Ubch9 conjugates SUMO but not ubiquitin

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Abstract Ubiquitin conjugating enzymes participate in the thioester cascade that leads to protein ubiquitination. Although Ubc9 is homologous to E2 ubiquitin conjugating enzymes we have shown that it is unable to form a thioester with ubiquitin, but can form a thioester with the small ubiquitin-like protein SUMO. Thus Ubc9 is a SUMO conjugating enzyme rather than a ubiquitin conjugating enzyme. Transacetylation of Ubc9 by SUMO is not mediated by the E1 ubiquitin activating enzyme, but by a distinct enzymatic activity. SUMO conjugation to target proteins is mediated by a different, but parallel pathway to ubiquitination.

Key words: Ubiquitination; Ubch9; SUMO; IκBα; Thioester formation

1. Introduction

Modification of proteins by ubiquitin is the critical step in targeting proteins for degradation via the proteasome. The first stage in this process is activation of ubiquitin by the ubiquitin activating enzyme (E1). In this ATP dependent reaction the C-terminus of ubiquitin is first adenylated with release of pyrophosphate. This is followed by formation of a thioester bond between the side chain of a cysteine residue in the same E1 and the C-terminal carboxyl group of the ubiquitin protein with release of AMP. The next stage in this process involves a transacetylation reaction which results in ubiquitin forming a thioester bond with a conserved cysteine residue in a family of ubiquitin conjugating enzymes (E2). Ubiquitin is then transferred from the E2 enzyme to the ultimate protein acceptor via an isopeptide linkage with the ε amino group of a lysine in the target protein. In many instances this final step requires the participation of ubiquitin protein ligase (E3) which may act either as the ultimate ubiquitin donor or in substrate recognition [1]. Polyubiquitinated proteins are bound by the 26S proteasome and processively degraded. In some instances protein ubiquitination functions not as a signal for degradation, but to alter the properties of the linked protein. Thus histone ubiquitination alters chromatin structure [2] while ubiquitination of a plasma membrane receptor modifies ligand-stimulated endocytosis [3]. Although covalent modification of proteins by ubiquitin is now well documented it is also clear that a number of other small protein molecules can be linked to target proteins in a similar fashion to ubiquitin. The protein UCRP, which contains two ubiquitin-like domains is conjugated to a number of intracellular proteins by a series of reactions that are separate from ubiquitination [4,5]. Recently a small ubiquitin-like protein variously known as sentrin, GMP1, SUMO, UBL1 and PIC1 has been found covalently linked to Ran GTPase activating protein 1 (RanGAP1) and associated with a variety of other proteins [6–10]. Covalent modification of RanGAP1 appears to be necessary for its interaction with the Ran-GTP binding protein RanBP2 at the cytoplasmic face of the nuclear pore complex [8,11]. Also found in the complex between SUMO modified RanGAP1 and RanBP2 is a protein designated Ubc9 which is homologous to the E2 class of ubiquitin conjugating enzymes [11]. In yeast Ubc9 is essential for cell cycle progression [12] and mammalian homologues have been isolated repeatedly from yeast two hybrid screens in association with a wide variety of proteins. However, it has not been possible to demonstrate that Ubc9 has the enzymatic activities that would be expected of a ubiquitin conjugating enzyme.

Here we demonstrate that while Ubc9 is unable to form a thioester with ubiquitin it is capable of forming a thioester with SUMO, indicating that Ubc9 is a SUMO conjugating enzyme rather than a ubiquitin conjugating enzyme. Furthermore, we show that transacetylation of Ubc9 by SUMO is not mediated by the ubiquitin activating enzyme E1, but by a distinct enzymatic activity. Thus SUMO is conjugated to its target proteins by a distinct, but parallel pathway to ubiquitination.

