Urm1 at the crossroad of modifications

'Protein Modifications: Beyond the Usual Suspects' Review Series

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The ubiquitin-like protein Urm1 can be covalently conjugated to other proteins, such as the yeast thioredoxin peroxidase protein Ahp1p, through a mechanism involving the ubiquitin E1-like enzyme Uba4. Recent findings have revealed a second function of Urm1 as a sulphur carrier in the thiolation of eukaryotic cytoplasmic transfer RNAs (tRNAs). Interestingly, this new role of Urm1 is similar to the sulphur-carrier activity of its prokaryotic counterparts, strengthening the hypothesis that Urm1 is a molecular fossil of the ubiquitin-like protein family. Here, we discuss the function of Urm1 in light of its dual role in protein and RNA modification.

Keywords: protein conjugation; sulphur carrier; tRNA modification; Urm1

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Introduction

Ubiquitin-like proteins (UBLs) comprise a diverse group of evolutionarily conserved small proteins that are covalently conjugated to target proteins, thereby regulating their function. Although they vary in their degree of similarity to ubiquitin, they all seem to be conjugated through a comparable enzymatic cascade: UBLs are activated initially through adenylation of their carboxy-terminal glycine–glycine motif and subsequently form a thioester bond with their specific activating E1 enzymes; they are then transferred to conjugating E2 enzymes by transthiosterification and are finally conjugated to lysine residues of specific target proteins with the help of E3 enzymes (Hochstrasser, 2000; Welchman *et al*, 2005). Structural and sequence data have led to the suggestion that a family of bacterial and archaeal sulphur carriers can be considered to be prokaryotic counterparts of the UBLs (Fig 1), although they do not share the protein-modification function (Iyer *et al*, 2006; Lake *et al*, 2001). For example, the bacterial MoaD and ThiS proteins are required for sulphur incorporation into molybdopterin and thiamine, respectively (Webb *et al*, 2007). Similarly to UBLs, MoaD and ThiS are activated by the adenylation of

their C terminus (Xi *et al*, 2001). However, after initial activation, the prokaryotic and eukaryotic pathways diverge (Fig 2A,B), and, despite the fact that several bacterial genomes encode proteins with similarity to the eukaryotic ubiquitin-conjugation machinery (Iyer *et al*, 2006), it remains unclear how the protein-modification function of the UBLs evolved.

In evolutionary terms, the eukaryotic ubiquitin-related modifier Urm1 occupies a special place. Similar to other UBLs, Urm1 contains a β -grasp-fold structural motif (Fig 1A) and covalently modifies other proteins (Furukawa *et al*, 2000; Goehring *et al*, 2003a,b). However, its sequence and structure resemble those of bacterial sulphur carriers more closely than UBLs (Fig 1B,D; Singh *et al*, 2005; Xu *et al*, 2006). This fact has led to the assumption that the first members of the UBL family were similar to Urm1, which has been called a molecular fossil (Xu *et al*, 2006).

Here, we summarize the findings on Urm1 as a canonical protein-modifying UBL, and discuss recent results that have revealed a role for Urm1 as a sulphur carrier in transfer RNA (tRNA) modification.

Urm1, the protein modifier

Urm1 was first described by Furukawa and co-workers (Furukawa *et al*, 2000). By searching a yeast sequence database for relatives of the bacterial sulphur carriers, they found Urm1p, a protein with 20% and 23% sequence similarity to ThiS and MoaD, respectively (Fig 1B). A yeast two-hybrid screen using Urm1p as bait identified Uba4p, which shows significant sequence similarity with the ubiquitin-activating E1 enzyme Uba1p. In addition to an unconjugated Urm1p band, higher molecular weight species were detected by Western blot analysis (Furukawa *et al*, 2000; Goehring *et al*, 2003a,b). As these species depend on functional Uba4p, it was concluded that Urm1p is a genuine UBL that can be conjugated to many proteins (Furukawa *et al*, 2000; Goehring *et al*, 2003b), and that Uba4p is its E1 enzyme. Furukawa and co-workers reported that Urm1p and Uba4p form a DTT-sensitive complex (DTT for DL-dithiothreitol) and concluded that—in analogy to other UBLs—they might be bound through a thioester bond. This hypothesis has recently been challenged by Schmitz and colleagues, who, after demonstrating the presence of a thiocarboxylate at the C terminus of Urm1p, suggested that the Uba4p–Urm1p complex observed by Furukawa and co-workers is probably bound through a disulphide bridge (Schmitz *et al*, 2008).

