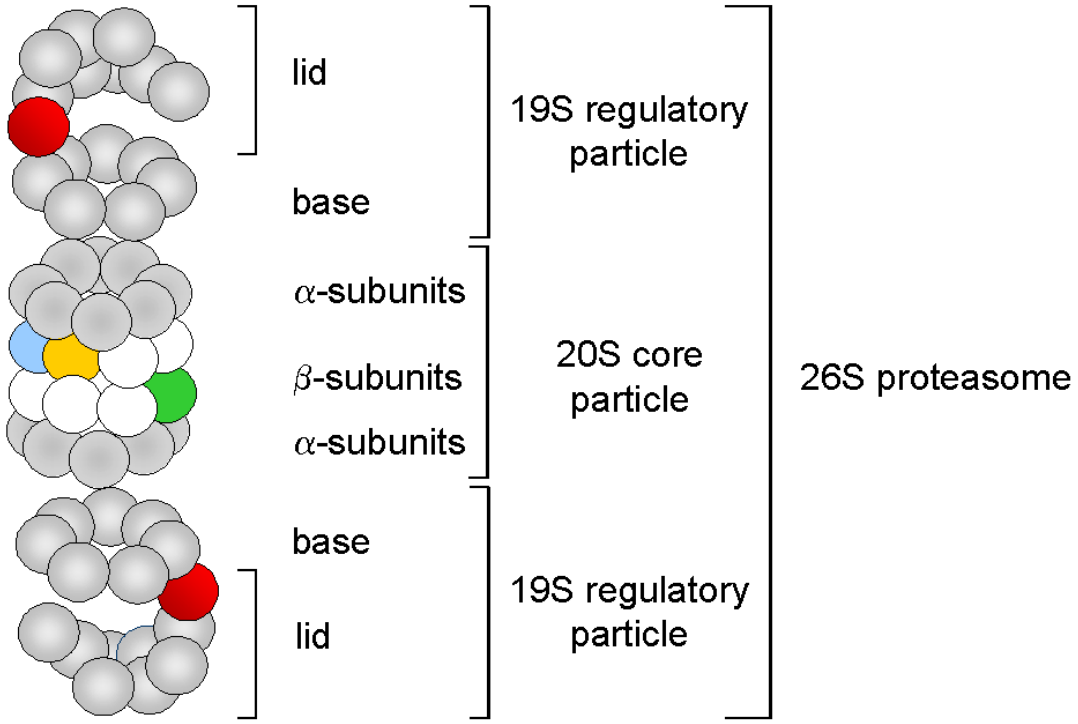

Figure 6. Scheme of the 26S proteasome.

The RP can bind to one or both ends of the 20S CP. Together the 20S CP and the 19S RP form the 26S proteasome holoenzyme. While the 20S CP does not need ATP to hydrolyze peptides, the degradation of ubiquitylated substrates by the 26S proteasome is an ATP-dependent process (Hough et al., 1987).

The 19S RP has to fulfil several duties to efficiently lead polyubiquitylated proteins to degradation. It has to recognize and bind substrates, cleave off the ubiquitin molecules, so that they can be recycled, unfold the proteins, and translocate them into the gated channel. It is still not completely clear which subunits of the 19S RP fulfil which functions, since a lot of the functions seem to be redundant and even the exact number of the 19S subunits is under discussion, because some subunits bind only transiently and may dissociate or not, depending on the purification procedure applied.

Under certain conditions, such as high salt concentration the 19S RP breaks into two subcomplexes, called the lid and the base. The base is made up of 10 subunits. Six of the subunits are ATPases of the AAA (ATPase associated with various cellular

activities) family of ATPases. These ATPases form a six membered ring and interact directly with the α-ring of the 20S CP. This ATPase ring fulfils several important functions: The binding of this ring to the 20S CP is responsible for the formation of the narrow pore that allows polypeptides to enter the 20S proteasome. This is called the gating function. In addition the ATPases are involved in the unfolding process that is necessary before a protein fits through the opening of the 20S CP. This is called the anti-chaperone activity. One of the ATPase subunits Rpt5/S6a is also able to bind multiubiquitin chains and may be involved in the substrate recognition and binding of the 26S proteasome (Lam et al., 2002).

The other four subunits of the base are non-ATPases. One of them is S5a/Rpn10 that in yeast also exits in a free, non proteasome bound form (van Nocker et al., 1996b) whereas in HeLa cells S5a was not found in the free state (Hendil et al., 2002). It contains a ubiquitin interacting motif (UIM) domain which is able to bind polyubiquitin chains but has only little affinity for monoubiquitin. Interestingly only free bovine S5a crosslinks to ubiquitin chains, whereas proteasome-associated S5a does not (Lam et al., 2002). S5a is also involved in the binding of UDPs to the 26S proteasome. S5a is not essential in yeast, and Δrpn10 mutants still degrade the bulk of short-lived proteins normally, but some substrates require it for their degradation (Rubin et al., 1997). This indicates that its functions are redundant. Of the other base subunits, one has a sequence homology to ubiquitin hydrolases (Uch2/UCH37), while the largest subunits S1/Rpn2 and S2/Rpn1, seem to play a structural role in linking the base and the lid and provide a platform to facilitate the transient interaction of a number of non-proteasomal proteins.

As already mentioned above, the lid subunit S13/Rpn11 contains a metalloprotease domain. It plays a key role in the recycling of ubiquitin, by cleaving the polyubiquitin chain from the protein substrate.

There also exist proteasomes composed of the 20S CP, one 19S RP and PA28 (Hendil et al., 1998). This suggests a model where proteins are bound by the 19S RP after ubiquitylation and are then unfolded and fed into the 20S CP. The PA28 complex at the other end might then change the cleavage pattern (Groettrup et al., 1995) to provide a spectrum of peptides that can be presented more efficiently on MHC than the fragments generated by the 26S protesome alone.

Ubiquitin independent proteasomal degradation

The standard model of proteasomal protein degradation assumes that ubiquitylation and degradation are linked with each other. Attachment of a certain (in most cases Lys-48 linked) polyubiquitin chain is a prerequisite for degradation by the 26S proteasome. There are however some cases where degradation can occur independently of ubiquitin modification.

One group of substrates consists out of chemical or physical denaturated proteins, like for example denatured ovalbumin, or proteins that lack a defined tertiary structure, like casein. Native ovalbumin is normally a stable protein, but after oxidation by perchloric acid and denaturation by guanidine treatment it can be degraded by the 26S proteasome without need for ubiquitin (Benaroudj et al., 2001). Casein is degraded by the proteasome in a ubiquitin independent manner, but conjugation with ubiquitin accelerates this process (Tanaka et al., 1983).

Another group consists out of proteins that are correctly folded and need to be unfolded by the proteasome. With the exception of ornithine decarboxylase (ODC) (see below for more details) all proteins of this group can also be found in ubiquitylated forms, so that is still a matter of discussion if their ubiquitin independent degradation is physiologically significant.

The Cdk inhibitor p21/WAf1/Cip1 is still degraded when all its lysine residues are mutated. It is also degraded, when mutant forms of ubiquitin without lysine residues, that can not form polyubiquitin chains, are expressed (Sheaff et al., 2000). It could also be shown that p21 directly binds to an α-subunit of the 20S proteasome, and that the interaction domain is necessary for its ubiquitin independent degradation by the 20S proteasome *in vitro* (Touitou et al., 2001). If the degradation *in vivo* is actually ubiquitin independent is still a matter of debate, since recently it could be shown that p21 is polyubiquitylated at its the free amino group of the N-terminal methionine, and that this is sufficient for proteasomal degradation (Bloom et al., 2003).

The T-cell antigen receptor (TCR) is a hetero-oligomeric membrane-complex of at least seven polypeptide chains. The α-subunit (TCR-α) is a type I membrane protein containing a short cytoplasmic domain and a 223-residue extracellular domain. When expressed in the absence of other TCR subunits, TCR-α is rapidly degraded by the proteasome. It could be demonstrated that a variant of TCR-α lacking lysines is

degraded with kinetics indistinguishable from those of the wild type protein (Yu et al., 1997). Experiments with a temperature sensitive E1 ubiquitin-activating enzyme, and with K48R ubiquitin, that cannot form Lys-48 linked polyubiquitin chains, showed that ubiquitylation events are necessary to degrade both wildtype and lysinless TCR-α (Yu and Kopito, 1999). In addition the E2 UBC7 is involved in this process, since overexpression of catalytically inactive UBC7 delayed the degradation events (Tiwari and Weissman, 2001).

IκBα is a protein that binds to and regulates NFκB function, and ubiquitylation targets IκBα for stimulation induced degradation. The basal turnover however could be ubiquitin independent, since lysineless mutants did not show any increase in steady state levels of the protein (Krappmann et al., 1996).

Other proteins that can be found ubiquitylated but where there are also hints of a ubiquitin independent degradation are the transcription factor c-Jun, that can be degraded by the 26S proteasome *in vitro* without ubiquitin being present (Jariel-Encontre et al., 1995) and Calmodulin, that in an aged form and without bound Calcium, is also degraded by the 26S proteasome in a ubiquitin independent fashion (Tarcsa et al., 2000).

Till today it seems that there is only one protein with a clearly defined tertiary structure that is degraded by the 26S proteasome, with no ubiquitylation events taking place, ornithine decarboxylase (ODC). ODC is the rate limiting enzyme in the polyamine biosynthesis. Since an excess of polyamines is toxic, it is necessary to control their levels. One method to do this is to change the amount of ODC in the cell. This is done in a very special fashion. Polyamine accumulation stimulates the production of antizyme, in inducing a frameshift that is necessary for the production of the functional protein. Then antizyme binds to ODC. This has two consequences: First antizyme acts as a stochiometric inhibitor, since the ODC/ antizyme heterodimer is catalytically inactive. Secondly, antizyme promotes the degradation of ODC about 8-fold by improving its affinity for the proteasome. While the inhibitory function requires a one to one molar ratio of antizyme and ODC, one molecule of antizyme can lead several molecules of ODC to destruction since it does not get degraded together with ODC (Coffino, 2001).

It seems that the antizyme/ ODC complex is recognized by the same element(s) of the proteasome that also mediate recognition of polyubiquitin chains, since a model polyubiquitylated protein or even a tetraubiquitin chain can act as competitive inhibitors of ODC degradation (Zhang et al., 2003). The mechanism of ODC degradation seems therefore to be a molecular mimicry of the ubiquitin dependent degradation.

ADDRESSED QUESTIONS AND AIMS OF THIS PROJECT

When I started this thesis the only known consequence of FAT10 conjugation to substrate proteins, was the induction of apoptosis (Raasi et al., 2001). The aim of this thesis was to further analyse the functional consequences of FAT10 conjugation.

In order to learn more about the function of FAT10 and to possibly identify enzymes involved in FAT10 conjugation, a two-hybrid interaction screen was performed.

None of the found FAT10 interaction partners showed homology to members of the ubiquitin conjugation pathway, but other interesting FAT10 interaction partners have been found. We choose to investigate the interaction of FAT10 with NUB1L, since the published data and the domain composition of this protein looked very interesting.

In addition to confirming the non-covalent physical interaction between NUB1L and FAT10, we also looked for functional consequences of the coexpression of the two proteins. Therefore the interaction was analyzed *in vivo* by coimmunoprecipitation from NUB1L and FAT10 coexpressing cells, and *in vitro* by GST pull down experiments. Parameters that have been checked after coexpression of FAT10 and NUB1L included FAT10 localization in the cell, half-life of FAT10 and interaction of FAT10 with other proteins.

The fast, proteasome dependent, turnover of FAT10 was another interesting feature that has been observed before (Liu et al., 1999; Raasi et al., 2001), and we decided to further investigate the degradation kinetics of FAT10, which are atypical for a ubiquitin-like modifier, and the possible mechanisms involved in its rapid degradation.

Moreover, using our inducible cell line, we also wanted to identify the FAT10 conjugates, which can be detected by western blot analysis.

2.) NEDD8 ULTIMATE BUSTER-1L INTERACTS WITH THE UBIQUITIN-LIKE PROTEIN FAT10 AND ACCELERATES ITS DEGRADATION

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SUMMARY

FAT10 is an interferon-γ inducible ubiquitin-like protein which consists of two ubiquitin–like domains. FAT10 bears a diglycine motif at its C-terminus which can form isopeptide bonds to so far unidentified target proteins. Recently we found that FAT10 and its conjugates are rapidly degraded by the proteasome and that the N-terminal fusion of FAT10 to a long lived protein markedly reduces its half life. FAT10 may hence direct target proteins to the proteasome for degradation. In this study we report a new interaction partner of FAT10 which may link FAT10 to the proteasome. A yeast two hybrid screen identified NEDD8 ultimate buster-1L (NUB1L) as a non-covalent binding partner of FAT10 and this interaction was confirmed by coimmunoprecipitation and glutathione-S-transferase pull down experiments. NUB1L is also an interferon inducible protein which has been reported to interact with the ubiquitin-like protein NEDD8 thus leading to accelerated NEDD8 degradation. Here we show that NUB1L binds to FAT10 much stronger than to NEDD8 and that NEDD8 can not compete with FAT10 for NUB1L binding. The interaction of FAT10 and NUB1L is specific as GFP fusion proteins containing ubiquitin or SUMO-1 do not bind to NUB1L. The coexpression of NUB1L enhanced the degradation rate of FAT10 eightfold while NEDD8 degradation was only accelerated twofold. Since NUB1 was shown to bind to the proteasome subunit RPN10 *in vitro* and to be contained in 26S proteasome preparations it may function as a linker that targets FAT10 for degradation by the proteasome.

INTRODUCTION

The covalent modification of proteins with lysine 48-linked polyubiquitin chains is a signal for their degradation by the 26S proteasome (Hershko et al., 2000; Voges et al., 1999). The 26S proteasome consists of a central proteolytic unit, the 20S proteasome, and one or two copies of the 19S regulator (also called PA700) which bind to the ends of the cylindrical 20S protease. The 19S regulator in turn can be dissociated into a proximal 'base' which is formed by a ring of 6 ATPases (designated Rpt1-6) and a distal 'lid' of at least 12 non-ATPase subunits (named RPN1-12) (Glickman et al., 1998). Four main functions can be assigned to the 19S regulator: it serves as a receptor for polyubiquitylated proteins (Deveraux et al., 1994; Lam et al., 2002; van Nocker et al., 1996b), it unfolds proteins before degradation (Braun et al., 1999; Strickland et al., 2000), it cleaves off the polyubiquitin chains so that they can be recycled (Verma et al., 2002), and it opens the gate of the 20S proteasome (Köhler et al., 2001). Two subunits of the 19S regulator, the non-ATPase subunit RPN10 (van Nocker et al., 1996a) and the ATPase subunit Rpt5 (Lam et al., 2002) have been shown to bind polyubiquitylated proteins and are thought to be substrate docking sites.

In addition to the direct binding of polyubiquitylated substrates to the proteasome, a second mode of substrate binding has been characterized. This association is mediated by adaptor proteins that possess ubiquitin-like domains which interact with the proteasome as well as ubiquitin association (UBA) domains which are able to bind polyubiquitylated proteins. Examples of these adaptor proteins are the RAD23 protein (Rao and Sastry, 2002), the hPLIC1 protein (Gao et al., 2003), and the protein NEDD8 ultimate buster-1 (NUB1) (Tanaka et al., 2003). Although the overexpression of these proteins has been shown to induce the degradation of non-covalently interacting proteins in several cases it is still a matter of debate whether these adaptors are mediators of proteasomal degradation or whether they serve to regulate the degradation process in a negative manner possibly by competing with the 26S proteasome for binding of polyubiquitylated substrates (Raasi and Pickart, 2003). Interestingly, it seems that UBA domains may also bind ubiquitin-like domains as has been shown for NUB1 which interacts *in vitro* with the ubiquitin-like protein NEDD8 and mediates its accelerated degradation (Tanaka et al., 2003).

It has often been emphasized that ubiquitin-like proteins, that become isopeptide linked to target proteins through selective cascades of activating enzymes (E1), conjugating enzymes (E2) and ligases (E3), serve purposes other than targeting for proteasomal degradation as for instance changes of intracellular localization, alterations of enzymatic activity or the modification of DNA binding. Recently, we and others have shown that a novel member of these so called ubiquitin-like modifiers that was first described under the name of 'diubiquitin' (Fan et al., 1996), and subsequently renamed to 'FAT10', is rapidly degraded by the proteasome (Liu et al., 1999; Raasi et al., 2001). FAT10 consists out of two ubiquitin-like domains that are joined by a short linker. It should be emphasized, however, that FAT10 is distinct from the ubiquitin-like modifier ISG15 (also called ubiquitin cross-reactive protein (UCRP)) that also consists out of two ubiquitin-like domains (Jentsch and Pyrowolakis, 2000).

The inducible expression of FAT10 in fibroblasts led to the appearance of the 18 kDa protein FAT10 as well as three closely migrating covalent conjugates at about 35 kDa which, like unconjugated FAT10, were degraded by the proteasome with a half life of one hour (Raasi et al., 2001).

Mutation of the C-terminal diglycine motif of FAT10 abolished the conjugate formation thus strongly suggesting that FAT10 becomes isopeptide linked via its C-terminal diglycine motif in analogy to ubiquitin and other ubiquitin-like modifiers as for instance SUMO, NEDD8 or ISG15 (Jentsch and Pyrowolakis, 2000). In contrast to these well characterized protein modifiers, the putative activating (E1) and conjugating enzymes (E2) for FAT10 have not been identified. Interestingly, the fusion of FAT10 to the N-terminus of the long lived green fluorescent protein (GFP) led to its rapid degradation in HeLa cells. FAT10 and ubiquitin were equally efficient at targeting GFP for degradation when fused to its N-terminus thus underlining the potency of FAT10 as a degradation signal. Taken together, it seems that FAT10 in contrast to other ubiquitin-like modifiers targets its conjugation substrates for proteasomal degradation (see next chapter).

Although several interesting properties of FAT10 have been described, the biological function of FAT10 has so far remained elusive. FAT10 is encoded in the major histocompatibility complex class I region and is inducible in many different cell lines

with the proinflammatory cytokines interferon (IFN)-γ and/or tumor necrosis factor (TNF)-α (Liu et al., 1999; Raasi et al., 1999).

Moreover, FAT10 is upregulated upon the activation of B cells and dendritic cells (Bates et al., 1997) which could hint at a function in antigen processing, but so far we have failed to substantiate this hypothesis experimentally. Interestingly, the induced expression of FAT10 causes apoptosis in fibroblasts suggesting that it may be functionally involved in the TNF-α triggered apoptosis pathway (Raasi et al., 2001). In seeming contradiction to this hypothesis, the FAT10 mRNA has recently been found to be highly upregulated in numerous gynecological and gastrointestinal tumors but it was not reported whether the fat10 gene was mutated in these tumors or not (Lee et al., 2003). It is therefore not yet clear whether FAT10 has the properties of an oncogene or a tumor suppressor. An involvement of FAT10 in cell cycle regulation was proposed by Liu et al. who reported a non-covalent interaction of FAT10 with the nuclear spindle assembly checkpoint protein MAD2 but the physiological consequences of this association have remained unclear (Liu et al., 1999).

In order to learn more about the function of FAT10 and to possibly identify enzymes involved in FAT10 conjugation, a two-hybrid interaction screen was performed which identified NUB1L as a FAT10 interaction partner and this firm non-covalent interaction was confirmed by coimmunoprecipitation and GST pull down experiments. Strikingly, NUB1L expression led to an accelerated degradation of FAT10 suggesting that NUB1L may target FAT10 for proteasomal degradation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents. The following antibodies were used: QIAexpress mAb anti-His_6 (Quiagen, Hilden, Germany), mAb anti-HA clone HA-7 (Sigma, St. Lewis, MS) mAb anti- Xpress (Invitrogen, Carlsbad, CA), and a FAT10 specific polyclonal antibody that was raised in rabbits by immunization with GST-FAT10 recombinant protein. For confocal microscopy the following fluorescence labeled mAb were used: FITC coupled anti-HA, (clone 3F10, Roche, Indianapolis, IN) and Alexa Fluor 594 coupled goat anti-mouse IgG (H+L) (Molecular Probes, Eugene, OR). Horseradish peroxidase coupled secondary antibodies were purchased from DAKO (Glostrup, Denmark).