2. Materials and methods

2.1. cDNA cloning

The yeast two hybrid system [13] was used to screen a human cDNA expression library for proteins that interact with N-terminal regulatory domain of IκBα (amino acids 1–74). The pV44ER.LexA expression vector was used to generate a fusion of the LexA DNA binding domain with the N-terminus of IκBα (1–74). A second vector pACT was used to express a fusion of Gal4 activation domain with proteins encoded by cDNAs in a library generated from human lymphocytes [14]. Interacting species were identified in yeast L40a displaying histidine independent growth and β-galactosidase activity. A full length Ubch9 cDNA was isolated from this cDNA library. The entire open reading frame of 474 nucleotides was amplified by polymerase chain reaction (PCR) using primers (5′-ACAAACGGATCCATGTCGGGGATCGCCCTCAGC-3′ and 5′-GCCGCGGAATTCTTATGAGGGCGCAAACTTCTTGGC-3′) with additional restriction enzyme cleavage sites to facilitate insertion into PGEX-2T such that a glutathione *S*-transferase-Ubch9 fusion protein can be produced in *Escherichia coli*. cDNA from human Jurkat cells was produced by reverse transcription of total RNA and the complete open reading frame of Ubch5 isolated by PCR amplification with specific primers (5′-ACAAACGGATCCATGGCGCTGAAGAGGATTCAG-3′ and 5′-GCGCGGGATCCTTACATTGCATATTTCTGAGTCC-3′) and inserted into PGEX-2T as described above. A cDNA encoding the complete open reading frame of SUMO was obtained by reverse transcription followed by PCR-amplification (5′-GCCGCGGGATCCCTAAACTGTTGAATGACC-3′ and 5′-ACAAACGGATCCATGTCTGACCAGGAGGCCAAA-3′) and inserted into PGEX-2T. PCR amplification, using a downstream primer containing a stop codon after nucleotide 291 of the open reading frame (5′-GCCGAGGGATCCCTAACCCCCCGTTTGTTCCTG-3′) was used to create a

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modified form of SUMO in which the C-terminus of the protein is G97. All GST-fusion constructions were used to transform *E. coli* DH5α to ampicillin resistance. Plasmid DNA was isolated and inserts sequenced (ABI377) by Alex Houston of the University of St. Andrews DNA sequencing facility.

2.2. Expression and purification of recombinant proteins

GST-Ubch9 and GST-Ubch5 were expressed in *E. coli* strain DH5α and both GST-Sumo constructions were expressed in *E. coli* strain B834. Induction of expression, glutathione agarose affinity chromatography and thrombin cleavage of fusion proteins were as described [15]. Both Sumo proteins were further purified over an anionic exchange column (FPLC, Mono Q H5/R5) equilibrated with 50 mM Tris pH 7.5 and eluted with a 0–1 M KCl gradient in 50 mM Tris pH 7.5. Human E1 was purified from HeLa cell extracts by covalent affinity chromatography on ubiquitin-Sepharose as described [16]. *Arabidopsis thaliana* E1 was produced in *E. coli* BL21DE3 using the pET expression system [17] and purified by ubiquitin affinity chromatography as described [16].

2.3. Iodination of ubiquitin and Sumo

Human ubiquitin from Sigma, full length SUMO and $SUMO_{1-97}$ expressed in *E. coli* were radiolabeled with carrier-free Na ^{125}I (Amersham) by the chloramine-T method [18]. [^{125}I]Sumo was fully reduced by incubation with 100 mM DTT and after removal of the reducing agent by passing through a Biogel P6 spin column, free sulphydryl groups were acetylated by incubation with 10 mM iodoacetamide. After 10 min at room temperature the iodoacetamide was quenched by the addition of DTT to 20 mM.