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Fig 1 | Multiple sequence alignment and phylogenetic trees of ubiquitin-like proteins and their E1 enzymes. (A) Nuclear magnetic-resonance structures of ubiquitin (Protein Data Bank identifier 1ubq) and Urm1 (Protein Data Bank identifier 2ax5). (B) Alignment of representative ubiquitin-like modifiers. With the exceptions of Urm1p (*Saccharomyces cerevisiae*), MoaD (*Escherichia coli*) and ThiS (*E. coli*), only human versions are shown. For the two-domain modifiers ISG15 and FAT10, only the relevant carboxy-terminal domain is shown. (C) Alignment of representative E1 enzymes that activate the modifiers shown in (B). Only the catalytically active ThiF domain is shown. (D) Neighbour-joining dendrogram of UBL modifiers derived from the alignment in (B). (E) Neighbour-joining dendrogram of E1 enzymes derived from the alignment in (C). FAT10, factor activated by TNF- α 10; ISG15, interferon-stimulated gene 15; MOCS2, molybdopterin cofactor synthesis gene 2; NEDD8, neural precursor cell expressed, developmentally down-regulated gene 8; SUMO, small ubiquitin-like modifier; Ub, ubiquitin; UBL, ubiquitin-like protein; UFM1, ubiquitin-fold modifier 1; URM1, ubiquitin-related modifier 1.

So far, the only known Urm1p-protein conjugate is the thiol-specific peroxiredoxin Ahp1p (Goehring *et al*, 2003a), which is a protein with an important role in the response to oxidative stress (Jeong *et al*, 1999; Lee *et al*, 1999). Given the obvious similarities between the Urm1p and UBL systems, it was assumed that an isopeptide bond formed between a lysine residue from Ahp1p and the C terminus of Urm1p. Interestingly, *ahp1Δ* cells do not share many phenotypes with *urm1Δ* and *uba4Δ* cells (see below), which indicates that other urmylation targets and/or an additional function of the *URM1* pathway must exist. The former is supported by the presence of high molecular-weight putative urmylated proteins in Western blot (Furukawa *et al*, 2000; Goehring *et al*, 2003a,b) and the latter has recently been identified.

A series of recent papers from independent groups has revealed a novel function for the *URM1* pathway in the modification of certain cytosolic tRNAs (Bjork *et al*, 2007; Dewez *et al*, 2008; Huang *et al*, 2008; Leidel *et al*, 2008; Nakai *et al*, 2008). tRNAs contain not only the four canonical bases—uridine, cytidine, adenosine and guanosine—but also modified forms thereof (Grosjean, 2005), which are thought to alter the folding, stability and turnover of tRNAs (Agris, 2008; Wang *et al*, 2007), and to fine-tune their interactions with messenger RNAs (mRNAs) and

Ncs6p belongs to the 'PP-loop' ATPases and is related to the TtcA protein family, which has an important role in cytidine thiolation (Fig 3B; Jager *et al*, 2004). Ncs6p binds to (Dewez *et al*, 2008; Leidel *et al*, 2008) and adenylates tRNA_{U34} *in vitro* (Dewez *et al*, 2008; Nakai *et al*, 2008). Ncs2p is another PP-loop ATPase and shares many sequence motifs with Ncs6p. The two ATPases might form a complex (Dewez *et al*, 2008; Leidel *et al*, 2008), although this is being debated (Nakai *et al*, 2008). Ncs2p is essential for the *in vivo* generation of s²U₃₄.