Expression constructs and recombinant proteins. Generation of HA-NUB1L, His-NUB1L, GST and GST-FAT10 proteins: NUB1L cDNA was PCR amplified from the yeast two hybrid prey plasmid pmg8-1 using 5'-CGGGAGGTACCTGGCGCAGGGATGGCAC-3' as forward and 5'-CTATCTAGATTAGTTTTTCTTTGTTGCTGAC-3' as reverse primer. The PCR product was cloned via Asp718 and XbaI sites into the plasmid pCDNA3.1. The sequence between the Hind III and Asp718 sites of the original vector was replaced by the sequence 5' AAG CTT ACC ATG GCC TAC CCC TAC GAC GTG CCC GAC TAC GCC GCG GTA CC 3' to create an in frame fusion protein of influenza HA-epitope and Nub1L (HA-NUB1L pcDNA). For the recombinant expression of a His_6-NUB1L fusion protein in *E. coli*, the sequence between HindIII and Asp718 was replaced by the sequence 5'AAG CTT TAA GAA GGA GAT ATA AAT ATG GCC CGG GGT TCT CAT CAT CAT CAT CAT CAT GGT ATG GCT AGC ATG ACT GGT GGA CAG CAA ATG GGT GCG GTA CCT 3' (His_6-NUB1L RBS-pcDNA). This plasmid was transformed into a BL21 (DE3)pLysS *E.coli* host strain. The expression of the His-NUB1L fusion protein was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified by Ni–affinity chromatography using a HiTrap affinity column (Amersham Biosciences, Uppsala) as recommended by the manufacturer. In order to produce the GST-FAT10 fusion protein, the FAT10 cDNA was retro transcribed from mRNA of JY B cells and amplified, using primers sense 5'- CCATGGATCCATGGCTCCCAATGCTTCCTGCCTC-3' and antisense 5'-

CCGTCTCGAGTCTCACCCTCCAATACAATAAGATGC-3', and cloned via BamHI and XhoI sites into the expression vector pGEX-4T-3 (Amersham). BL21 cells were transformed with this construct and the GST-FAT10 fusion protein was induced with IPTG and purified by Glutathione Sepharose 4B chromatography (Amersham) according to protocols provided by the supplier.

Generation of His_6-Xpress-FAT10-pBI, HA-FAT10-pBI, HA-NUB1L-pBI, His_6-Xpress-FAT10-HA-NUB1L-pBI and HA-FAT10-HA-NUB1L-pBI: His_6-Xpress-FAT10-pcDNA3.1 and HA-FAT10-pcDNA3.1 had been described previously (Raasi et al., 2001). The two differently tagged versions of FAT10 as well as HA tagged NUB1L were excised from pcDNA3.1 via HindIII and XbaI and cloned into the vector pBK-CMV (Stratagene, Amsterdam). HA-NUB1L was excised from pBK-CMV with NotI and Pst I and cloned into the MCSII of the vector pBI (BD Biosciences Clontech). The two FAT10 clones were excised with BssHII and XbaI from pBK-CMV and each of them was cloned into the multiple cloning site of pBI and HA-NUB1L-pBI via MluI and NheI.

Generation of GFP Fusion Proteins: In order to create fusion proteins of ubiquitin or fat10 with the N-terminus of GFP, we used the pcDNA3 based plasmids HA-FAT10 (Raasi et al., 2001) and HA-Ubiquitin (M. Basler, unpublished). The primer 5'-AGA CGG AAG CTT ACC ATG GCC TAC CCC-3' was used as forward primer for all PCRs. For Fat 10 we used the reverse primer 5'-ATT GCG GGA TCC GCC ACT GCA ATA CAA TAA G-3' to replace the diglycine by Ala-Val in order to prevent a potential proteolytic processing. Ubiquitin was amplified with the reverse primer 5'-ATA TGG ATC CGC CAC TGC AGA GTC CGC TTC CTG-3' which also replaces the diglycine by Ala-Val. After amplification, the PCR products were cloned via Hind III and Bam HI restriction sites into pEGFP-N1 vector (Clontech, Heidelberg). In order to obtain a SUMO-1 cDNA we prepared human RNA from HeLa cells according to standard procedures. First strand c-DNA synthesis and reverse transcriptase PCR was performed according to the instructions provided by the supplier (Roche). We used the primers 5'-CGC CGC GGT ACC TAT GTC TGA CCA GGA G-3' and 5'-ATA TGG ATC CGC CAC TGC CGT TTG TTC CTG ATA-3' to mutate the C-terminal diglycine to Ala-Val. The PCR product was digested

with Asp718 and BamHI and cloned into pEGFP N1. All sequences were verified by dideoxy sequencing.

GST pull down assay. GST-FAT10 and GST proteins were expressed in *E.coli* strain BL21. Bacteria were pelleted and lysed in PBS plus 150mM NaCl and 1% Triton X-100. After centrifugation the supernatant was incubated with 250µl of Glutathione Sepharose 4B for 4h at 4°C on an end-over-end rotor. An aliquot was tested for similar binding of GST and GST-FAT10. After extensive washing 575µl of Ni-affinity purified NUB1L-His (50µg/ml) were added and incubated for 2h at 4°C end-over-end. The beads were washed three times with PBS before boiling in a sample buffer containing 5% SDS and 10% β-mercaptoethanol. Graded aliquots of the lysate were analysed on western blots probed with an anti-His_6 mAb.

Confocal fluorescence microscopy. HEK293 cells were grown on cover slips to about 30 % confluence at the time of transfection. The plasmids HA-NUB1L-pBI, His_6-Xpress-FAT10-pBI, His_6-Xpress-FAT10-HA-NUB1L-pBI were introduced into different aliquots of cells together with the plasmid p-Tet-splice-tTA (Shockett et al., 1995), to induce the expression of the genes. We used FuGENE transfection reagent (Roche) for transfection at a reagent to DNA ratio of 3:1. After 16 hours of incubation the cells were fixed with 4% paraformaldehyde, permeabilised with PBS containing 2mM EDTA, 2% FCS, 2mM azid and 0.1% saponin. The differently transfected cells and the mock transfectants were stained with anti-HA-FITC (the His_6-Xpress-FAT10-pbi transfectant served as negative control), anti-Xpress and Alexa Fluor 594 goat anti-mouse (the HA-NUB1L-pBI transfectant served as negative control) or with all three antibodies, followed by analysis with a laser scanning confocal fluorescence microscope (LSM 510; Zeiss, Oberkochen, Germany).

Yeast two-hybrid screen. The yeast two-hybrid screen using the FAT10 bait was carried out by Dualsystems Biotech AG, Zurich, Switzerland. The bait construct for yeast two-hybrid screening was made by subcloning the full length human FAT10 cDNA into the vector pLexA-DIR (Dualsystems Biotech AG, Zurich, Switzerland). The bait construct was transformed into the strain L40 (*MATa his3Δ200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4*) using standard

procedures (Gietz and Woods, 2001). Correct expression of the bait was verified by western blotting of cell extracts using a mouse monoclonal antibody directed against the LexA domain (Santa Cruz Biotechnology, Santa Cruz, California, USA). The absence of self-activation was verified by cotransformation of the bait together with a control prey and selection on minimal medium lacking the amino acids tryptophane, leucine and histidine (selective medium). For the yeast two-hybrid screen, the bait was cotransformed together with a human adult whole lymph node cDNA library (Complexity $1x10^6$, Clontech, Basel) into L40. A number of $8.2x10^5$ transformants were screened, yielding 86 transformants that grew on selective medium. Positive transformants were tested for β-galactosidase activity using a filter assay (Serebriiskii and Golemis, 2000). Out of 86 initial positives, 38 clones showed β-galactosidase activity and were considered to be true positives. Library plasmids were isolated from positive clones and retransformed into L40 with 1.) the bait plasmid and 2.) a control bait encoding a LexA-lamin C fusion. Only 6 out of 38 positives showed β-galactosidase activity when coexpressed with the bait but not when coexpressed with the control bait and were considered to be bait dependent positive interactors. The identity of positive interactors was determined by sequencing.

Pulse-Chase Experiments and Immunoprecipitation. For the experiments presented in figure 9 we used the plasmids His_6-Xpress-FAT10-pBI, HA-NUB1L-pBI and His_6-Xpress-FAT10-HA-NUB1L-pBI. The data generated for figure 11 were obtained with the plasmids HA-FAT10-pBI, HA-NUB1L-pBI and HA-FAT10-HA-NUB1L-pBI. HEK293 cells were transfected with the plasmids indicated in the figure legends and the inducer pTetsplice- tTa. 16 hours after transfection the cells were starved for 1h in Met/Cys-free RPMI- 1640 medium (Sigma) supplemented with L-glutamine, penicillin/streptomycin and 10% dialysed FCS, followed by labeling for one hour with 0.25 mCi/ml [^{35}S] Met/Cys (Translabel, Amersham). Subsequently cells were washed three times, aliquoted and chased for the indicated time periods. For the data in figure 9, the labeled cells were lysed in 20mM Tris/HCl pH 8.0, 0.1% Triton X-100 for 30 minutes on ice. After centrifugation for 15 minutes at 15000 x g one volume of 20mM Tris/HCl pH 8.0, 300mM NaCl was added to the supernatant and an aliquot analysed with a β-counter. For the experiment presented in figure 11, cells were lysed in RIPA-Buffer (50mM Tris/HCl pH 8.0, 150 mM NaCl, 1% NP40,

0.5% deoxycholate, 0.1% SDS, 1µg/ml aprotinin, 1 µg/ml pepstatin A, 1µg/ml leupeptin) and centrifuged and counted as described above. Equal amounts of radioactivity were used for immunoprecipitation with either 5µg of anti-HA-antibody or 20µg of anti FAT10 antibody and Protein G or Protein A SepharoseTM CL-4B (Amersham). The samples were incubated over night at 4°C with agitation. After five washes with the respective lysis-buffer, the immunoprecipitate was analysed by SDS-PAGE/autoradiography on a Fuji BAS1500 radioimager.

RESULTS

Identification of NUB1 as a FAT10 interaction partner by yeast two hybrid screen

To identify putative novel interaction partners of human FAT10 we screened a human lymph node cDNA library using a LexA based yeast two-hybrid system (Gyuris et al., 1993). Screening of $8.2x10^5$ transformants yielded 6 bait dependent clones which were analyzed further. Sequencing the insert of the plasmid recovered from one of the six transformants yielded the full length in frame cDNA of NUB1L.

NUB1 was first described by Yeh and colleagues as a protein of 601 amino acids and a molecular mass of 69.1 kDa which interacts with the ubiquitin-like protein NEDD8 (GenBank accession number AF300717) (Kamitani et al., 2001; Kito et al., 2001). Sequencing of the NUB1 insert recovered from our two hybrid screen revealed that the predicted amino acid sequence contained 14 amino acids more than the published sequence which were inserted between amino acid 451 and 452 of the published sequence for human NUB1 (Fig. 7). The original human NUB1 sequence contains two UBA domains of 37 amino acids spanning residues 376-413 and 477-514. Interestingly, the 14 amino acid insertion at residue 451 as predicted for our FAT10 interaction partner completes a partial UBA domain situated in between the residues 432-455 of human NUB1. It hence appears that like the published NUB1 homologues of mice (accession number: AF534114), *Drosophila* (accession number: AE003752), and *Arabidopsis* (accession number: AC007295), the human NUB1 protein variant found to interact with FAT10 contains three UBA domains in its C-terminal half. While we were preparing this manuscript, Yeh and colleagues reported the existence of a longer splice variant of human NUB1, which they named NUB1L (Tanaka et al., 2003). Since our FAT10 interaction partner is identical to NUB1L except for an

alanine residue at position 200 which was not found in our sequence, we adopted the name for this report.

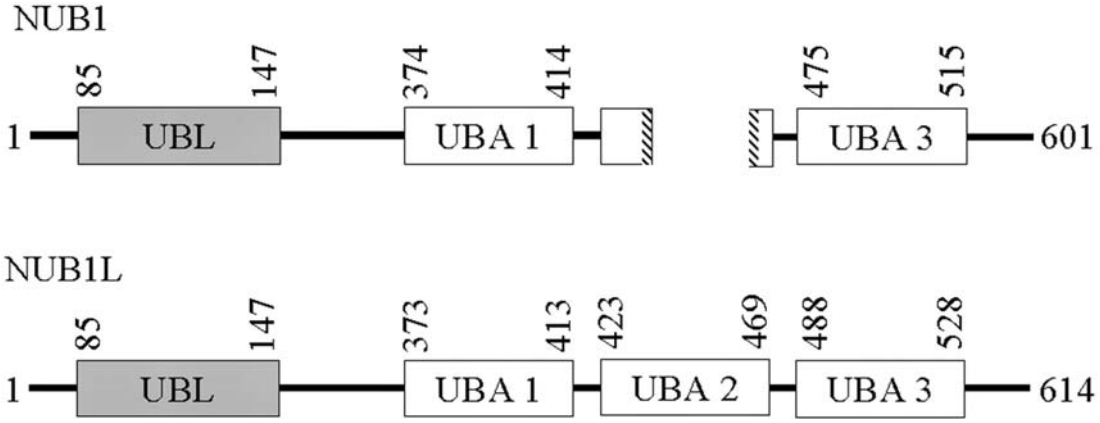

FIG. 7. Scheme of domain composition of NUB1 and NUB1L. The additional 14-amino acid insert in NUB1L creates a third UBA domain that spans residues 423–469. Both NUB1 and NUB1L contain a UBL and two other UBA domains, UBA1 and UBA3. NUB1 and NUB1L also contain a bipartite nuclear localization signal (NUB1-(414–431) and NUB1L-(413–430)) and a PEST domain at the C terminus.

Recombinant GST-FAT10 and His-NUB1L fusion proteins interact specifically in vitro

NUB1L has interesting properties which are consistent with an interaction with FAT10. Like FAT10, it is inducible with IFN-γ and it has three UBA domains which could potentially mediate binding to one or both of the ubiquitin-like domains of FAT10. We hence decided to focus on NUB1L as a FAT10 binding protein. At first we tried to confirm the yeast two hybrid result in a GST pull down experiment. A bacterial expression construct for a human GST-FAT10 fusion protein was generated and the bacterially expressed GST-FAT10 protein was purified over a glutathione sepharose column. A His_6-tagged version of human NUB1L was cloned into a bacterial expression vector, expressed in bacteria, and purified over a nickel-sepharose matrix. GST-FAT10 as well as GST protein as negative control were bound to glutathione sepharose and incubated for 2 hours with purified His-NUB1L protein. The glutathione matrix was washed and bound proteins were eluted and analysed in five fold dilutions on anti-His western blots. As shown in figure 8A, His-NUB1L specifically bound to the GST-FAT10 matrix but not to the GST control thus indicating that FAT10 and NUB1L directly bind to each other *in vitro*.

Since NUB1 was originally described as a binding partner of NEDD8 we tested whether recombinant NEDD8 would be able to compete with FAT10 for binding to NUB1L.

GST-FAT10 was immobilized on glutathione matrix and incubated with recombinant human NUB1L in the presence of commerically available recombinant human NEDD8 at molar ratios varying from 0.01 to 10 (Fig. 8B). Even a tenfold molar excess of NEDD8 was unable to compete with FAT10 for binding to NUB1L. This result may indicate that NEDD8 binds to NUB1L at a different site than FAT10 or that NEDD8 binds to the same site of NUB1L but with much lower affinity than FAT10. In order to compare the binding of FAT10 and NEDD8 to NUB1L, we modified the binding assay in that His-NUB1L was incubated with comparable concentrations of GST, GST-FAT10 or NEDD8 followed by immunoprecipitation of His-NUB1L and western analysis of the precipitate and the supernatant with anti-GST and anti-NEDD8 antibodies (Fig. 8C). In this assay, we consistently failed to demonstrate a specific interaction of recombinant NEDD8 and NUB1L while binding of GST-FAT10 to NUB1L was readily observed. This result suggests that NEDD8 binds either not at all or much weaker to NUB1L as compared to FAT10.

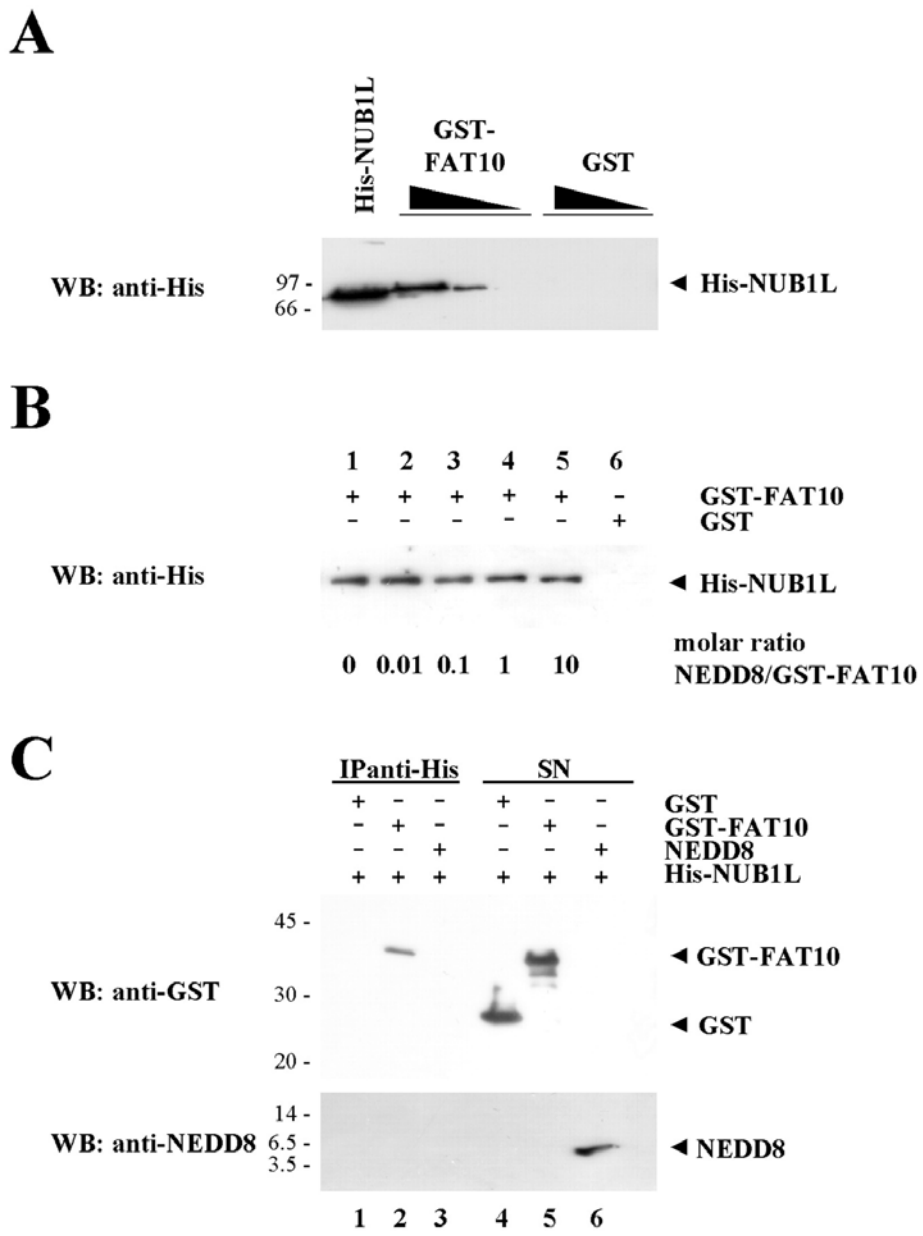

FIG. 8. GST-FAT10 and NUB1L recombinant proteins directly interact *in vitro*. **(A)** purified recombinant GST and GST-FAT10 proteins were bound to glutathione-Sepharose™ 4B matrix and incubated with purified recombinant His-NUB1L for 2 h. After washing the beads, the interacting proteins were analyzed by anti-His Western blot (*WB*). Three different dilutions of the sample (100, 20, and 4%) were loaded. *Lane 1* contains the His-NUB1L input (100%). **(B)** the same GST pull-down experiment as in *A* was performed but in the presence of increasing concentrations of recombinant NEDD8. The indicated molar ratios of NEDD8 and GST-FAT10 were estimated from Coomassie-stained SDS-PAGE. **(C)** coprecipitation experiment to compare the association of GST-FAT10 and NEDD8 with His-NUB1L. Purified recombinant His-NUB1L was incubated with comparable concentrations of purified recombinant GST, GST-FAT10, and NEDD8. Subsequently, His-NUB1L was immunoprecipitated (*IP*) with an anti-His mAb, and the precipitates as well as 3% of the supernatants (*SN*) were analyzed by Western blots using anti-GST as well as anti-NEDD8 antibodies. Molecular mass markers are indicated in kDa at the *left*, and the positions of indicated proteins are marked with *arrowheads*. Representative examples out of three independent experiments are shown.