These findings support a model (Fig 3B) in which Nfs1p would extract a sulphur atom from a cysteine residue and transfer it to the RHD domain of Uba4p (Marelja *et al*, 2008; Nakai *et al*, 2004), either directly or with the participation of Yor251cp, the sequence of which is similar to that of mercaptopyruvate sulphurtransferases. The N-terminal nucleotide-binding domain of Uba4p adenylates the C terminus of Urm1p, followed by its conversion to a thiocarboxylate

A *URM1*-like pathway allows *Thermus thermophilus* to grow at high temperatures by stabilizing the bacterial tRNA through thiolation (Shigi *et al*, 2006a; Shigi *et al*, 2006b). Recent work by Sinha and co-workers (Sinha *et al*, 2008) established a similar function of the *URM1* pathway in yeast. These findings indicate that the temperature sensitivity of *urm1Δ* cells is mediated by alterations in tRNA modification.

Table 1 | Genes of the URM1 pathway in yeast and humans

Yeast	Human	Architecture	Function
<i>URM1</i>	<i>URM1</i>	Ubiquitin-like	Conjugated to Ahp1p (in yeast), sulphur carrier for tRNA modification
	<i>MOCS2</i>		Sulphur carrier for molybdopterin biosynthesis
<i>UBA4</i>	<i>MOCS3</i>	E1-like NBD, RHD	E1-like activating enzyme for URM1 (and possibly for MOCS2)
<i>NCS6</i>	<i>ATPBD3</i>	PP-loop ATPase	Adenylates uridine in tRNA, thereby activating it for modification
<i>NCS2</i>	<i>C16ORF84</i>	PP-loop ATPase	Not known, forms a complex with Ncs6p
<i>YOR251C</i>	<i>TST</i>	2 × RHD (first inactive, second catalytic)	Thiosulphate sulphur transferase
	<i>MPST</i>		Mercaptopyruvate sulphurtransferase
<i>NFS1</i>	<i>NFS1</i>	Class V aminotransferase	Cysteine desulphurase
<i>AHP1</i>	–	Thioredoxin-fold	Only known urmylation target, enzyme with redox-active cysteine

ATPBD3, ATP-binding domain 3; *C16ORF84*, chromosome 16, open reading frame 84; *MOCS*, molybdopterin cofactor synthesis; *MPST*, mercaptopyruvate sulphurtransferase; NBD, nucleotide-binding domain; *NFS1*, *nifS*-homologue 1; RHD, rhodanese-homology domain; tRNA, transfer RNA; *TST*, thiosulphate sulphurtransferase; *URM1*: ubiquitin-related modifier 1.

Table 2 | Bacterial counterparts of URM1 system components

Bacteria	Eukaryotic homologue	Architecture	Function
<i>MoaD</i>	<i>URM1</i> , <i>MOCS2</i>	Ubiquitin-like	Sulphur carrier for molybdopterin biosynthesis
<i>ThiS</i>	–	Ubiquitin-like	Sulphur carrier for thiamin biosynthesis
<i>MoeB</i>	<i>UBA4</i> (NBD)	E1-like NBD	E1-like activating enzyme for MoaD
<i>ThiF</i>	<i>UBA4</i> (NBD)	E1-like NBD	E1-like activating enzyme for ThiS
<i>ThiI</i>	<i>NCS6</i> (ATPase), <i>UBA4</i> (RHD)	PP-loop ATPase, RHD	Required with ThiF for ThiS loading
<i>IscC</i>	<i>NFS1</i>	Class V aminotransferase	Cysteine desulphurase

Abbreviations the same as in Table 1.

URM1-pathway mutants also share some phenotypes with TORC1 (target of rapamycin complex 1) mutants, including sensitivity to rapamycin and caffeine. Interestingly, in *urm1Δ* and *uba4Δ* cells, Gln3p and Gat1p—two transcription factors downstream of the TOR (target of rapamycin) kinase—are mislocalized, and the TORC1-regulated genes *CIT2* and *GAP1* are aberrantly regulated (Rubio-Teixeira, 2007). Together, these data seem to indicate an important role of Urm1p in controlling the genes responsible for sensing and regulating levels of amino acids. As urmylation has not been detected for any of the candidate factors, and tRNA overexpression rescues rapamycin and caffeine sensitivity, it will be interesting to test whether tRNA overexpression can also rescue the other TORC1-related phenotypes of *urm1Δ* cells. Finally, it is important to note that the phenotypes of *ahp1Δ* strains are different from those of *urm1Δ* and *uba4Δ* cells, which indicates that a lack of Ahp1p urmylation is not the cause of the phenotypes of *URM1*-pathway mutants. For example, *ahp1Δ* cells do not exhibit a defect in invasive growth, and deletion of *URM1/UBA4* results in increased diamide sensitivity relative to that of cells lacking Ahp1p. In addition, although *urm1Δ* and *uba4Δ* cells are not sensitive to tert-butyl hydroperoxide, *ahp1Δ* cells are sensitive (Goehring et al, 2003a).

In summary, resistance to *K. lactis* γ-toxin (Huang et al, 2008), sensitivity to rapamycin, caffeine and diamide (Leidel et al, 2008), and probably temperature sensitivity, can all be attributed to a lack of tRNA thiolation. It will be important to test whether the remaining

phenotypes can also be rescued by tRNA overexpression or, rather, are related to defects in protein urmylation.

Conclusions

The recently discovered function of Urm1p as a sulphur carrier in the modification of tRNA bases has challenged its accepted function as a classical protein-modifying UBL and has raised several important questions (see also Sidebar A).

The first concerns the exact nature of the Urm1p–protein bond. The similar architecture of Urm1 and the canonical ubiquitin-like modifiers, along with their similar mechanism of activation (Fig 2), indicates that an isopeptide bond is formed between Urm1p and its targets. However, a modified lysine from Ahp1p has not yet been identified, and the presence of a thiocarboxylate at the C terminus of Urm1p raises the possibility that an acyl disulphide bond is formed instead. In this respect, it is interesting that *N*-ethyl maleimide—an inhibitor of cysteine-based proteases and thiol-containing reducing agents—is required to detect Urm1–protein conjugates. If urmylation of Ahp1p really involves an acyl disulphide bond, the two proteins might reversibly inactivate each other by masking both active-site cysteine residues. Interestingly, two recent publications reported non-lysine conjugation of ubiquitin to proteins (Cadwell & Coscoy, 2005; Williams et al, 2007). In both cases, the authors identified β-mercaptoethanol-sensitive conjugation of ubiquitin to a cysteine residue, and concluded that a thioester bond is involved. In light

of the new Urm1p findings, it seems worthwhile characterizing the exact nature of these complexes. Finally, it would be interesting to investigate whether similar complexes exist in prokaryotes, where no protein conjugation of UBLs has been described so far; it is possible that labile disulphide-bound complexes have eluded detection.

A second, related question deals with the components involved in the protein-modifying and tRNA-modifying branches of the *URM1* pathway (see Tables 1,2). Several studies have identified a set of five *URM1* pathway components involved in tRNA thiolation: Urm1p, Uba4p, Ncs2p, Ncs6p and Yor251cp (Huang et al, 2008; Leidel et al, 2008; Nakai et al, 2008). In addition, Nfs1p is thought to act upstream of the pathway (Marelja et al, 2008; Nakai et al, 2004). It is likely that all of the proteins required for U₃₄ thiolation have been accounted for, although bacterial s²U-modification requires a more intricate sulphur-relay system (Ikeuchi et al, 2006), which is absent in eukaryotes. Much less is known about the protein-conjugation branch. No Urm1p-specific E2-conjugating and E3-ligating enzymes have been identified so far. Uba4p might act as a dual-role E1-like and E2-like enzyme; however, it is also possible that E2 and E3 are not even required, given that urmylation has never directly been shown to target lysine residues or to form an isopeptide bond. It is also unclear whether some proteins from the *URM1* pathway are required only for one of the two functions. For example, *NCS6* and *NCS2*—two genes with a strong evolutionary link to tRNA metabolism—have been described to influence the urmylation level of proteins. It will be of substantial interest to find out how the two branches differ and whether they regulate each other, for example by competing for a Urm1p pool or by using Ahp1p modification as a reversible Urm1p buffer.