FAT10 and NUB1L strongly interact intracellularly

The intracellular localization of FAT10 and NUB1 is only partially overlapping as FAT10 has been shown to be predominantly localized in the cytoplasm (Liu et al., 1999; Raasi et al., 2001) whereas NUB1 as well as NUB1L are mainly found in the nucleus (Tanaka et al., 2003). In order to determine if the two proteins can nevertheless substantially interact in intact cells and to confirm FAT10-NUB1L interaction in the intracellular environment we performed coimmunoprecipitation experiments. Mammalian expression constructs for His_6 and Xpress tagged human FAT10 (HisX-FAT10) and HA tagged human NUB1L (HANUB1L) were generated and transiently transfected into HEK293 human embryonal kidney cells either alone or together. In addition, FLAG-tagged NEDD8 was transiently expressed alone or together with HA-NUB1L in order to investigate their intracellular interaction in HEK293 cells. The cells were metabolically labeled and NUB1L was precipitated with anti-HA mAb. As depicted in figure 9A, HisX-FAT10 was prominently coprecipitated with HANUB1L when both proteins were coexpressed (Fig. 9A, lane 5) but no 23 kDa band corresponding to HisX-FAT10 was detected when either HA-NUB1L or HisX-FAT10 were expressed individually (Fig. 9A, lanes 2 and 3). A specific interaction of HA-NUB1L and NEDD8, in contrast, was not apparent in our experiments. A faint NEDD8 band appeared when immunoprecipitation was performed with the anti-HA mAb and a washing buffer containing 0.1% Triton X-100, but this band was equally prominent in the absence or presence of HA-NUB1L (Fig. 9A, lanes 4 and 6) suggesting unspecific binding. Using more stringent washing conditions, which were permissive for the NUB1L-FAT10 interaction (0.1% SDS, 1%NP40), eliminated NEDD8 from all immunoprecipitates (data not shown) although the intensity of radiolabeled NEDD8 in the lysates was at least as high as that of FAT10 (Fig. 9A, bottom panel). In a series of coprecipitation experiments in HEK293 as well as HeLa cells we determined the portion of HA-NUB1L associated HisX-FAT10 to be about 1/3 of the total amount of HisX-FAT10 thus indicating that the association of FAT10 and NUB1L under the chosen experimental conditions is robust and extensive (Fig. 9A and unpublished data).

To provide further evidence for the specificity of the NUB1L-FAT10 interaction we generated FAT10-GFP, ubiquitin-GFP, and SUMO1-GFP expression constructs and transfected each of them either alone or together with the HA-NUB1L expression

construct into HEK293 cells. The cells were metabolically labeled and the GFP fusion proteins were immunoprecipitated with an anti-GFP antibody in order to monitor whether the HA-NUB1L protein would associate with the respective fusion proteins. The autoradiography shown in figure 9B illustrates that the FAT10-GFP precipitate but neither the ubiquitin-GFP nor the SUMO-GFP precipitate contained bound NUB1L although the NUB1L expression was comparable in the transfected cells (Fig. 9B, bottom panel). Taken together, our data indicate that the intracellular binding of NUB1L to FAT10 is highly specific.

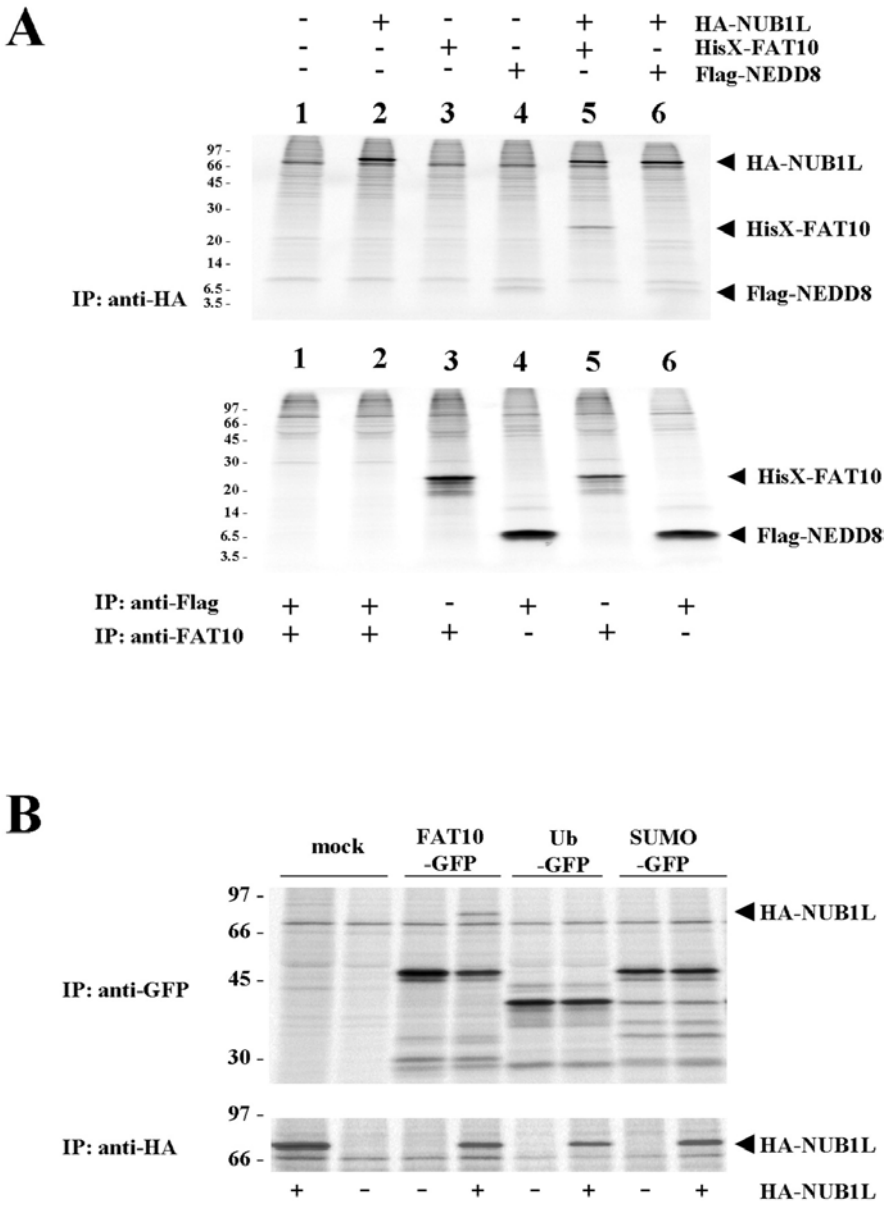

FIG. 9. Coprecipitation of HisX-FAT10 and HA-NUB1L in transiently transfected cells. (A) His_6- and Xpress-tagged human FAT10 (*HisX-FAT10*), FLAG-tagged NEDD8 (*FLAG-NEDD8*), and HA-tagged human NUB1L (*HA-NUB1L*) were transiently expressed in HEK293 cells either alone or together as indicated at the *top*. The cells were labeled for 1 h with [^{35}S]Cys/Met prior to HA-specific immunoprecipitation, SDS-PAGE, and autoradiography. The positions of molecular mass markers in kDa are indicated at the *left*, and the positions of HA-NUB1L, HisX-FAT10, and FLAG-NEDD8 are shown at the *right*. From the supernatants of the experiment shown in the *upper panel*, proteins were immunoprecipitated with anti-FLAG and anti-FAT10 antibodies as indicated below the *bottom panel.* **(B)** NUB1L specifically interacts with the ubiquitin-like protein FAT10. Expression constructs for FAT10-GFP, ubiquitin (*Ub*)-GFP, and SUMO-GFP were transiently transfected into HEK293 cells either alone or together with an HA-NUB1L expression plasmid as indicated *below each lane*. Cells were then radiolabeled and lysed prior to immunoprecipitation (*IP*) with anti-GFP and anti-HA mAb as indicated at the *left*. Shown are autoradiographies of the immunoprecipitates separated on SDS-PAGE. Representative examples out of 3–5 independent experiments are shown.

The coexpression of FAT10 and NUB1L does not mutually change their intracellular localization

Since other ubiquitin-like modifiers as for instance SUMO-1 alter the intracellular localization of their target proteins, we examined whether FAT10 and NUB1 would mutually influence their localization. Previously we and Liu et al. have shown in mouse fibroblasts and human B cells that FAT10 is predominantly localized in the cytoplasm (Liu et al., 1999; Raasi et al., 2001). This finding was recently challenged by Lee et al. who found that FAT10 was localized in the nucleus of the transiently transfected human embryonic liver cell line WRL68 and in murine NIH/3T3 fibroblasts adenovirally transfected for FAT10 expression (Lee et al., 2003). In order to reexamine this issue we transiently transfected HEK293 cells with expression constructs encoding either His-Xpress tagged human FAT10 or HA-tagged human NUB1L. In addition we coexpressed both proteins together from a vector encoding both proteins under a bidirectional promotor. The analysis of the cells with confocal microscopy revealed that in agreement with our previous studies, FAT10 was predominantly localized in the cytoplasm irrespective of whether it was expressed alone or together with NUB1L (Fig. 10). The FAT10 immunoreactivity in the nucleus was faint but clearly detectable while nuclear bodies which had the appearance of nucleoli were spared. NUB1L, in contrast, was clearly more prominent in the nucleus than in the cytoplasm but also absent from nuclear bodies. However, similar to FAT10, the distribution of NUB1L was not exclusively confined to one compartment as the cytoplasmic staining for NUB1L was well detectable in contrast to mock transfected controls.

We therefore conclude that the localization of NUB1L and FAT10 is predominant in the nucleus and cytoplasm, respectively, but that a significant overlap prevails as illustrated by the merged images. This overlap is consistent with their interaction observed by coimmunoprecipitation in the same cells (Fig. 9). Nevertheless, an impact of FAT10 coexpression on the intracellular localization of NUB1L and vice versa was not apparent when comparing the single and double transfectants shown in the upper and lower panels of figure 10.

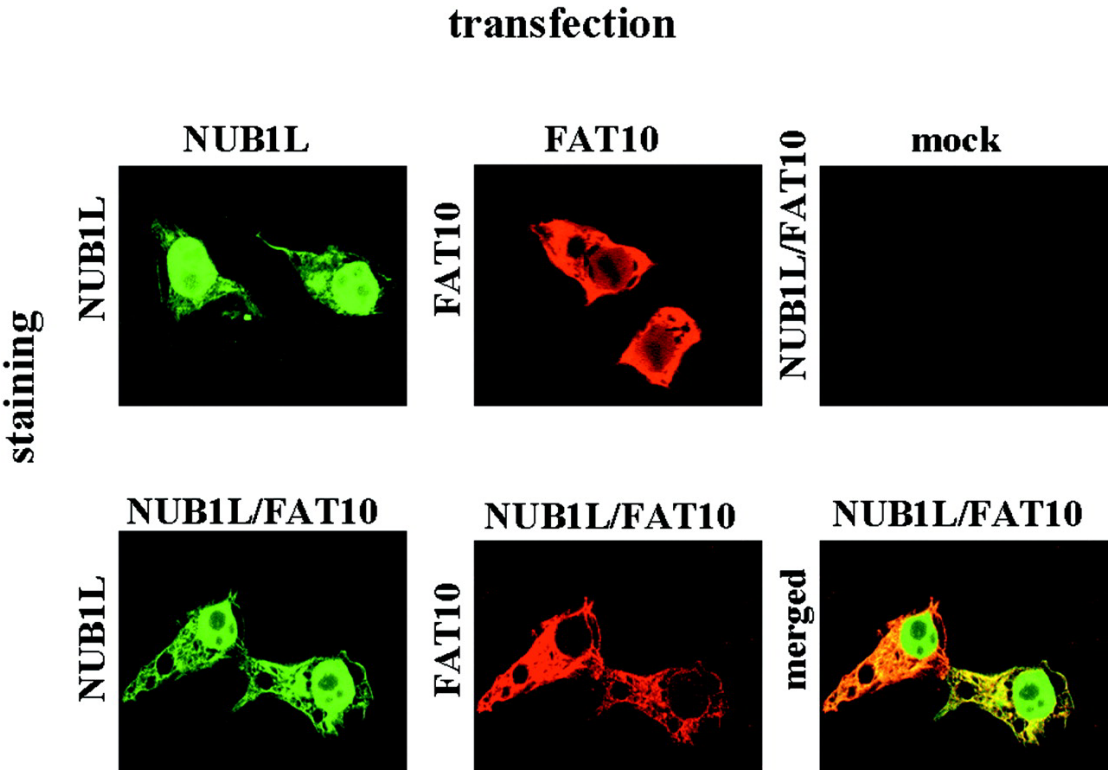

FIG. 10. FAT10 and NUB1L do not mutually affect their intracellular localization. HEK293 cells were transiently transfected with either HA-tagged human NUB1L or His_6- and Xpress-tagged human FAT10 or mock-transfected (single transfectants, *top panels*). Alternatively, tagged NUB1L and FAT10 were coexpressed in HEK293 cells from a bidirectional vector (double transfectants, *bottom panels*). Antibodies against the Xpress or HA tag were used to visualize the intracellular localization of HA-NUB1L (*green*) and HisX-FAT10 (*red*) by confocal fluorescence microscopy. The *rightmost panel* on the *bottom* is a merge of the two images to the *left*.

NUB1L strongly downregulates FAT10 expression levels by accelerating its degradation

NUB1L contains a ubiquitin-like domain and was shown to bind to the RPN10 subunit of the 26S proteasome *in vitro* (Kamitani et al., 2001). Moreover, NUB1L was reported to accelerate the degradation of the ubiquitin-like modifier NEDD8. We hence investigated whether NUB1L would also increase the degradation rate of FAT10. HEK293 cells were transiently cotransfected with an expression construct for HA-FAT10 or HA-tagged NEDD8 either alone or together with an HA-NUB1L expression construct. To ensure that FAT10 and NUB1L were expressed in the same cells both mRNAs were transcribed from the same plasmid under the control of a bidirectional promotor in some of the experiments. The cells were pulse labeled with [^{35}S] methionine for 1 hour and then chased for up to 4 hours. Immunoprecipitation with anti-HA mAb revealed that the coexpression of human HA-NUB1L led to a strong downregulation of HA-FAT10 protein levels and to an about eightfold

acceleration of HA-FAT10 degradation (Fig. 11A). Quantification of the FAT10 signal on a radioimager revealed that the coexpression of HA-NUB1L reduced the half life of HA-FAT10 in HEK293 cells from 4 hours to less than 30 minutes (Fig. 11B). Also for NEDD8 we consistently observed an acceleration of degradation when NUB1L was coexpressed (Fig. 11C) but the effect was much less prominent with an about twofold increase in degradation rate (Fig. 11D). Taken together, our data suggest that the rapid degradation of FAT10 is a potential functional consequence of the herein described interaction of FAT10 with NUB1L.

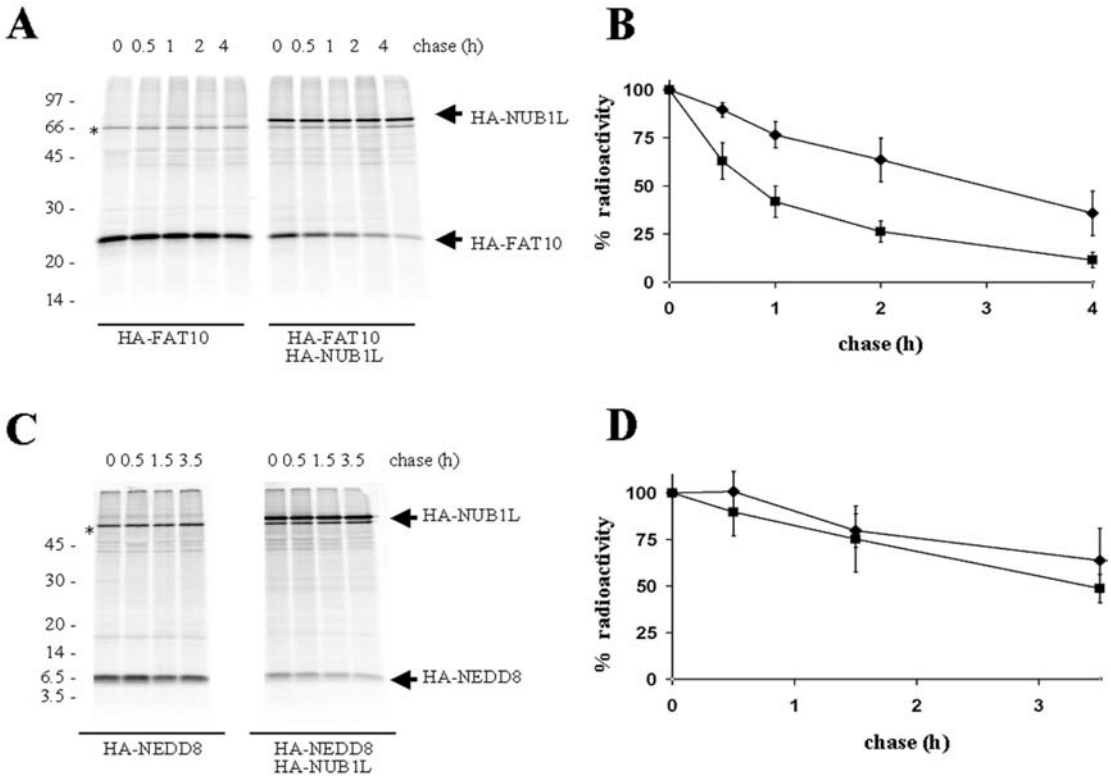

FIG. 11. NUB1L expression reduces the half-life of FAT10. (A) HA-FAT10 either alone (*left panel*) or together with HA-NUB1L (*right panel*) was transiently expressed in HEK293 cells. The cells were labeled for 1 h with [^{35}S]Cys/Met and chased for the indicated times prior to HA-specific immunoprecipitation, SDS-PAGE, and autoradiography. Molecular mass markers are indicated to the *left* in kDa, and immunoprecipitated proteins are indicated with an *arrowhead*. The *asterisk* denotes an unspecific band. **(B)** the HA-FAT10 bands from three experiments were quantified on a radioimager and plotted as percent radioactivity based on values of the pulsed cells. *Filled boxes* denote degradation in the presence of NUB1L; *filled diamonds* denote degradation in the absence of NUB1L. **(C)** HA-NEDD8 either alone (*left panel*) or together with HA-NUB1L (*right panel*) was transiently expressed in HEK293 cells, metabolically labeled, chased as indicated, and immunoprecipitated with anti-HA mAb. **(D)** quantitative evaluation of three autoradiographies similar to that shown in *C* with a radioimager. Typical experiments out of 3–4 independent experiments are shown.

DISCUSSION

In this study we identify NUB1L as a new non-covalent interaction partner of the ubiquitin-like modifier FAT10. This interaction was initially found in a yeast two-hybrid screen using full length human FAT10 as a bait and subsequently confirmed by coimmunoprecipitation from NUB1L and FAT10 coexpressing cells. The binding of FAT10 and NUB1L appears to be direct because recombinant purified GST-FAT10 and His-NUB1L interacted in GST pull down assays. The functional consequence of FAT10-NUB1L interaction does not seem to be a change in localization because no such change was observed by confocal microscopy when both proteins were coexpressed. Instead, NUB1L coexpression led to an eightfold acceleration of FAT10 degradation resulting in a fulminant downregulation of FAT10 expression level in NUB1L coexpressing cells.