A third question deals with the scope of Urm1 RNA modification capabilities and their functional consequences. A range of prokaryotic and eukaryotic RNA species contain sulphur-modified bases. For example, mitochondrial tRNA is also thiolated, which is a process that does not depend on *URM1* (Leidel et al, 2008); however, a role of *URM1* in the modification of other sulphur-containing RNAs remains possible.

To gain a better understanding of the *URM1*-deletion phenotypes, it will be necessary to study the impact of uridine 2-thiolation on protein translation. Can some of the *URM1* phenotypes be explained by the improper translation of a small set of proteins? Even more intriguingly, thiolation might be regulated in response to external or internal stimuli, thereby providing the cell with a way to fine-tune protein expression.

The dual role of Urm1 might also have biomedical implications. Although most work on Urm1 has been done in yeast, the pathway seems to be conserved in other eukaryotes, as implied by experiments in *Caenorhabditis elegans* (Dewez et al, 2008; Leidel et al, 2008) and in humans (Schmitz et al, 2008). As shown in the alignment and phylogenetic tree (Fig 1B,D), the human genome encodes two proteins that are similar to Urm1p, one of which is closely related to yeast Urm1p and is therefore known as URM1. A second protein, which is encoded by the *MOCS2* gene, is more similar to the bacterial sulphur carriers and is required for molybdopterin biosynthesis (Stallmeyer et al, 1999). Humans have a single *UBA4*-like E1 enzyme, *MOCS3*, which is probably also responsible for activating human URM1, pointing to an overlap between the molybdopterin and *URM1* pathways. Defects in molybdopterin-biosynthesis genes are associated with several health conditions (Reiss & Johnson, 2003). A re-evaluation of these conditions in light of the new role of Urm1 in tRNA thiolation might be warranted.

Sidebar A | In need of answers

- (i) How does protein urmylation work? What type of bond is involved? What other proteins are urmylated?
- (ii) Are there interrelationships between the two pathways?
- (iii) What are the functional significances of transfer RNA (tRNA) and protein modifications?
- (iv) How does the tRNA thiolation proceed after URM1 activation?
- (v) What is the interdependence between mcm⁵ and s²U₃₄ modifications of tRNA?
- (vi) Does the URM1 pathway have medical relevance, for example for human immunodeficiency virus (HIV) reverse transcription, molybdopterin deficiency or familial dysautonomia?

Finally, some retroviruses use the tRNA species tK^{lys3}_{UUU} as a primer to reverse transcribe their RNA genomes (Abbink & Berkhout, 2008). *In vitro* experiments have shown that unmodified tK^{lys3}_{UUU} renders human immunodeficiency virus (HIV)-reverse transcriptase inactive (Isel et al, 1993, 1996). The identification of the pathway responsible for thiolation of cytoplasmic tRNAs will allow the experimental validation of this result *in vivo*. Members of the *URM1* pathway might be particularly interesting drug candidates, as they are crucial for thiolation but are not essential genes in yeast.

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Polyglutamylation: a fine-regulator of protein function?

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The O-linked N-acetylglucosamine modification in cellular signalling and the immune system

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The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy

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Function and regulation of protein neddylation

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The expanding field of poly(ADP-ribosyl)ation reactions

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