Recently, we have determined the half life of FAT10 and a covalent conjugate of FAT10 to be in the order of 1 hour and this degradation was prevented by proteasome inhibitors thus strongly suggesting that the proteasome is in charge of FAT10 catabolism (see next chapter).

Moreover, the fusion of FAT10 to the long lived protein GFP led to its rapid degradation indicating that FAT10 may serve as a degradation tag. Ubiquitin-like proteins as for instance RAD23 and hPLIC1/2 were shown to act as adaptors by associating with the 26S proteasome via their ubiquitin-like domains (Kleijnen et al., 2000; Schauber et al., 1998) and it is possible that NUB1L serves a similar purpose for FAT10 and, to a lesser degree, for NEDD8. NUB1 appears to copurify with the 26S proteasome and an interaction of NUB1 recombinant protein with an RPN10 fusion protein has been shown by Kamitani *et al.* in GST pull down assays (Kamitani et al., 2001). NUB1 contains a ubiquitin-like domain in its N-terminus and this could potentially bind to the ubiquitin interaction motif (UIM) of RPN10 or the leucine-rich-repeat-like domain of the base component RPN1 which are bound by the ubiquitin-like domains of the adaptors hPLIC1/2 and RAD23, respectively (Elsasser et al., 2002; Walters et al., 2002). In its C-terminal domain, NUB1L contains three UBA domains. Domains of this class have been shown to bind to polyubiquitylated proteins and are therefore likely candidates for FAT10 interaction domains. We hence hypothesize that NUB1L bridges FAT10 and its covalent conjugates to the 26S proteasome and mediates their degradation. If this assumption is valid, FAT10-

proteasome interaction may be inapparent when the expression levels of NUB-1 are low as seems to be the case in most tissues (Kito et al., 2001). Our results therefore predict that the rate of FAT10 degradation in tissues and cell lines is regulated by NUB1.

In this respect it is interesting that NUB-1 is inducible with the antiviral cytokines IFN-β and IFN-γ (Kito et al., 2001). FAT10 is induced in a broad array of cell lines with TNF-α and IFN-γ but not with IFN-α/β (Raasi et al., 1999). This overlapping but not identical pattern of cytokine inducibility implies that the T cell derived cytokines TNF-α and IFN-γ induce at the same time high levels of the ubiquitin-like protein FAT10 as well as the putative adaptor NUB-1 which facilitates the rapid degradation of FAT10. This scenario is well compatible with a role of FAT10 in selectively targeting so far unidentified substrate proteins for proteasomal degradation in a situation where cells are stimulated by T helper type 1 derived cytokines. The pattern of inducibility also suggests that NUB-1 must have a function other than FAT10 degradation in a situation when cells produce type I interferons (IFN-α and IFN-β) leading to the induction of NUB-1 but not of FAT10. This prediction is also consistent with the fact that NUB1 is expressed in organisms like *Arabidopsis* or *Drosophila* which lack a specific immune system whereas FAT10 has only been found in mammals endowed with a specific immune system. The promotion of NEDD8 degradation is one example of a FAT10 unrelated function of NUB1 given that homologues of NEDD8 are also expressed in lower eukaryotes.

NUB1 has been discovered two years ago by Yeh and colleagues as a protein which interacts with the ubiquitin-like modifier NEDD8 *in vitro* and accelerates its proteasome dependent degradation (Kito et al., 2001). It is therefore somewhat surprising that now a second ubiquitinlike modifier, FAT10, is downregulated by the same protein. Both, in our GST pull down experiments (Fig. 8) as well as in our coimmunoprecipitation experiments (Fig. 9) we failed to detect a specific interaction between human NUB1L and human NEDD8 even when using buffers without detergents and physiological salt conditions. Yeh and colleagues reported a specific interaction of RH-tagged NUB1 as well as RH-NUB1L with GST-NEDD8 but not GST when using a washing buffer containing 0.1% NP-40 as a detergent in GST pull down assays (Tanaka et al., 2003). When we used buffers containing 0.1% NP40 we lost the interaction of His-NUB1L with untagged NEDD8. We have used different

tags than Yeh et al. and slightly different conditions so it may be that for technical reasons we missed a significant interaction of NUB1L and NEDD8. Hence, we can not claim that the NUB1L-NEDD8 interaction does not exist at all. We can, however, conclude from our experiments that the binding of NEDD8 to NUB1L is too weak to compete with the FAT10-NUB1L interaction and that even a tenfold excess of NEDD8 does not affect the amount of His-NUB1L that can be pulled down with GST-FAT10 (Fig. 8B). It appears that the NUB1L-FAT10 interaction that withstands even washing with RIPA buffer (containing 1% NP40, 0.5% deoxycholate, and 0.1% SDS) is much more robust and extensive than the NEDD8-NUB1L interaction. These results from binding assays are also in accordance with our finding that NUB1L coexpression accelerates the degradation of FAT10 in HEK293 cells by a fourfold greater factor than that of NEDD8 (Fig. 11).

Tanaka et al. have very recently performed an extensive analysis of domains within NUB1L which are responsible for NEDD8 interaction and degradation (Tanaka et al., 2003). Interestingly, it is only the second of three consecutive UBA domains in NUB1L which can bind to NEDD8 thus leaving the first and third UBA domain as potential interaction domains for FAT10. We are currently performing numerous domain shuffling experiments in order to find out which parts of NUB1L and FAT10 are responsible for their binding (see also supplementary data in this chapter). We may be in for surprises given that Yeh and colleagues unexpectedly found that NEDD8 binds to the PEST sequence in the ultimate C-terminal part of NUB1L as well, and that interaction via the PEST sequence is pivotal for NEDD8 degradation (Tanaka et al., 2003). Another important question which we are currently addressing is whether NUB1L is able to target FAT10 for binding and degradation by the isolated 26S proteasome *in vitro*. This experiment will also help to clarify whether polyubiquitylation of FAT10 is required for its degradation or whether it becomes degraded in a ubiquitin-independent manner as has previously been shown for ornithine decarboxylase (Murakami et al., 1992). In the latter case, the covalent modification with FAT10 would be a cytokine inducible and self limiting alternative to polyubiquitylation as a marker for proteasomal degradation.

ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

(M.Hipp, unpublished)

Material and Methods

Expression constructs and recombinant proteins. The generation of the plasmid HA–Nub1long pCDNA3.1 and of recombinant FAT10-GST and GST have been described elsewhere (Hipp et al., 2004). The plasmids of the Nub1long deletion mutants were a kind gift of Petra Bochtler, Elvira Weber and Gunter Schmidtke. The generation and sequence of these mutants is described in the diploma theses of Petra Bochtler and Elvira Weber.

GST pull down assay. GST-FAT10 and GST proteins were incubated with Glutathione Sepharose 4B for 4h at 4°C on an end-over-end rotor. An aliquot was tested for similar binding of GST and GST-FAT10. After extensive washing *in vitro* transcribed/ translated and [^{35}S] labeled NUB1L or the indicated NUB1L mutants were added, and incubated over night at 4°C end-over-end. After four washes with RIPA-buffer (50mM Tris/HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS), the immunoprecipitate was analysed by SDS-PAGE/autoradiography on a Biorad radioimager with the Quantity One 4.1.1 software (Biorad, Hercules,CA).

Results and Discussion

Bacterial expressed human GST-FAT10 fusion protein was generated, as well as GST protein as negative control, bound to glutathione sepharose, and incubated over night with *in vitro* transcribed/ translated and [^{35}S] labeled NUB1L, or with one of several NUB1L mutants. In these mutants either the ubiquitin like domain (-ubl), or one of the UBA domains (-uba1, -uba2, -uba3) or all three UBA domains (-uba) is deleted.
As shown in figure 12A NUB1L specifically bound to the GST-FAT10 matrix but not to the GST control thus indicating that the observed interaction is specific for FAT10 and not due to unspecific binding of NUB1L to GST or the glutathione sepharose. Also the NUB1l mutant with the deleted ubiquitin like domain (-ubl) is still able to bind more than 50% of the FAT10 that is bound by the wildtype NUB1L. If instead

all three UBA domains are deleted, only about 10% of the UBA deleted NUB1L is bound.

Figure 12C shows a further analysis of the necessity of the UBA domains. If either the UBA domain 1 or 3 is missing only about 10% the mutants is bound compared with the wildtype. This is quite similar to the bound amount of the mutant where all three UBA domains are deleted.

If the UBA domain 2 is missing (-uba2) still more than 25% of the amount of the wildtype is bound. This indicates that the UBA2 domain plays only a minor role in the FAT10 – NUB1L interaction. It is noteworthy that NUB1, the NUB1L splicing variant described by Yeh and colleagues also does not have this UBA2 domain (Kito et al., 2001).

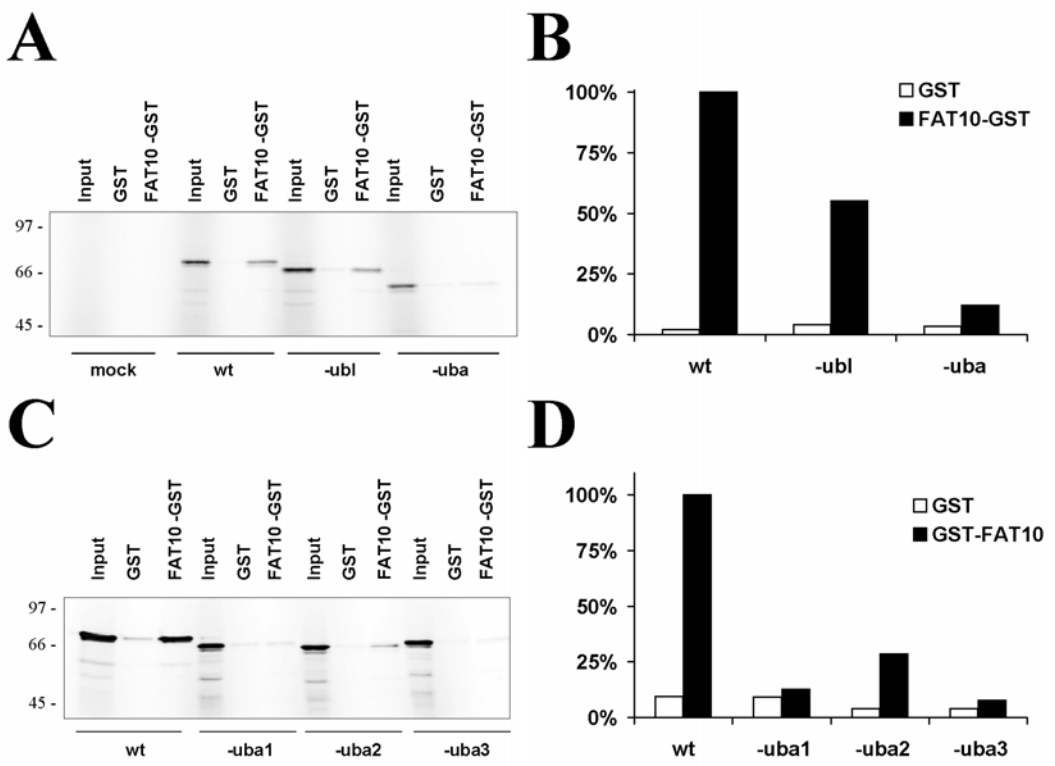

Fig. 12. The *in vitro* interaction of GST-FAT10 and NUB1L is dependent of the UBA domains UBA1 and UBA3. (A) Purified recombinant GST and GST-FAT10 proteins were bound to Glutathione Sepahrose™ 4B matrix and incubated with *in vitro* transcribed/ translated and [^{35}S] labeled wild type NUB1L (wt) or with one of several NUB1L mutants. NUB1L without the ubiquitin like domain is termed "-ubl". NUB1L without all three UBA domains is called "-uba". **(B)** The bands shown in (A) were quantified on a radioimager and plotted as per cent radioactivity based on values of the wild type activity pulled down. **(C)** The same GST pull down experiment as in (A) was performed but with NUB1L mutants where only one UBA domain is missing. NUB1L without the UBA domain 1, 2 or 3 is called "-uba1", "-uba2" or "-uba3" respectively. **(D)** The bands shown in (B) were quantified on a radioimager and plotted as per cent radioactivity based on values of the wild type activity pulled down.

The data presented here fit nicely into a model that is proposed for other UDPs with a ubiquitin-like domain and additional UBA domains, like Rad23/Rhp23 and Dsk2/Dph1. These proteins have the ability to interact with the 26S proteasome via their N-terminal UBL domains (Elsasser et al., 2002; Schauber et al., 1998), while using their C-terminal UBA domain for binding ubiquitin chains. This hints that they may function in the transport of ubiquitylated proteins to the 26S proteasome (HartmannPetersen et al., 2003b). NUB1L could be an interesting member in this group with the noteworthy difference that here the UBA domains, are responsible for the binding of UBLs like FAT10 and perhaps NEDD8 or for ubiquitin-precursors like UbC1 (Tanaka et al., 2004). While it is published for NEDD8 and UbC1 that only single UBA domains are involved in the protein-protein interaction (Tanaka et al., 2003; Tanaka et al., 2004), it seems that for the FAT10 – NUB1L interaction *in vitro* at least two domains are necessary (Figure 12). Unpublished in vivo coimmunoprecipitation studies even suggest that all three UBA domains are necessary for this interaction (Bochtler and Schmidtke, unpublished results).

Taken together the data presented here suggest a model where FAT10 degradation is accelerated by the binding of NUB1L that guides FAT10 faster to the proteasome. NUB1L would function as a carrier of FAT10 or FAT10 modified proteins (see next chapter) to the proteasome.

3.) FAT10 – A UBIQUITIN-INDEPENDENT SIGNAL FOR PROTEASOMAL DEGRADATION

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SUMMARY

FAT10 is a small ubiquitin-like modifier that is encoded in the major histocompatibility complex and is synergistically inducible by tumor necrosis factor-α and interferon-γ. It is composed of two ubiquitin-like domains and possesses a free C-terminal diglycine motif that is required for the formation of FAT10 conjugates. Here we show that unconjugated FAT10 and a FAT10 conjugate were rapidly degraded by the proteasome with a similar half-life of approximately one hour. Fusion of FAT10 to the N-terminus of very long-lived proteins reduced their half-lives as potently as achieved by fusion with ubiquitin. FAT10-green fluorescent protein fusion proteins were not cleaved but entirely degraded suggesting a lack of FAT10-specific deconjugating enzymes. Interestingly, the prevention of ubiquitylation of FAT10 by mutation of all lysines or by expression in ubiquitylation-deficient cells did not affect FAT10 degradation. Thus, conjugation with FAT10 is an alternative and ubiquitin-independent targeting mechanism for degradation by the proteasome, which in contrast to polyubiquitylation, is cytokine-inducible and irreversible.

INTRODUCTION

The ubiquitin-proteasome pathway is the main system for the targeted degradation of intracellular proteins (Voges et al., 1999). Depending on the metabolic and functional requirement of a cell, regulatory proteins like cell cycle regulators, transcription factors or key enzymes can be specifically selected for degradation by the 26S proteasome. The basis for selectivity does not lie in the protease itself, but rather in the selective covalent modification of target proteins with Lys-48 linked polyubiquitin chains. Polyubiquitylation is achieved by an enzymatic cascade of a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3). The specificity of substrate recognition is afforded by the numerous ubiquitin ligases which selectively bind a substrate as well as an E2 enzyme thus facilitating the formation of isopeptide bonds between the ε-amino group of lysines in a substrate protein and the diglycine motif at the carboxy-terminus of ubiquitin (Hershko and Ciechanover, 1998). Monomeric ubiquitin is processed from precursor proteins through ubiquitin specific proteases which recognize the C-terminal diglycine motif along with the ubiquitin domain and which cleave after the diglycine motif irrespective whether it is isopeptide-linked or linked through a conventional peptide bond (Wilkinson, 2000). Before degradation, the ubiquitin chains are removed from the substrate and disassembled into monomeric ubiquitin which can be reused. Ubiquitin levels are hence kept at a steady state level and the ubiquitin protein itself is long lived (Haas and Bright, 1987; Neff et al., 1981).

A growing number of proteins have been discovered over the past five years which contain domains with significant homology to ubiquitin. These ubiquitin-like proteins can be assigned either to the group of 'ubiquitin domain proteins' (UDPs) which contain a ubiquitin homology domain but which do not become covalently linked to target proteins or to the group of 'ubiquitin-like modifiers' (UBLs) which become isopeptide linked to target proteins (Jentsch and Pyrowolakis, 2000). Prominent members of the UBL family like SUMO, APG12 or ISG15 have been investigated in detail and specific E1, E2, and/or E3 enzymes have been identified. However, none of these modifiers target proteins for proteasomal degradation.

FAT10 is a fairly new member of the UBL family. It was recognized as a ubiquitin-like protein after chromosomal sequencing of the human MHC class I locus (Fan et

al., 1996). FAT10 is an 18 kD protein that consists of two ubiquitin-like domains with 29% and 36% identity to ubiquitin in its N- and C-terminal parts, respectively. Unlike other UBLs, FAT10 is synthesized with a free diglycine motif at its C-terminus which implies that it can become conjugated immediately after translation and folding. FAT10 is constitutively expressed in mature dendritic cells and B cells (Bates et al., 1997; Fan et al., 1996) but it is also inducible by the proinflammatory cytokines IFN-γ and TNF-α in cells of various tissue origins (Liu et al., 1999; Raasi et al., 1999). The ectopic expression of murine FAT10 led to the induction of caspase dependent apoptosis, which would be consistent with a role of FAT10 in the TNF-α mediated induction of apoptosis. Evidence that FAT10 becomes covalently linked to target proteins via its C-terminus was obtained in inducible FAT10 transfectants because in addition to monomeric FAT10, a prominent band of about 35 kD appeared upon FAT10 induction that was detected with FAT10-specific antibodies and resisted boiling in SDS under reducing conditions. This band was not observed when the diglycine motif of FAT10 was mutated thus strongly suggesting that it represented a covalent FAT10 conjugate (Raasi et al., 2001).

The functional consequences of FAT10 conjugation have so far not been thoroughly investigated but it is noteworthy that the inhibition of proteasome activity lead to an accumulation of FAT10 (Liu et al., 1999; Raasi et al., 2001). Moreover, we have recently identified a non-covalent interaction partner of FAT10 named NEDD8 ultimate buster1L (NUB-1L), that bound to the proteasome and markedly accelerated FAT10 degradation (Hipp et al., 2004). In this study we show that monomeric FAT10 as well as its conjugates are rapidly degraded by the proteasome in a ubiquitin-independent manner. Interestingly, ubiquitin and FAT10 turned out to be equally efficient at targeting long lived proteins for degradation, thus indicating that FAT10 is the first ubiquitin-like modifier which, like polyubiquitylation, functions as a protein signal for rapid degradation of substrate proteins through the proteasome.

MATERIALS AND METHODS

Cell lines, Tissue culture and Transfectants. The tetracycline inducible mouse FAT10 transfectant TB1N has been described before (Raasi et al., 2001). TB1N cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Invitrogen), 100 U/ml penicillin/streptomycin (Sigma, Taufkirchen, Germany), 400µg/ml hygromycin (Calbiochem, San Diego, CA), 5µg/ml puromycin (Calbiochem) and 1µg/ml tetracycline (Sigma). The epitheloid cervix carcinoma cell line HeLa and the human embryonic kidney line HEK293T were obtained from ATCC (Manassas, VA). Stimulation with 100U/ml recombinant human interferon-γ (Roche, Basel) and 100U/ml recombinant human tumor necrosis factor-α (Roche, Basel) was performed for at least 12 hours. The E1 thermosensitive cell line E36-ts20 and E36-ts20 cells retransfected with a wild type E1 (E36-ts20/E1) were kindly provided by M. Piechaczyk and have been described previously (Bossis et al., 2003; Handley-Gearhart et al., 1994; Kulka et al., 1988). E36-ts20 and E36-ts20/E1 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM**,** Invitrogen) supplemented with 20% FCS (Invitrogen) and 100 U/ml penicillin/streptomycin. Cells were kept at 32°C (permissive temperature for the ts20 cell line). For studies at the restrictive temperature, cells were incubated for 120 min at 42°C (to inactivate the E1 in the ts20 cells) and then switched to 39.5°C for up to 5h. For transfection experiments, HEK293T or E36-ts20 cells were grown in 100-mm dishes to 30-40% confluence before transfection with 5 µg cDNA/dish, using the Fugene reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol.

Chemicals and Antibodies. Lactacystin was purchased from Biomol (Plymouth Meeting, PA; PI-104), LLnL (Calpain Inhibitor I) from Roche (Mannheim, Germany), Iodacetamide and N-Ethyl-Maleimide from Sigma, and ubiquitin-aldehyde from Calbiochem (Darmstadt, Germany). The following antibodies were used: monoclonal anti-HA, clone HA-7(Sigma), monoclonal anti-β-galactosidase, clone GAL-13 (Sigma), monoclonal anti GFP-antibodies, clone 7.1 and 13.1 (Roche), monoclonal anti-His_6 (clone BMG-His-1 (Roche)), polyclonal anti-ubiquitin, code no. Z0458 (DAKO, Glostrup, Denmark) and monoclonal anti-HA high affinity matrix (Roche). Horseradish peroxidase coupled secondary antibodies were purchased from DAKO.

Generation of FAT10 polyclonal Antibodies. A FAT10 specific polyclonal antibody was raised in rabbits by immunization with a GST-FAT10 recombinant protein. For production of the GST-FAT10 fusion protein, the FAT10 cDNA was retro-transcribed from mRNA of JY B cells and amplified, using primers sense 5'-CCATGGATCCATGGCTCCCAATGCTTCCTGCCTC-3' and antisense 5'-CCGTCTCGAGTCTCACCCTCCAATACAATAAGATGC-3' and cloned via BamHI and XhoI sites into the expression vector pGEX-4T-3 (Amersham Biosciences, Uppsala). BL21 cells were transformed with this construct and the GST-FAT10 fusion protein was induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) and purified by Glutathione Sepharose™ 4B column chromatography (Amersham). The GST-FAT10 fusion protein (5mg) was used for immunisation of four different rabbits. One of the raised antibodies was very specific for detection of human and mouse FAT10 in western analysis and immunoprecipitation.

Pulse-Chase Experiments and Immunoprecipitation. Pulse chase experiments and immunoprecipitations were performed as previously described (Hipp et al., 2004). In the immunoprecipitation experiments shown in figure 16 and 17, we included 50 mg/ml ubiquitin aldehyde, 25 mM iodoacetamide, 25 mM N-Ethyl-Maleimide, and 5 mM EDTA in the lysis and washing buffers in order to inhibit ubiquitin specific proteases.

Plasmids and Generation of Expression Constructs. The plasmids pcDNA3.1/HA-FAT10, pcDNA3.1/His_6-FAT10, pcDNA3.1/HA-Ub, pcDNA3.1/HA-UbK48R, and pcDNA3.1/HA-UbK48RΔGG have been described previously (Hipp et al., 2004; Raasi et al., 2001). The vector pEGFP N1 was purchased from Clontech (Heidelberg, Germany). The generation of the vectors encoding HA-Fat10-GG-GFP, HA-Fat10-AV-GFP, HA–Ubiquitin-AV-GFP, HA-Sumo-AV-GFP, and HA–Nub1long pCDNA3.1 has been described elsewhere (Hipp et al., 2004). The vector containing DHFR-HA-UBK48R was a kind gift from F. Levi, Lausanne (Levy et al., 1996). HA-Fat10-AV-DHFR, and HA–Ubiquitin-AV-DHFR were generated by PCR amplification of DHFR and replacement of the GFP-gene in the corresponding vectors

by the DHFR-gene using conventional cloning methods. The vector expressing HA-Ubiquitin K48R was generated by replacing ubiquitin with PCR amplified ubiquitin K48R using pcDNA3.1/HA-UbK48R as template. The mutant of fat10 in which all lysines were replaced by arginines (HA-FAT10-K0) was generated by consecutive site directed mutagenesis using PCR; the sequences of the 18 primers used for this purpose are made available by us upon request. All sequences were verified by dideoxy sequencing.

RESULTS

FAT10 and its endogenous conjugates are rapidly degraded by the proteasome

Previously we and others have shown that the inhibition of proteasome activity resulted in an accumulation of FAT10 suggesting that FAT10 is rapidly degraded by the proteasome (Liu et al., 1999; Raasi et al., 2001). To determine the half-life of FAT10, we performed a pulse chase experiment with the inducible FAT10 transfectant TB1N. The induction of FAT10 in this cell line leads to the appearance of an additional major band at 35 kD which we have previously shown to be a covalent FAT10 conjugate (Raasi et al., 2001). FAT10 was induced for 24h by tetracycline removal and the cells were then labeled for one hour and chased for the indicated time periods (Figure 13A). Quantification of the FAT10 monomer band on a radioimager demonstrated that the half life of FAT10 was 0.9 hours and was prolonged to 3.3 hours when the cells were chased in the presence of 80μM of the proteasome inhibitor lactacystin (Figure 13B). Interestingly, the 35 kD FAT10 conjugate which was apparent in the induced but not in the uninduced cells, had a similar half life as FAT10 itself (t1/2 of 1.4 h) and was similarily stabilized by lactacystin treatment (t1/2 of 3.4 h). Since our quantifications provided no evidence for the release of FAT10 from the conjugate over time, the data strongly suggests that both, FAT10 and its target protein were degraded by the proteasome.

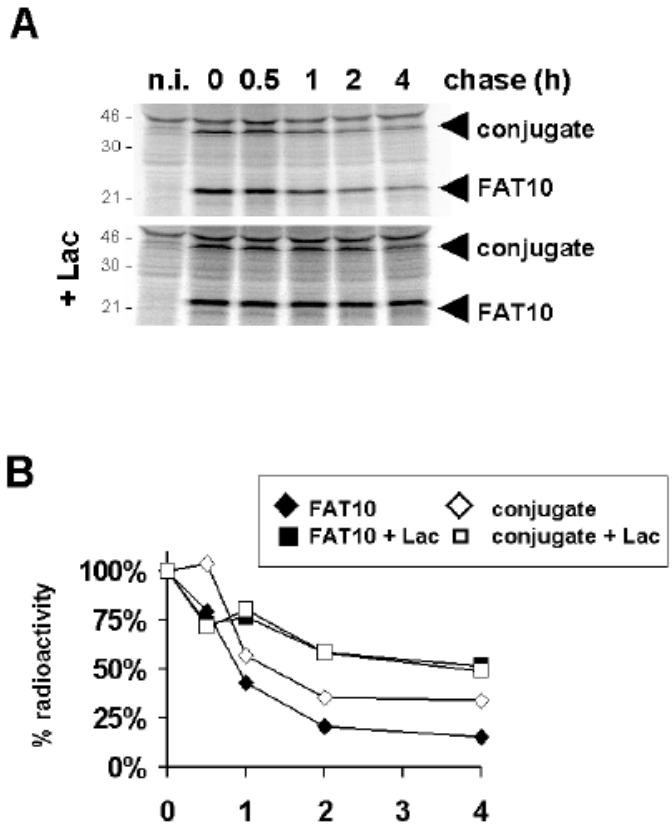

Figure 13. FAT10 and FAT10-conjugate have a short half life and are degraded in a proteasome dependent manner. (A) The tetracycline inducible FAT10 transfectant TB1N was labeled with ^{35}S Met/Cys and chased for indicated time periods in the absence (top panel) or presence (bottom panel) of 80μM lactacystin (+Lac) followed by immunoprecipitation against HA-FAT10. Lane 1 represents non induced TB1N cells (n.i.). **(B)** The bands shown in (A) were quantified on a radioimager and plotted as per cent radioactivity based on values obtained after the pulse.

FAT10 and ubiquitin are equally potent at reducing the half life of long-lived proteins when fused to their N-termini

Since FAT10 and its conjugate were both degraded by the proteasome at a similar rate, we decided to test whether FAT10 may function as a degradation signal. The N-terminal fusion of ubiquitin to long lived proteins is known to markedly reduce their half lives (Johnson et al., 1992). This requires, however, that the two C-terminal glycine residues are replaced by different amino acids because otherwise ubiquitin-specific proteases will rapidly cleave off the ubiquitin moiety. Hence, we created expression constructs for fusion proteins between the long lived green fluorescent protein (GFP) at the C-terminus and either HA-tagged FAT10, ubiquitin, or SUMO-1

at the N-terminus. In these constructs the diglycine motif was replaced with Ala and Val and for SUMO-1 the C-terminal extension was removed. The constructs were transiently transfected into HeLa cells and the degradation of the three fusion proteins as well as GFP alone was monitored in pulse chase experiments (Figure 14A, top panel). As expected, GFP was not degraded within 7 hours and also the HA-SUMO-AV-GFP fusion protein, which served as our negative control, remained stable. Interestingly, quantification of the bands with a radioimager revealed that FAT10 and ubiquitin were equally potent in targeting GFP for fast degradation (Figure 14A, bottom panel). This demonstrates that FAT10, like ubiquitin, but in contrast to SUMO-1 and other UBLs, functions as a degradation signal.

Subsequently, we tested whether this property of FAT10 is also observed with another long-lived protein and chose dihydrofolate reductase (DHFR) for this purpose. We generated HA-FAT10-AV-DHFR expression constructs and compared the half life of the encoded fusion protein to that of HA-Ub-AV-DHFR as a positive control. As a negative control we used a DHFR-HA-UbK48R construct because it has been shown that the attachment of a single ubiquitin to the C-terminus of DHFR does not lead to accelerated degradation (Levy et al., 1996; Turner and Varshavsky, 2000). A quantitative evaluation of the experiment shown in figure 14B and two additional experiments indicated that the N-terminal fusion of FAT10 and ubiquitin similarly reduced the half life of DHFR.

NUB1L targets a FAT10-AV-GFP fusion protein but not Ubiquitin-AV-GFP for accelerated degradation

Previously, we have shown that the coexpression of NUB1L caused a marked acceleration of FAT10 degradation (Hipp et al., 2004). In order to examine whether fusion proteins containing FAT10 were likewise degraded in an accelerated manner, we performed a similar pulse chase experiment as described above but this time HeLa cells were transiently transfected with a FAT10-AV-GFP plasmid either alone or together with an expression construct for HA-NUB1L (Hipp et al., 2004). Interestingly, the coexpression of NUB1L markedly accelerated the degradation of FAT10-AV-GFP as revealed by the quantitative analysis of three independent experiments (Figure 14C). Moreover, a similar NUB1L-dependent acceleration of FAT10-AV-GFP degradation was observed when the same transfection experiments

were performed in HEK293T cells (data not shown). Evidently, NUB1L also accelerated the degradation of FAT10 when it was covalently linked to another protein. This function appeared to be FAT10 specific, as NUB1L co-expression had virtually no effect on the proteolysis of a Ub-AV-GFP fusion protein (Figure 14C).

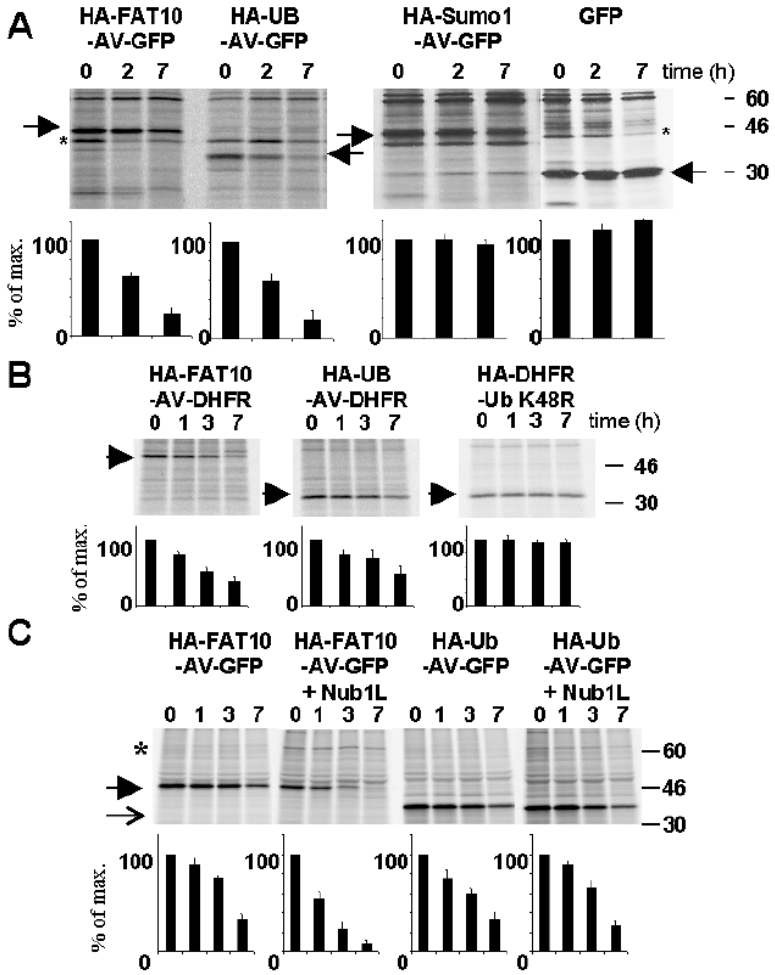

Figure 14. FAT10 and ubiquitin are equally potent in targeting for degradation. **(A)** FAT10, SUMO, and ubiquitin were transiently expressed as uncleavable AV/GG mutated and HA-tagged GFP fusion proteins in HeLa cells. The cells were labeled for 1h with ^{35}S Cys/Met, and chased for the indicated time periods prior to HA-specific immunoprecipitation, SDS/PAGE, autoradiography, and quantification on an radioimager. As a control, GFP was expressed and immunoprecipitated with an anti-GFP antibody. The arrowhead denote the different GFP fusion proteins and GFP as indicated above, an asterisk labels an unspecific band (**B)** Fusion proteins of DHFR with HA-tagged FAT10 and ubiquitin as well as the DHFR-HA-UbK48R control were expressed in HeLa cells and the pulse chase experiment was performed as in (A). The arrowheads denote the respective DHFR fusion proteins as indicated above the panels. **(C)** HA-FAT10-GFP and HA-ubiquitin-GFP were transiently expressed in HeLa cells without or together with HA-NUB1L. A pulse chase analysis was performed as in (A) and (B). An arrow denotes HA-Ub-AV-GFP, an arrowhead denotes HAFAT10-AV-GFP. All bands were quantified on a radioimager and plotted as per cent radioactivity based on values obtained after the pulse below each panel. The data represent means of three independent experiments +/- SEM.

No evidence for a FAT10-specific protease

In order to investigate whether a FAT10 specific protease exists, we compared two FAT10-GFP fusion proteins, one with the regular diglycine C-terminus of FAT10 and another one where the diglycine motif was replaced by Ala and Val. This mutation can abolish the cleavage at the C-terminus of most other UBLs and ubiquitin itself (Wilkinson, 2000). HeLa cells were transiently transfected with GFP and the two FAT10-GFP fusion proteins and the fate of these proteins was analyzed in pulse chase experiments. Both FAT10-GFP fusion proteins were degraded with the same kinetics and neither GFP nor FAT10 was released from the HA-FAT10-GG-GFP fusion protein. This result indicates that a FAT10-specific processing protease which ought to remove FAT10 from isopeptide-linked or N-terminally fused target proteins was not active neither in untreated (Figure 15) nor in IFN-γ treated HeLa or HEK293T cells (data not shown).

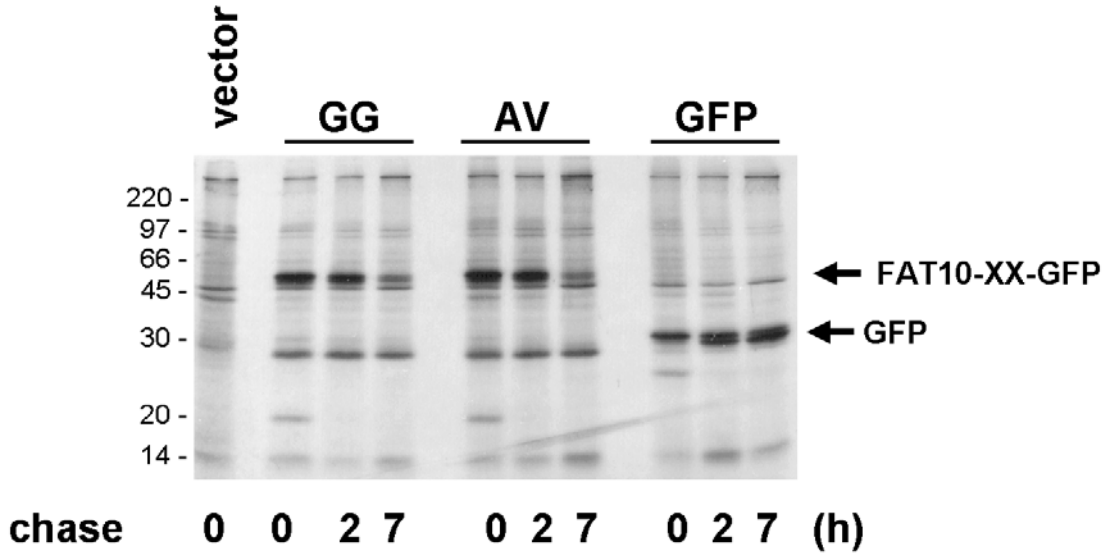

Figure 15. Lack of evidence for a FAT10 specific processing protease. GFP and FAT10-GFP fusion proteins with either wild type FAT10 or with an GG to AV mutation of the FAT10 C-terminus were transiently expressed in HeLa cells. The cells were labeled for 1h with ^{35}S Cys/Met, and chased for the indicated time periods prior to GFP-specific immunoprecipitation, SDS/PAGE and autoradiography. The leftmost lane is a vector control.

Determination of the ubiquitylation status of FAT10

Next we investigated whether targeting to the proteasome is mediated by the two ubiquitin-like domains of FAT10 itself, or whether FAT10 needs to be polyubiquitylated for degradation by the 26S proteasome. For these and other experiments we generated a polyclonal antibody by immunizing rabbits with a GST-FAT10 fusion protein. This antibody prominently detected HA-FAT10 in immunoblots of HEK293T cells that were transiently transfected with an HA-FAT10 expression construct but not in untransfected cells (Figure 16A). To determine the ubiquitylation status of FAT10, we transiently expressed human His_6-FAT10 in HEK293T cells in the absence or presence of HA-tagged ubiquitin in its wild type form, in a K48R mutant or a K48R/ΔGG double-mutant form. After treatment with the proteasome inhibitor LLnL, FAT10 was immunoprecipitated and the amount of FAT10 was determined by anti-His_6 western blot. In addition, ubiquitin-FAT10 conjugates were visualized by anti-HA western blot (Figure 16B). From the anti-His_6 western blot it was apparent that roughly the same amount of FAT10 was immunoprecipitated in all transfection experiments. The anti-HA western blot revealed that bands became visible that correspond to one, two, and three ubiquitin molecules linked to FAT10, but high molecular weight polyubiquitin conjugates were not very prominent. Since none of the ubiquitin-conjugates were visible when HA-UbK48R/ΔGG was coexpressed, we can be confident that the apparent bands do not represent ubiquitin molecules that have themselves been modified by FAT10 conjugation.

The low level of polyubiquitylated FAT10 in figure 16B casted some doubt whether FAT10 needs to be polyubiquitylated for proteasomal degradation. Moreover, when we examined the degree of polyubiquitylation of GFP fusion proteins immunoprecipitated from HEK293T cells transiently transfected with expression constructs for FAT10-AV-GFP, Ub-AV-GFP, SUMO-AV-GFP, UbK48R-AV-GFP, and GFP (Figure 16C), we noted that the degree of polyubiquitylation of the respective fusion proteins did not correlate with their rate of degradation (Figure 14A). Hence we decided to investigate whether the degradation of FAT10 could be ubiquitin-independent.

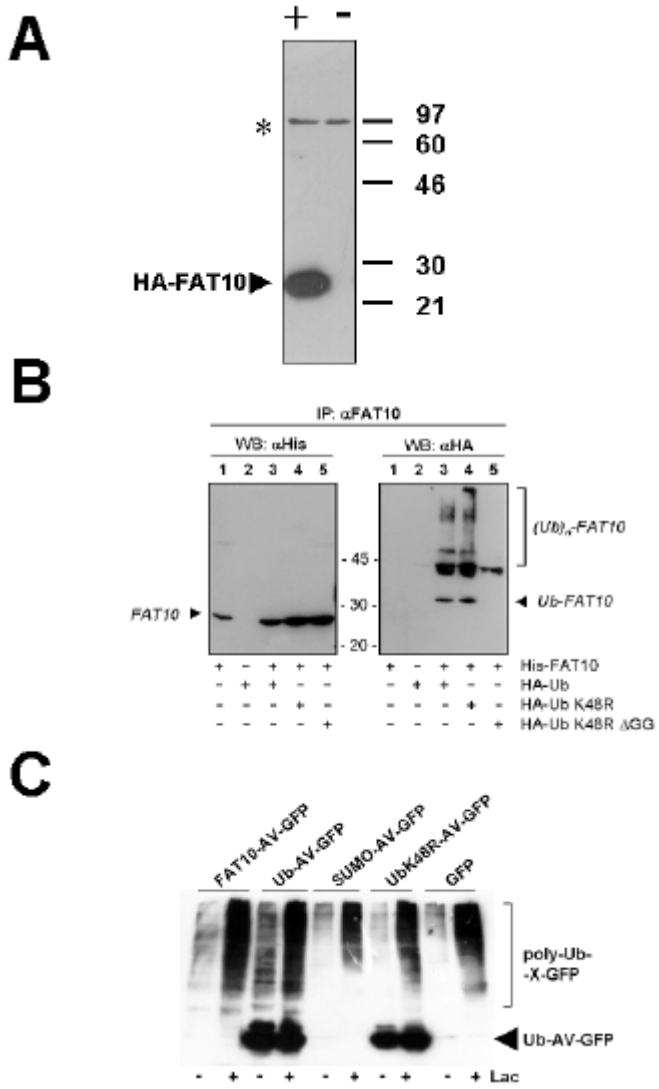

Figure 16. Ubiquitylation status of FAT10. (A) Characterization of a polyclonal anti human FAT10 antibody. HEK293 cells were transiently transfected with an HA-FAT10 expression construct (+) or a vector control (-). A western blot with a polyclonal antibody raised in rabbits against a GST-FAT10 fusion protein was then performed. The HA-FAT10 protein is labeled with an arrowhead, an unspecific band also present in untransfected cells is indicated with an asterisk **(B)** HEK293T cells were transiently transfected with His_6-FAT10, HA-Ub, HA-UbK48R or HA-UbK48RΔGG. Prior to lysis, cells were incubated with 100µM LLnL for 6 hours. After an immunoprecipitation (IP) with the anti-FAT10 characterized in (A), the precipitates were analyzed by western-blot with either anti-His_6 (left panel) or anti-HA (right panel) antibodies. **(C)** HEK293T cells were transiently transfected with constructs encoding FAT10-AV-GFP, Ubiquitin-AV-GFP, SUMO-AV-GFP, UbK48R-AV-GFP or GFP. Four hours before lysis cells were treated with 50µM of the proteasome inhibitor lactacystin (Lac) where indicated. Lysates were immunoprecipitated with anti-GFP antibody and immunoprecipitates analysed by western blot with anti-ubiquitin antibody. The arrowhead indicates the signal for Ub-AV-GFP and UbK48-AV-GFP, polyubiquitin conjugates of the respective proteins are labeled poly-Ub-X-GFP.

Ubiquitylation of FAT10 is not necessary for FAT10 degradation

An expression construct was generated in which all 17 lysines of human FAT10 were replaced by arginines (designated HA-FAT10-K0). HA-FAT10 and HA-FAT10-KO were transiently expressed in HEK293T cells and their half-life was determined in a pulse chase experiment (Figure 17A). A quantitative evaluation of the results revealed that the degradation rate of HA-FAT10 and HA-FAT10-K0 was identical. Moreover, the coexpression of NUB1L accelerated HA-FAT10-K0 degradation to the same extent as we had shown before for wild type FAT10 (Hipp et al., 2004) and the FAT10-AV-GFP fusion protein (Figure 14C). The degradation of HA-FAT10-K0 also appeared to be proteasome dependent, as the protein could be stabilized with the proteasome inhibitor lactacystin even in the presence of NUB1L (Figure 17A, bottom).

A concern with these experiments could be that HA-FAT10-K0 may not be folded properly. Several observations argue against this possibility. First, we could efficiently immunoprecipitate HA-FAT10-K0 with our polyclonal anti-FAT10 antibody from transiently transfected HEK293T cells (Figure 17B, first lane from the left). Second, NUB1L was as efficiently co-immunoprecipitated with HA-FAT10 as with HA-FAT10-K0 from transiently transfected HEK293T cells (Figure 17B, second and sixth lane from the left). Third, NUB1L accelerated HA-FAT10-K0 degradation as efficiently as that of HA-FAT10 (Figure 17A and (Hipp et al., 2004)). It is hence unlikely that the rapid degradation of HA-FAT10-K0 is caused by misfolding.

In general, lysine residues are required for the conjugation of substrate proteins with ubiquitin. In some proteins, however, the amino-termini were found to be ubiquitylated (Ciechanover and Ben-Saadon, 2004). To test whether the HA-FAT10-K0 protein can be ubiquitylated, we expressed HA-FAT10 or HA-FAT10-K0 alone or together with HA-ubiquitin in transiently transfected HEK293T cells. FAT10 was immunoprecipitated with an anti-FAT10 antibody and the immunoprecipitates were analysed by immunoblot with an anti-HA antibody. Although similar amounts of the FAT10 proteins were expressed, and although polyubiquitin conjugates were readily detected in the lysates of the transfected cells (Figure 17C bottom), we detected mono- and polyubiquitylation only for HA-FAT10 but not for HA-FAT10-K0 even after prolonged exposure (Figure 17C top). This indicates that ubiquitin conjugation of FAT10 is not required for its proteasome-dependent degradation.

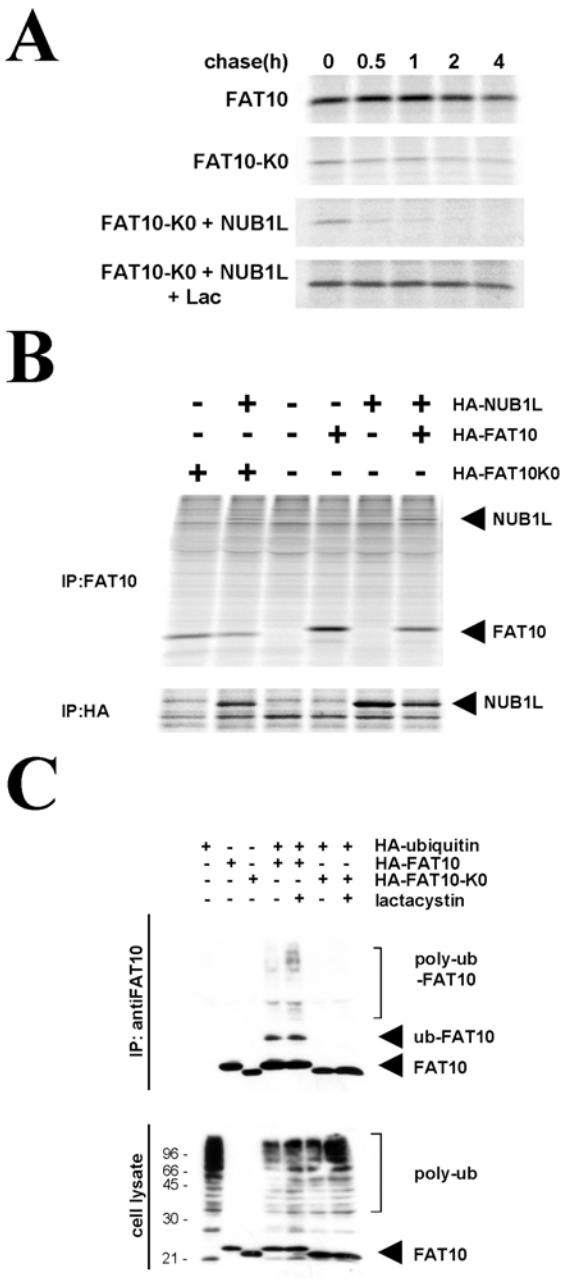

Figure 17. Ubiquitylation of FAT10 is not necessary for FAT10 degradation. (A) FAT10, a lysineless FAT10 mutant (FAT10-K0), and NUB1L were transiently expressed as HA-tagged proteins in HEK293T cells and treated with 50μM of lactacystin (Lac) during the labeling and the chase where indicated. The cells were labeled for 1h with ^{35}S Cys/Met, and chased for the indicated time periods prior to HA-specific immunoprecipitation, SDS/PAGE, and autoradiography. **(B)** HEK293T cells were transfected with HA-FAT10, HA-FAT10-K0 and HA-NUB1L as indicated on the top. After labeling, lysates were immunoprecipitated with anti-FAT10 or anti-HA antibodies as indicated, and analysed by SDS/PAGE and autoradiography. **(C)** HEK293T cells were transfected with HA-FAT10, HA-FAT10-K0, and HA-ubiquitin as indicated on the top. Four hours before lysis cells were treated with 50μM of lactacystin where indicated. In the upper panel lysates were immunoprecipitated with anti-FAT10 antibody and immunoprecipitates were analyzed by western blot with anti-HA antibody. The lower panel shows an anti-HA western blot of total lysates.

FAT10 degradation occurs normally in E1 temperature sensitive mutants

A prerequisite for ubiquitin conjugation is the ATP-dependent activation of the carboxy terminus of ubiquitin by the ubiquitin-activating enzyme (E1). An extensively characterized mutant cell line which expresses a temperature sensitive form of E1 is the Chinese hamster ovary line E36-ts20 (Kulka et al., 1988). We used this mutant cell line and E36-ts20/E1 cells that are reconstituted with a transfected wild type E1 enzyme to investigate if the degradation of FAT10 or FAT10-GFP fusion proteins is in fact independent of ubiquitin conjugation to FAT10. For this purpose, E36-ts20 and E36-ts20/E1 cells were transiently transfected with a FAT10-AV-GFP expression construct and a pulse chase experiment was performed at the restrictive temperature (Figure 18A) and quantitatively evaluated (Figure 18C). Interestingly, the FAT10-AV-GFP protein was degraded at the same rate in both cell types, indicating that FAT10-AV-GFP catabolism occurred even when ubiquitin conjugation was defective (Handley-Gearhart et al., 1994). To confirm that the E36-ts20 line was defective in ubiquitylation, we transfected E36-ts20 and E36-ts20/E1 cells with an HA-DHFR-ubiquitin-arg-β-galactosidase expression plasmid. This construct encodes a fusion protein which is cleaved by ubiquitin specific proteases into a stable N-terminal HA-DHFR-ubiquitin part that was used as a transfection control (data not shown) and a C-terminal arg-β-galactosidase (arg-β-gal) part which is an N-end rule substrate and therefore rapidly degraded in a ubiquitin-dependent manner (Levy et al., 1996). As shown in figure 18B and C, the arg-β-gal protein was stable in the E36-ts20 mutant while it was degraded in the E36-ts20/E1 transfectant. Taken together, these results agree with the data obtained with the lysine-deficient FAT10 mutant (Figure 17) in that they establish FAT10 as a ubiquitin-like modifier that can target proteins for degradation by the proteasome without the need for polyubiquitylation.

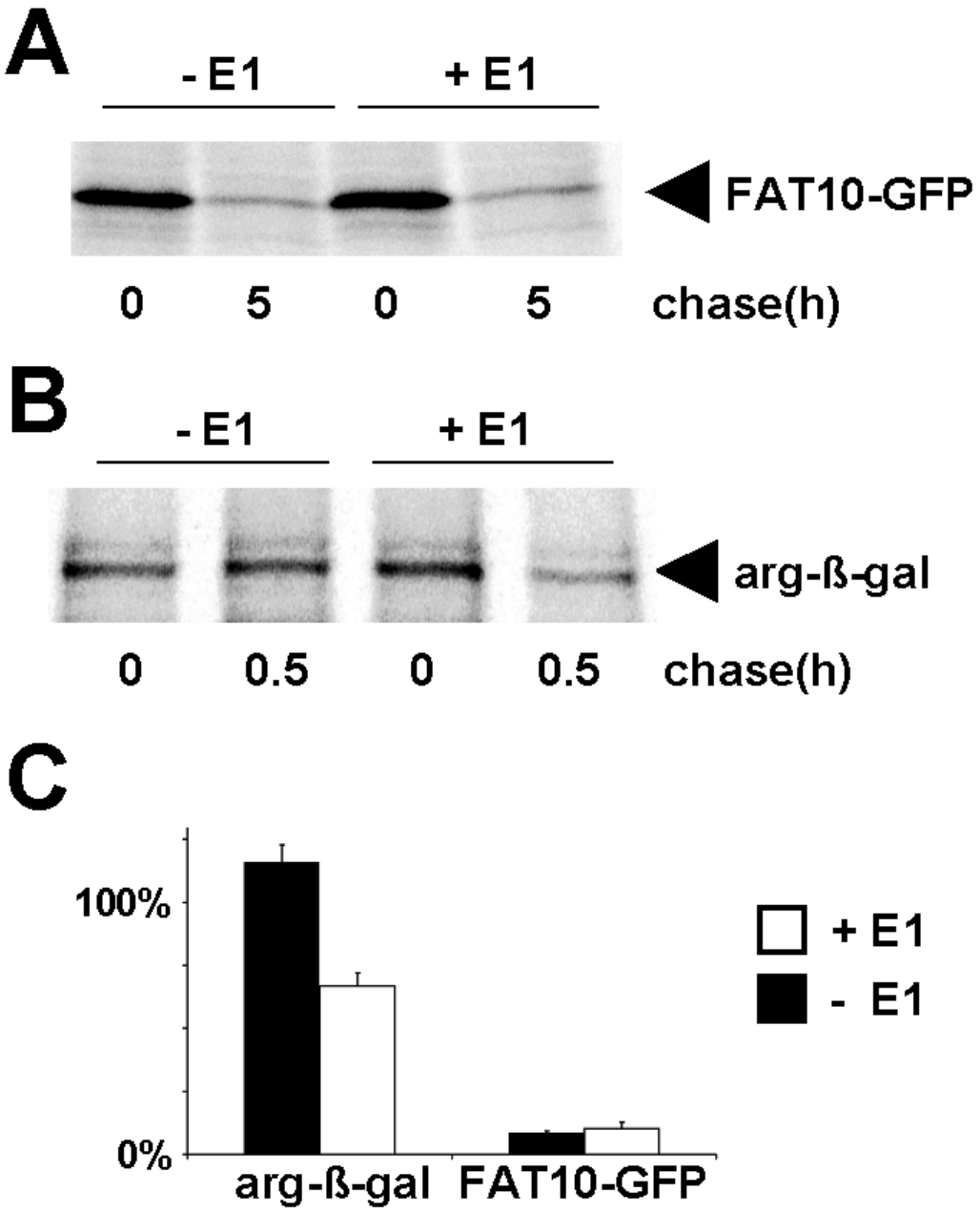

Figure 18. Degradation of FAT10 is ubiquitin independent. (A) The E1 thermosensitive cell line E36-ts20 (-E1) and E36-ts20 cells retransfected with a wild type E1 (+E1) were transiently transfected with HA-tagged FAT10-AV-GFP. After inactivation of the thermolabile E1 due to temperature shift, the cells were labeled for 1h with ^{35}S Cys/Met, and chased for the indicated time periods prior to HA-specific immunoprecipitation, SDS/PAGE, and autoradiography. **(B)** Similar experiments were performed transfecting a shortlived HADHFR-ubiquitin-arg-ß-galactosidase construct. The immunoprecipitation was performed with an anti-ß-gal antibody. **(C)** The bands were quantified on a radioimager and plotted as per cent radioactivity based on values of the pulsed cells. The means of three independent experiments are shown, error bars indicate the standard error of the mean.

DISCUSSION

When ubiquitin-like modifiers (UBLs) are described it is usually emphasized that they form isopeptide linkages with target proteins just like ubiquitin, but that they do not serve proteolytic functions. In this work we show that FAT10 is the first UBL which, like ubiquitin, targets conjugated proteins for degradation through the proteasome. Similar to ubiquitin, FAT10 served as a degradation signal when it was fused to the N-terminus of two long lived proteins and it is remarkable that FAT10 was as potent as ubiquitin in accelerating their proteolysis (Figure 14A). Since proteasomal targeting by FAT10 did not rely on the ubiquitin conjugation system, modification with a single FAT10 molecule appears to be an independent alternative to polyubiquitylation.

When we initially observed that FAT10 can act as a transferable degradation tag, two scenarios seemed possible: Firstly, FAT10 could, like other degrons, bind to an E3 ubiquitin ligase and by that means initiate the assembly of polyubiquitin chains, and, secondly, FAT10 itself may act like polyubiquitin in functioning as a proteasome targeting signal. First we addressed the question whether FAT10 is ubiquitylated in cells and we observed that FAT10 was primarily modified with only 1-3 copies of ubiquitin that do not mediate an efficient targeting to the 26S proteasome (Thrower et al., 2000) (Figure 16B). This was an incentive for us to further investigate the ubiquitin-dependence of FAT10 degradation.

Subsequent experiments revealed that FAT10 is rapidly degraded by the proteasome without requiring the ubiquitylation system in general, and polyubiquitylation of FAT10 itself in particular. In figure 18, we compared the degradation rate of FAT10-AV-GFP in E36-ts20 cells that express a temperature sensitive ubiquitin activating enzyme E1 with the degradation rate in the reconstituted E1 transfectant E36-ts20/E1. Consistently, we observed no differences in FAT10-AV-GFP degradation between E36-ts20 and E36-ts20/E1 cells. It should be emphasized though, that while ubiquitin conjugation in E36-ts20 is largely deficient at the restrictive temperature, some 20% of polyubiquitin conjugate formation remains that, nevertheless, did not suffice to maintain viability or to degrade the bulk of short lived proteins (Gropper, 1991; Handley-Gearhart et al., 1994; Kulka et al., 1988). Since FAT10-AV-GFP degradation occurred at a normal pace in these mutants, it is fair to conclude that FAT10 degradation does not rely on polyubiquitylation in any similar extent as other

short lived proteins like p53 (Chowdary et al., 1994) or FOS (Stancovski et al., 1995). We can not, however, rule out the unlikely scenario that the 20% polyubiquitylation capacity remaining at the restrictive temperature contributed to FAT10 degradation.

To further investigate this issue, we generated a FAT10 mutant in which all 17 lysines were replaced by arginines. This mutant protein, designated HA-FAT10-K0, bound normally to anti-FAT10 polyclonal antibodies and to the FAT10 interacting protein NUB1L (Figure 17B), arguing that HA-FAT10-K0 was properly folded. Since HA-FAT10-K0 was degraded at the same rate as HA-FAT10, both in the presence and absence of NUB1L, we conclude that isopeptide linkage to ubiquitin at lysine residues was not required for HA-FAT10-K0 degradation. In a few cases, however, the α-amino group at the N-terminus of proteins was shown to be linked to the C-terminus of ubiquitin by formation of a conventional peptide bond (Ciechanover and Ben-Saadon, 2004). Lysine-less variants of proteins have been shown to be polyubiquitylated at the N-terminus and degraded, although in some of these proteins the degradation was significantly slowed down when all lysines were mutated. We found that wild type HA-FAT10 was ubiquitylated but no ubiquitylation was detected for the lysine-less variant HA-FAT10-K0 neither in the absence nor in the presence of proteasome inhibitors (Figure 17C). One could argue that a very minor portion of HA-FAT10-K0 was ubiquitylated that escaped our detection but if this was the case, such trace amounts are very unlikely to mediate HA-FAT10-K0 degradation at the same efficiency as lysine proficient HA-FAT10. Moreover, the HA-FAT10-K0 protein bears an HA tag at the N-terminus which most likely is acetylated since it bears an alanine in position 2 that, after cleavage of the N-terminal methionine by methionine aminopeptidases, is predicted to become acetylated by the N-terminal acetyltransferase type A (Polevoda and Sherman, 2003). N-terminal acetylation interferes with N-terminal ubiquitylation and is hence another strong argument against N-terminal ubiquitylation of HA-FAT10-K0. Taken together, the data shown in figures 17 and 18 indicates that FAT10 and FAT10 conjugated proteins can be efficiently degraded in a ubiquitin-independent manner.

These results raise the question how FAT10 can mediate degradation by the proteasome. Ubiquitin independent degradation by the proteasome has been described before for ornithine decarboxylase (Murakami et al., 1992) which is targeted to the 26S proteasome by a polyamine induced protein called antizyme 1 (Zhang et al.,

2003). Also the cell cycle inhibitor p21 appears to be degraded by the proteasome without the need for ubiquitylation (Sheaff et al., 2000), as unmodified p21 can be efficiently degraded by the 20S and 26S proteasome *in vitro* (Liu et al., 2003).

Ubiquitin-like domains can mediate binding to the 26S proteasome as has been demonstrated for the UDPs Rad23, Dsk2, and BAG-1 (Elsasser et al., 2002; Funakoshi et al., 2002; Luders et al., 2000; Schauber et al., 1998). Rad23 and Dsk2 may act as adaptor proteins in that they bind to the Rpn1 subunit of the 26S proteasome through their ubiquitin-like domain and to polyubiquitin chains through their UBA domains. In this respect it is intriguing that we recently identified NUB1L as a non-covalent interaction partner of FAT10 (Hipp et al., 2004). NUB1L contains three UBA domains in its C-terminal part and a ubiquitin-like domain at the N-terminus (Hipp et al., 2004; Tanaka et al., 2003). In addition, NUB1L was found to be associated with the 26S proteasome and to bind to the 19S regulator subunit Rpn10 *in vitro* (Kamitani et al., 2001). Given that NUB1L, which is also interferon-γ inducible (Kito et al., 2001), markedly accelerated the degradation of FAT10 (Hipp et al., 2004) and FAT10 fusion proteins (Figure 14C), it is attractive to hypothesize that NUB1L functions as an adaptor that ties FAT10 and FAT10 conjugated proteins to the proteasome. A recent study demonstrated that proteins that were targeted to the proteasome with an artificial tagging system were rapidly degraded by the proteasome (Janse et al., 2004). Proximity to the proteasome may therefore be sufficient for degradation and polyubiquitin chains may not be the only signal for proteasomal degradation.

A striking difference between ubiquitin and FAT10 is their rate of turnover. Although investigations on the half life of ubiquitin yielded quite different results ranging from 320 hours (Neff et al., 1981) to 28 hours (Haas and Bright, 1987), it is clear that ubiquitin is much more stable than FAT10 for which we determined a half life of about 1 hour in human and murine cells. Since FAT10 conjugates are degraded at the same speed as monomeric FAT10 and since both are similarly stabilized through lactacystin we consider it very likely that FAT10 is being degraded along with its substrate. This notion is supported by the observation that monomeric FAT10 did not accumulate during our pulse chase experiments (Figure 13) thus indicating that FAT10 was not liberated from FAT10 conjugates. Liberation of FAT10 from its conjugates would require the existence of FAT10-specific proteases. Using FAT10-

GFP fusion proteins we have examined this possibility, but no evidence for cleavage of the fusion protein could be obtained (Figure 15). A lack of FAT10-specific processing proteases would be in striking contrast to ubiquitin, NEDD8, SUMO-1, or ISG15 for which specific processing proteases have been described. This feature would, however, be in accordance with another unique trait of FAT10, i.e. the direct biosynthesis with a free diglycine motif at the C-terminus which does not need further processing before conjugation. Interestingly, in mutants that lack the proteasome associated deubiquitinating enzymes Ubp6 or Doa4, the half life of ubiquitin is dramatically shortened which was attributed to a failure to remove ubiquitin from polyubiquitylated substrates before they are degraded by the 26S proteasome (Leggett et al., 2002; Swaminathan et al., 1999). In these mutants ubiquitin appears to have roughly the same half life and fate as FAT10.

The rapid proteolysis of FAT10 conjugates may also explain why it has been difficult to detect these conjugates. Liu et al. reported that using a FAT10 specific antibody, they did not detect proteins that could represent FAT10 conjugates (Liu et al., 1999). The first time we could detect a FAT10 conjugate with the molecular mass of about 35 kD was after the tetracycline induced overexpression of HA-FAT10 in mouse fibroblasts (Raasi et al., 2001). Recently, Lee *et al.* published that FAT10 expression was markedly upregulated in 90% of hepatocellular carcinoma tissue as well as several tumors of the gastrointestinal tract and female reproductive organs (Lee et al., 2003). Interestingly, the anti-FAT10 antibody generated in that study also detected a 35 kD band in addition to monomeric FAT10 in NIH3T3 fibroblasts. It will now be crucial to identify FAT10 conjugated target proteins in order to learn more about the biological functions of FAT10 and to understand why these proteins are rapidly and irreversibly targeted for proteasomal degradation without the involvement of the ubiquitin system.

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SUPPLEMENTARY DATA

(M.Hipp, unpublished)

Materials and Methods

Cell lines, Tissue culture and Transfectants

The tetracycline inducible mouse FAT10 and FAT10ΔGG fibroblast transfectants TB1N and mTB14 have been described previously (Raasi et al., 2001). TB1N and mTB14 cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM, Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (Invitrogen), 100 U/ml penicillin/streptomycin, 400µg/ml hygromycin (Calbiochem, San Diego, CA), 5µg/ml puromycin (Calbiochem) and 1µg/ml tetracycline (Sigma, Taufkirchen, Germany). The colon carcinoma cell line SW620 was obtained from ATCC (Manassas, VA) and grown in IMDM supplemented with 10% FCS, 2mM L-glutamine, 100 U/ml penicillin/streptomycin. Stimulation with 100U/ml recombinant human interferon-γ (Roche, Basel) and 100U/ml recombinant human tumor necrosis factor-α (Roche, Basel) was performed for at least 12 hours.

Chemicals and Antibodies

Lactacystin was purchased from Biomol (Plymouth Meeting, PA; PI-104). The following antibodies were used: Monoclonal anti-HA, clone HA-7 (Sigma), anti-HA affinity matrix (immobilized anti-HA high affinity rat monoclonal antibody (clone 3F10)) (Roche), and polyclonal antiFAT10-GST (see above). Horseradish peroxidase coupled secondary antibodies were purchased from DAKO (Glostrup, Denmark).

Metabolic labeling and Pulse-Chase Experiments

For labeling cells with [^{35}S] Met/Cys, the cells were starved for 1h in RPMI-1640 medium (Sigma) supplemented with L-glutamine, penicillin/streptomycin and dialysed FCS, but without Met and Cys, followed by labeling with 0.1 mCi/ml [^{35}S] Met/Cys. Cells were then washed and chased in media containing cold Met/Cys for the indicated time periods. The labeled cells were lysed in Ripa buffer (50mM

Tris/HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 1µg/ml aprotinin, 1 µg/ml pepstatin A, 1µg/ml leupeptin).

For labeling cells with [^{32}P], the cells were starved for 1h in phosphate free RPMI (P-RPMI, ICN) supplemented with L-glutamine and dialysed FCS, followed by labeling with 0.1mCi/ml $^{32}P_i$ for 4h. During the last 30 minutes the phosphatase inhibitor Calyculin A (Sigma) was added in a final concentration of 0.1µM. The cells were lysed in buffer R (20mM Tris pH7.8, 150mM NaCl, 1mM $MgCl_2$, 2% Triton X-100, 1µg/ml aprotinin, 1 µg/ml pepstatin A, 1µg/ml leupeptin) plus 1mM EDTA. In the samples where later no phosphatases were added, the lysisbuffer was supplemented with 20mM $Na_4P_2O_7$, 2mM $Na_3VO_4^-$, 2mM NaF, 10mM β-Glycerophosphate and 10µM okadaic acid.

Immunoprecipitation

Postnuclear lysates were measured and balanced, and corresponding aliquots of lysate were agitated over night at 4°C with the indicated antibody and Protein A or Protein G Sepharose™ CL-4B (Amersham). Where indicated the samples were treated with 200 U/ml phosphorylase phosphatase PP1 (Calbiochem) at 30°C for 1.5h, followed by treatment with 1000 U/ml protein tyrosine phosphatase YOP (Calbiochem) at 30° for 1h, according to the protocols supplied by the manufacturer. After extensive washing, the final immunoprecipitate was either analyzed by SDS-PAGE/autoradiography and evaluation of bands on a Fuji BAS1500 imager, or by SDS-PAGE followed by matrix-assisted laser desorption ionization fourier transform-ion cyclotron resonance (MALDI-FT-ICR) mass spectrometry (described below), or by non-equilibrium pH gradient gel electrophoresis (NEPHGE)/SDS-PAGE described previously (Groettrup et al., 1995), followed by western blotting.

Silver staining, Proteolytic digestion of proteins in gels, Desalting and MALDI-FT-ICR mass spectrometry

The silver staining method used for figure 23 was based on that of Heukeshoven and Dernick (Heukeshoven and Dernick, 1985), but has omitted the use of glutaraldehyde in the sensitization step.

Protein spots were manually excised, washed with MilliQ water, dehydrated with acetonitrile, and dried in a Speed-Vac centrifuge. Gel pieces were destained in 30 mM

$K_3[Fe(CN)_6]$/100 mM $Na_2S_2O_3$, 1:1 (v/v) for 10 min and washed extensively with MilliQ water. Gel pieces were then dehydrated with acetonitrile and dried in vacuum. The protein digestion was performed by swelling the gel pieces in protease containing buffer (12.5 ng/µl trypsin in 50 mM NH_4HCO_3) at 4 °C for 45 min, and then by incubation in 50 mM NH_4HCO_3 at 37 °C for 15 h. The peptides were subsequently extracted with 20 mM NH_4HCO_3/acetonitrile, 1:2 (v/v) for 3-4 h. The eluates were lyophilised to dryness and dissolved just before MALDI-MS analysis in 5 µl of acetonitrile: 0.1% trifluoroacetic acid in water (2:1).

The ZipTip cleanup procedure was performed using $ZipTip_{C18}$ pipette tips (Millipore, Billerica, MA, USA) according to the instructions supplied by the manufacturer.

MALDI-FT-ICR and analysis of the achieved fragments was performed as described elsewhere (Damoc et al., 2003). The fragments were analysed using the MS-Fit database (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm), and the search was carried out within the Swiss-Prot and NCBInr protein databases.

Results and Discussion

Endogenous FAT10 is rapidly degraded by the proteasome

Previously it has been shown that the treatment of cells with the specific proteasome inhibitor lactacystin lead to the accumulation of FAT10 in IFN-γ treated JY cells and in the FAT10 transfectant TB1N (Liu et al., 1999; Raasi et al., 2001). Pulse chase analysis of overexpressed FAT10 in the transfectant cell line TB1N revealed a half life of FAT10 of about 0.9 hours which was prolonged to 3.3 hours when the cells were chased in the presence of 80μM lactacystin. Interestingly, the FAT10 conjugate which was apparent in the induced but not in the uninduced cells, had a similar half life as FAT10 itself (t1/2 of 1.4 h) and was also greatly stabilized by lactacystin treatment (t1/2 3.4 h) strongly suggesting that both, FAT10 and its conjugate, are degraded by the proteasome (Figure 13)

In order to confirm the half-life of FAT10 in cells endogenously expressing FAT10, SW620 cells were treated with IFN-γ and TNF-α for one day before they were used for metabolic labeling and pulse chase analysis. In cytokine treated SW620 cells, the half-life of FAT10 was determined to be 1.3 h in the absence and 3.4 hours in the presence of lactacystin (Figure 19), which is in good agreement with the data obtained for mouse FAT10 in TB1N cells. These results show, that the short half-life of FAT10 that we can observe in our other systems is not an artefact due to overexpression or misfolding of the tagged versions of FAT10.

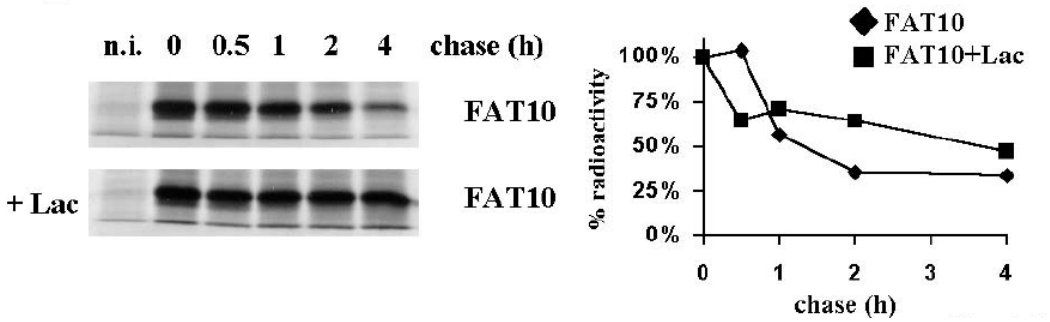

Figure 19. Endogenous FAT10 has a short half-life and is degraded in a proteasome dependent manner. SW620 cells were induced with TNFα and IFNγ for 24h and were then labeled with [^{35}S] Met/Cys and chased for indicated time periods in the absence (top panel) or presence (bottom panel) of 80μM lactacystin followed by immunoprecipitation against FAT10. The bands were quantified on an imager and plotted as per cent radioactivity based on values of the pulsed cells. Lane 1 represents non induced cells (n.i.).

Conjugation to a substrate is not necessary for fast degradation of FAT10

It is important to distinguish between degradation of monomeric FAT10, or a conjugation event that is followed by fast degradation of the substrate together with FAT10, because this could also explain the reduction of the signal for monomeric FAT10. Therefore a mutant of FAT10 was used that cannot be conjugated any more to its target proteins (Raasi et al., 2001). Pulse chase analysis of the cell line mTB14 revealed a half life of FAT10ΔGG of less than 0.5h (Figure 20), so it can be concluded that monomeric FAT10 is degraded directly, and no conjugation event is necessary.

This is an interesting difference to ubiquitin, since Lys-48 linked tetraubiquitin chains, that are sufficient as a proteasomal degradation signal, are recognized differently when conjugated to target proteins in comparison to unanchored tetraubiquitin (Thrower et al., 2000).

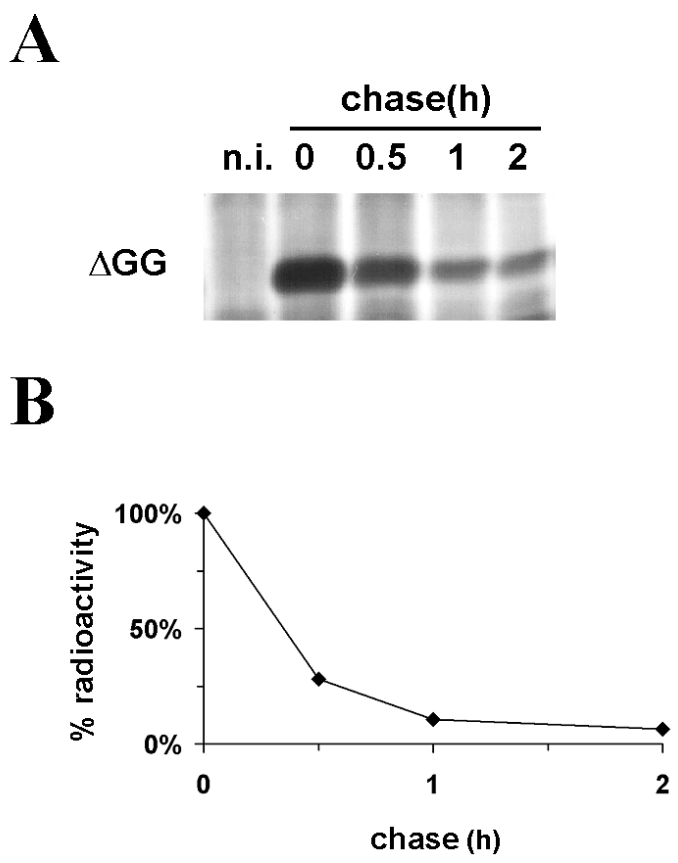

Figure 20. FAT10ΔGG has a short half life The FAT10 transfectant mTB14 was labeled with [^{35}S] Met/Cys and chased for indicated time periods followed by immunoprecipitation against HA-FAT10. Lane 1 represents non induced mTB14 cells (n.i.). The bands were quantified on an imager and plotted as per cent radioactivity based on values of the pulsed cells

FAT10 is a ubiquitin-like modifier that gets phosphorylated

When performing NEPHGE/SDS-PAGE analysis of HA-FAT10 protein expression in the wild type FAT10 transfectant (TB1N) followed by an anti-HA Western blot, it could be observed that the FAT10 monomer migrated as a series of differently charged spots, and it was suggested that this could be due to a covalent modification of FAT10 by phosphorylation (Raasi et al., 2001). If the differently charged spots were due to several phosphate residues conjugated to FAT10, it would be expected that these spots disappear when phosphatases cleave them off and only one spot of unmodified FAT10 remains. To address this question, FAT10 expression was induced through tetracycline removal for 12h, followed by immunoprecipitation with a polyclonal antiFAT10-GST antibody. The immunoprecipitate was then treated with the phosphatases PP1 and YOP, where indicated, and after extensive washing, the final precipitate was analyzed by NEPHGE/SDS-PAGE, followed by western blotting against the HA-tag of FAT10. In figure 21 it can be observed that in the sample treated with the phosphatases the series of differently charged spots disappears and only one spot, resembling unphosphorylated FAT10, remains.

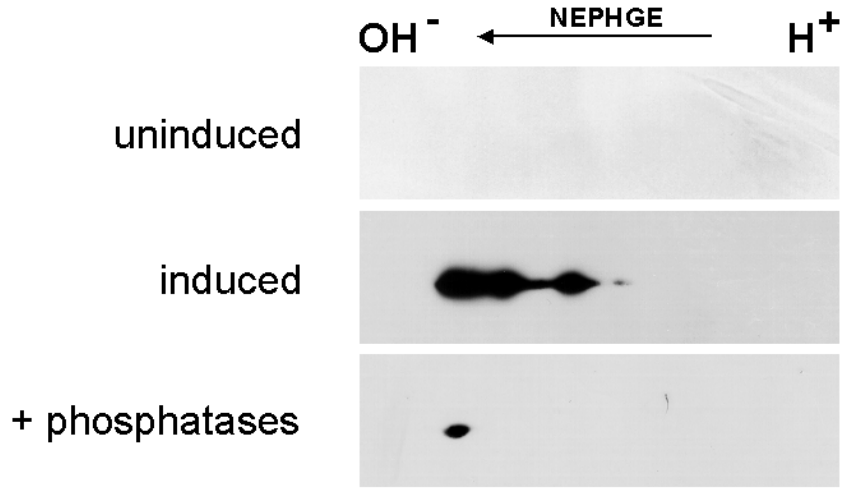

Figure 21 After treatment with phosphatases only a single FAT10 signal is detectable in 2D gel electrophoresis. FAT10 expression was induced through tetracycline removal for 12h prior to cell lysis, and immunoprecipitation with a polyclonal antiFAT10-GST antibody. The two lower panels (induced, +phosphatases) show the analysis of cells expressing FAT10 proteins, whereas the panel at the top (uninduced) shows the analysis of cells lacking FAT10 expression. In the panel in the middle FAT10 appears in multiple differently charged forms, which disappear after treatment with the phosphatases PP1 and YOP.

To confirm this result, the phosphorylation was analyzed with the help of ^{32}P labeled orthophosphate (P_i). When cells were labeled with P_i prior to immunoprecipitation against the HA-tag of FAT10, a band could be observed in the cells expressing FAT10 that runs at the expected molecular weight for FAT10 (Figure 22 lane 2). If the immunoprecipitate was treated with phosphatases this band was not present anymore (Figure 22 lane 3). Lane 4 and 5 of figure 22 represent TB1N cells labeled with [^{35}S] Met/Cys, as a control for FAT10 induction.

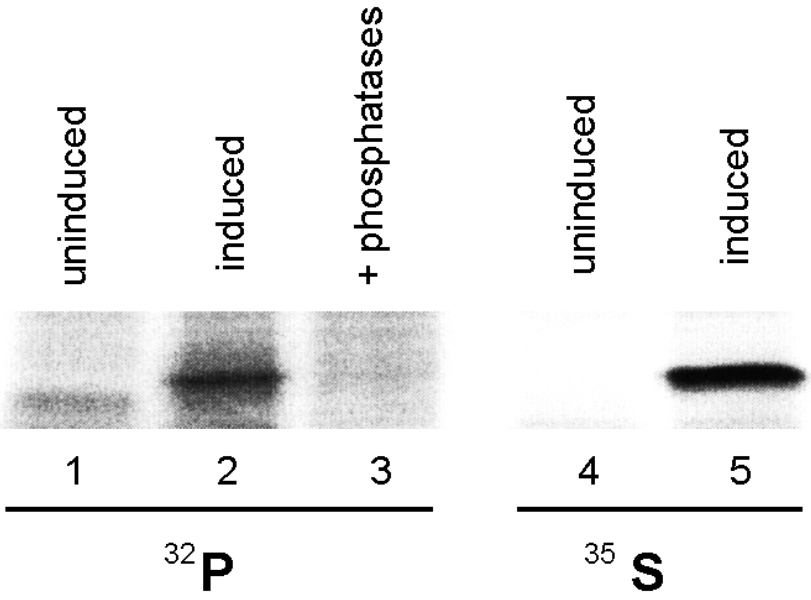

Figure 22 FAT10 is covalently modified by phosphorylation ^{32}P orthophosphate (left) or ^{35}S Met/Cys (right) was used to label cells prior to cell lysis, and immunoprecipitation with an antibody against the HA-tag of FAT10. Where indicated (+phosphatases) the immunoprecipitate was treated with PP1 and YOP. Lane 1 and 4 represent cells lacking FAT10 expression (uninduced), lane 2,3 and 5 show cells expressing FAT10 (induced).

When the same experiment was repeated using only one phosphatase, it could be observed that the FAT10 signal only disappeared after treatment with PP1, but not after treatment with the tyrosine phosphatase YOP (data not shown).

This is in accordance with the prediction by the neural network-based program for the prediction of potential phosphorylation sites NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos) (Blom et al., 1999), which suggest several potential phoshorylation sites at threonine or serine residues, but none at the tyrosine residues of FAT10.

Phosphorylation events play important roles in protein degradation. Phosphorylation of many substrates is required for their recognition by their E3s, followed by ubiquitylation and subsequent substrate degradation. Conversely, similar modification of many other proteins prevents this (Ciechanover et al., 2000). Nevertheless this regulation seems to play a role before the ubiquitylation event takes place and does not affect the fate of the substrates once the polyubiquitin chain is added.
To our knowledge the only other UBL that can get phosphorylated is ubiquitin itself, but no function of this modification is known. The phosphorylation seems to happen at serine 57 of ubiquitin (Peng et al., 2003). Serine 57 is nonessential for viability, but the crystal structure of tetraubiquitin shows that it is solvent exposed. Interestingly the corresponding residue in the N-terminal ubiquitin-like domain of FAT10 is also a serine, and is conserved in mouse and human. This serine residue in FAT10 is predicted by the NetPhos 2.0 to get phosphorylated.
Phosphorylation might add an additional layer of regulation to the already tightly regulated FAT10 protein expression, but till now we do not have any hints if phosphorylation events regulate the half life of FAT10 or its interaction with other proteins.

FAT10 interacts non-covalently with Hsp70

In order to examine the identity of the FAT10 conjugate we performed an immunoprecipitation against the HA-tag of the FAT10 in our inducible cell line TB1N.

After extensive washing, the final immunoprecipitate was analyzed by SDS-PAGE, followed by silver staining. Bands that appeared in the induced sample, but not in the non induced control, where cut out, and a proteolytic digestion of the proteins in the gel was performed, followed by desalting of the sample and MALDI-FT-ICR mass spectrometry.

The band cut out at the expected molecular weight for FAT10 could be identified by database search as murine FAT10. The matched peptides covered 37% of the protein.
A band running at 70kD could be identified as murine HSP70, with the matched peptide covering 23% of the protein.
For the other bands it was not possible to find matches in the databases.

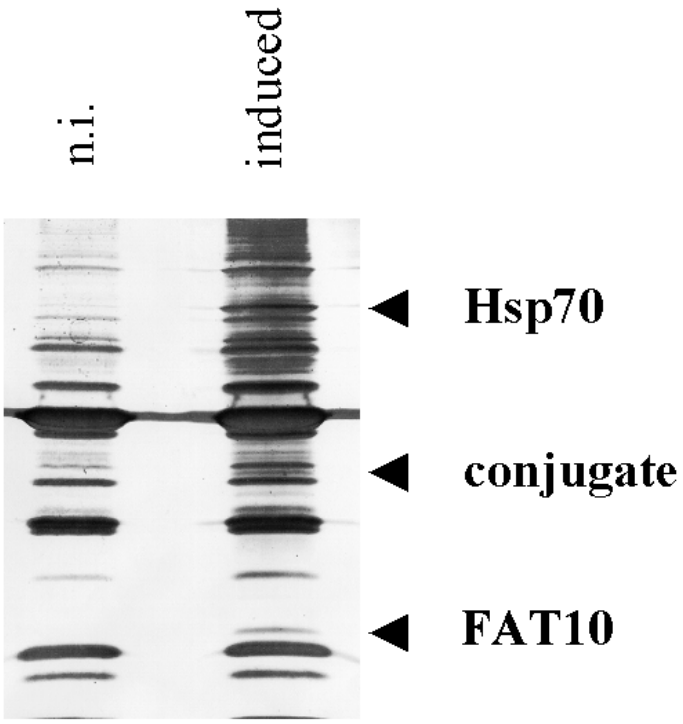

Figure 23 Silver staining of proteins coprecipitating with FAT10 HA-FAT10 expression was induced in the tetracycline inducible FAT10 transfectant TB1N, followed by immunoprecipitation against HA and silver staining. The left lane represents non induced TB1N cells (n.i.). At the positions indicated by the arrowheads, bands where cut out and treated like described in material and methods, before analyzing them by MALDI-FT-ICR.

The fact that we were able to identify monomeric FAT10 tells us that our technical approach to identify FAT10 conjugates is working, and we are repeating this experiment under varying conditions in order to achieve better results.

Since 70kD is the expected molecular weight for unmodified HSP70, we conclude that FAT10 is interacting non-covalently with HSP70.

Because HSP70 is known to bind to many proteins, and because it seemed that the washing conditions necessary for the removal of tetracycline induce HSP70 in control cells, not encoding FAT10 (data not shown), we decided not to follow up this interaction.

4.) DISCUSSION

When ubiquitin-like modifiers are described, it is usually emphasized that they form isopeptide linkages with target proteins just like ubiquitin, but that they do not serve proteolytic functions.

However there are several points of contact between ubiquitin-like modifiers and protein degradation. Examples are NEDD8 modification of SCF-E3 ubiquitin ligases, where the presence or absence of NEDD8 modifies the ubiquitylation activity of the ubiquitin ligase, and thereby determine certain SCF-E3-ligases to destroy or spare their targets (Pan et al., 2004; Parry and Estelle, 2004). Another meeting point of ubiquitin with an UBL is the SUMO modification of some lysines, which prevents their ubiquitylation. A prominent example for this is Lys-21 of IκBα, which can be used for ubiquitylation or sumoylation (Desterro et al., 1998). However all these contacts are indirect, and no UBL was known to target its targets directly to degradation.

In this thesis I present a potential function for the ubiquitin-like modifier FAT10 in protein degradation.

We show that FAT10 is the first UBL, which, like ubiquitin, targets conjugated proteins for degradation through the proteasome. Similar to ubiquitin, FAT10 served as a degradation signal when it was fused to the N-terminus of two long lived proteins and it is remarkable that FAT10 was as potent as ubiquitin in accelerating their proteolysis (Figure 14A). Also the isopeptide-linked FAT10 conjugate found in TB1N cells has a short half life, but since the nature of this FAT10 substrate is unknown at the moment, we can not make a statement if this substrate is degraded in an accelerated manner.

When we initially observed that FAT10 can act as a transferable degradation tag, two scenarios seemed possible:

Firstly, FAT10 could, like other degrons, bind to an E3 ubiquitin ligase and by that means initiate the assembly of polyubiquitin chains. In this scenario a potential role of NUB1L could have been to similarly bind to FAT10, conjugated to its substrate, and to NEDD8, conjugated to a SCF-E3 ligase, which then would polyubiquitylate FAT10.

Secondly, FAT10 itself may act like polyubiquitin in functioning as a proteasome targeting signal.

Unexpectedly we found that FAT10 is able to fulfil its role as a degradation signal without any involvement of cells regular degradation signal ubiquitin. Although we found ubiquitylated forms of FAT10 in cells, these forms where primarily modified with only 1-3 copies of ubiquitin that do not mediate an efficient targeting to the 26S proteasome (Thrower et al., 2000) (Figure 16B).

Further experiments, using either cells with a defective ubiquitylation machinery (Figure 18), or with a FAT10 mutant that could not be modified by ubiquitin any more (Figure 17), revealed that FAT10 is rapidly degraded by the proteasome without requiring the ubiquitylation system in general, and polyubiquitylation of FAT10 itself in particular.

It is also not necessary for FAT10 to build dimers and so to mimic tetraubiquitin-chains, since both FAT10ΔGG, which cannot be conjugated to target proteins, and FAT10-K0, which cannot be substrate to a targeting event are degraded fast (Figures 17 and 20).

These results raise the question how FAT10 can mediate degradation by the proteasome.

This question might be answered through the identification of NUB1L as a new non-covalent interaction partner of FAT10. This interaction was found in a yeast two-hybrid screen and was confirmed by coimmunoprecipitation from NUB1L and FAT10 coexpressing cells (Figure 9). The binding of FAT10 and NUB1L appears to be direct because recombinant purified GST-FAT10 and His-NUB1L interacted in GST pull down assays (Figure 8).

NUB1L coexpression led to an eightfold acceleration of FAT10 degradation resulting in a strongly reduced FAT10 expression level in NUB1L coexpressing cells (Figure 11).

Ubiquitin-domain proteins as for instance Rad23/Rhp23 and Dsk2/Dph1 were suggested to act as adaptors by associating with the 26S proteasome via their ubiquitin-like domains (Elsasser et al., 2002; Schauber et al., 1998), while using their C-terminal UBA domain for binding ubiquitin chains (Wilkinson et al., 2001). This would enable these proteins to function in the transport of ubiquitylated proteins to the

26S proteasome (HartmannPetersen et al., 2003b). It is possible that NUB1L serves a similar purpose for FAT10 and, to a lesser degree, for NEDD8.

NUB1 appears to copurify with the 26S proteasome and an interaction of NUB1 recombinant protein with an RPN10 fusion protein has been shown by Kamitani *et al.* in GST pull down assays (Kamitani et al., 2001). It has not been shown, but it is tempting to speculate that it is the N-terminal ubiquitin-like domain of NUB1 that binds to the ubiquitin interaction motif (UIM) of RPN10.

The three UBA domains in the C-terminal domain of NUB1 seem not to be involved in the binding of ubiquitin (Figure 9B and (Tanaka et al., 2004)), and it seems that only the second UBA domain in NUB1L is responsible for NEDD8 interaction and degradation (Tanaka et al., 2003). The main NEDD8 binding site, which is also present in NUB1, where the UBA-2 domain is missing, was found to be located at the C-terminus of the protein, in a region outside of the UBA domains, which contains one half of a PEST domain (Tanaka et al., 2003).

The first UBA domain of NUB1 and NUB1L might be involved in the interaction with UbC1, which could be observed in a yeast two hybrid assay and in a GST-pulldown. UbC1 is a molecule that is composed of nine tandem repeats of a ubiquitin unit connected through α-peptide bonds. It is a precursor of ubiquitin, and is processed into single ubiquitin units. It is therefore not clear if this interaction has any physiological relevance, since the level of unprocessed endogenous UbC1 in a cell will be very low.

For the interaction of FAT10 with NUB1L, we could show in *in vitro* GST-pulldown experiments, that at least the UBA domains 1 and 3 together are necessary for a stable NUB1L-FAT10 interaction (Figure 12) and it seems that for coimmunoprecipitation *in vivo* even all three UBA domains are necessary (Bochtler and Schmidtke, unpublished results). What does this mean for the ability of UBA-2 deficient NUB1 to interact with FAT10 is at the moment an unresolved question, since, in the UBA-2 deletion mutant used in these experiments, also regions flanking the UBA domain have been deleted, which may be important for the overall structure of NUB1.

Given that NUB1L, which is also interferon-γ inducible (Kito et al., 2001), markedly accelerates the degradation of FAT10 (Figure 11) and FAT10 fusion proteins (Figure 14), it is attractive to hypothesize that NUB1L functions as an adaptor that ties FAT10 and FAT10 conjugated proteins to the proteasome. A recent study demonstrated that

proteins that were targeted to the proteasome with an artificial tagging system were rapidly degraded by the proteasome (Janse et al., 2004). NUB1L mediated proximity to the proteasome may therefore be sufficient for degradation.

The model of UBL-UBA domains working as a carrier of polyubiquitin- or FAT10-modified substrates, raises the question why this degradation-tags (either K48-linked polyubiquitin or monomeric FAT10) are not recognized directly by the proteasome. A possible explanation would be that with the help of the adaptors other "degradation signal recognition subunits" of the proteasome could be accessed, which have different properties than the "normal" polyubiquitin receptors. Another role could be that the adaptors either protect the modifier-substrate isopeptide linkage from deconjugating enzymes (HartmannPetersen et al., 2003a; Raasi and Pickart, 2003) or recruit additional enzymatic activities.

We believe that NUB1L has more functions than only to be an adaptor between FAT10/ FAT10 modified proteins and the proteasome. Several points argue in favour of that: Firstly, the cytokine pattern that induces FAT10 and NUB1L is similar but not identical. Secondly NUB1L homologues are also present in organisms where no FAT10 is found. And thirdly NUB1L interacts also with several other proteins, like NEDD8, UbC1 and AIPL1 (Akey et al., 2002; Tanaka et al., 2003; Tanaka et al., 2004).

It will be very interesting to further investigate the role of NUB1L in FAT10 mediated degradation. We may be in for surprises given that, preliminary results achieved in our group hint that the ubiquitin-like domain of NUB1L may play a more important role in the acceleration of FAT10 degradation than all three UBA domains together (Bochtler, Weber and Schmidtke, unpublished results) and that FAT10 is able to directly interact with the proteasome (Kalveram and Hipp, unpublished results).

Not only the UBLs but also the other class of ubiquitin-like proteins is linked with degradation and the ubiquitin-proteasome system. It seems that UDPs interact with the 26S proteasome through their ubiquitin-like domain and also the other domains of these proteins are quite often linked to protein degradation or to ubiquitylation (HartmannPetersen and Gordon, 2004).

It is tempting to speculate that FAT10 with its two ubiquitin-like domains is a blend of both of these protein classes, especially since the two domains share only about 20% identity with each other. The C-terminal domain would fulfil functions of a

UBL, since the diglycine motif is localized here, whereas the N-terminal ubiquitin-like domain would have mainly the protein-protein interaction functions of the ubiquitin-like domain of the UDPs. The observation that the N-terminal domain of FAT10 is sufficient for NUB1L binding fits into this model (Weber and Schmidtke, unpublished Results).

How would an organism benefit from possessing an alternative to ubiquitin-dependent degradation?

The facts that FAT10 is encoded in the MHC locus, is inducible through proinflammatory cytokines, and is only present in species that possess an adaptive immune system, suggest a role of FAT10 in antigen processing and presentation.

In this field it indeed would be important to have an alternative to ubiquitin, since several viruses try to manipulate and exploit the ubiquitylation machinery of the host cells. Examples for this are the human papillomaviruses that exploit the ubiquitin-proteasome system to degrade a number of important negative cell regulatory proteins (Scheffner and Whitaker, 2003), the Kaposi's sarcoma-associated herpesvirus that encodes E3 ubiquitin ligases to downregulate cell surface molecules involved in the immune recognition of infected cells (Coscoy et al., 2001) or the adenovirus L3 23K proteinase, which can function as a DUB in vitro and in vivo (Balakirev et al., 2002).

A second system that leads proteins to degradation would have several advantages; firstly it could serve as a backup system in case the “regular” system can not work properly any more, and secondly it could alter the overall pattern of protein degradation and of the epitopes generated.

A role of FAT10 as an alternative to general degradation is unlikely, since the overall pattern of polyubiquitylated proteins does not change upon FAT10 induction (Raasi et al., 2001). Also the fact that we and others only observe few conjugates argues against a function of FAT10 in degradation of the bulk of cellular or pathogen encoded proteins (Figure 13) and (Lee et al., 2003; Raasi et al., 2001).

When we examined the role of FAT10 expression in MHC class I cell surface expression, no significant difference could be observed in the cell surface expression of the MHC class I molecules H-2L^{d}, H-2D^{d}, and H-2K^{d} in the presence or absence of FAT10 induction (Raasi et al., 2001). We also could not observe a change in the antigen presentation of two different epitopes, since the H-2L^{d}-restricted presentation of the mouse cytomegalovirus (MCMV) immediate early gene product pp89

(MCMV-pp89$_{168-176}$) and of the lymphocytic choriomeningitis virus (LCMV) nucleoprotein-derived epitope NP118 was not affected by FAT10 overexpression (Raasi et al., 2001); S.Basta and M.Hipp, unpublished). Although additional epitopes may have to be tested to reach a definitive conclusion, it seems likely that the FAT10 expression level is at least not limiting in MHC class I-restricted antigen presentation of viral proteins.

Nevertheless, FAT10 seems to have antiviral functions, since FAT10 is able to inhibit hepatitis B virus expression in a hepatoblastoma cell line (Xiong et al., 2003).

A potential antiviral role of FAT10 could be, that proteins, which are needed for viral replication, are degraded specifically. If these proteins were not of viral, but of host origin, the long time effects of this would of course also harm the host. This would be consistent with the induction of cell death through FAT10 expression. In this case cell death would be a costly, but necessary consequence of the FAT10 function.

On the other hand, there are also scenarios possible where apoptosis would be the main aim of the FAT10 conjugation and not just an adverse side effect.

Regulated death of cells of the immune system is essential for its function. Lymphocytic homeostasis can only be kept, when activated lymphocytes undergo apoptosis after an antigen has been eliminated. This process is triggered by TNF-α and other ligands for the TNF-receptor family, like fas-ligand. The fact that expression of FAT10 was found in activated cells of the immune system, like mature B cells and dendritic cells, and that FAT10 is inducible by TNF-α, would favour such a function of FAT10 (Bates et al., 1997; Raasi et al., 1999).

The degradation promoting nature of FAT10 and the fact that expression of FAT10 is lethal, render the biggest open question, the identity of the FAT10 substrates, difficult to address. The results we achieved from immunoprecipitation experiments could not be confirmed with other methods, but we hope that it will be possible with our inducible cell lines and antibodies to soon identify the first FAT10 substrates.

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RECORD OF ACHIEVEMENT/ EIGENABGRENZUNG

The results described in the figures 8, 10, 12, 13, 18, 19, 20, 21,and 22 were performed by myself.

For the results described in figure 23, the cell work, the immunoprecipitation and the silver staining was performed by myself, the in gel digest, the desalting and the MALDI-FT-ICR mass spectrometry, was performed by Eugen Damoc

I contributed to the figures 9, 11, 14 and 17 by providing expression constructs.

The work performed by Birte Kalveram in the chapter: “FAT10 – a ubiquitin independent signal for proteasomal degradation” was performed in her diploma thesis under my direct supervision.