2.4. Thioester assay

Formation of thioester adducts between recombinant E2s (GST-Ubch5 and Ubch9) and ubiquitin was determined essentially as described [19]. Reactions contained the indicated amount of *A. thaliana* E1 or human E1, 1U of inorganic pyrophosphatase (Sigma), 0.5 μg of [^{125}I]ubiquitin and recombinant E2s in a final volume of 20 μl of 50 mM Tris-HCl pH7.6, 10 mM $MgCl_2$, 1 mM ATP. Reactions were incubated at 30°C for 10 min and terminated either by boiling for 10 min in the presence of 2% (w/v) sodium dodecyl sulphate and 4% (v/v) 2-mercaptoethanol, or by incubating the samples at 30°C for 15 min in the same buffer containing 4 M urea instead of 2-mercaptoethanol. Samples were subjected to SDS-PAGE (12.5%) and dried gels analysed by phosphorimaging (Fujix BAS1000, MacBAS software). Formation of thioester adducts between recombinant E2s and [^{125}I]Sumo was determined using the above procedure with 0.5 μg of [^{125}I]Sumo and instead of purified E1 enzyme, a HeLa cell fraction containing Sumo E1 activity was used.

2.5. Preparation of HeLa cell fractions

HeLa cell extracts were prepared and fractionated on Q-Sepharose into Fr I and Fr II.1–II.5 as described [20].

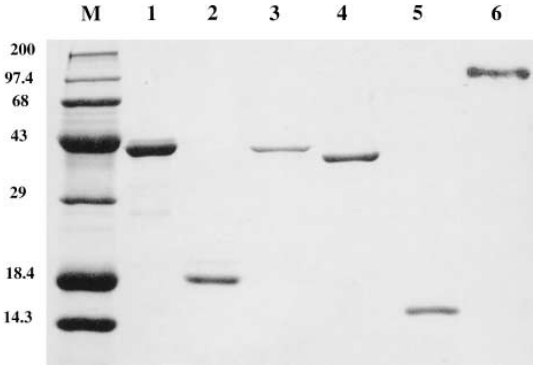

Fig. 1. Purification of recombinant proteins. Recombinant proteins were purified from *E. coli* as described in Section 2, subjected to SDS-PAGE (12.5%) and stained with Coomassie blue. Lane 1, 1.5 μg GST-Ubch9 (44 kDa); lane 2, 1 μg Ubch9 (18 kDa); lane 3, 1 μg GST-Ubch5 (42 kDa); lane 4, 1.5 μg GST-$SUMO_{1-97}$ (38 kDa); lane 5, 1 μg $SUMO_{1-97}$ (12 kDa); lane 6, 1 μg *A. thaliana* E1 (123 kDa). The molecular weight of protein markers (M) is shown at the left.

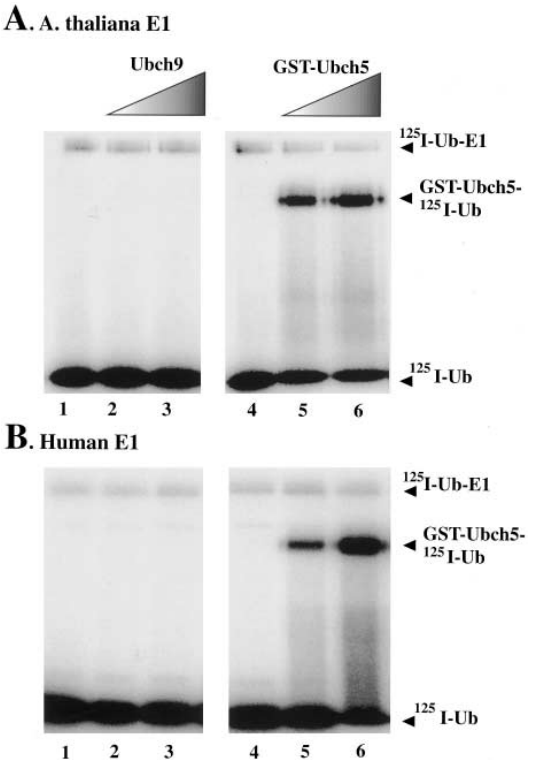

Fig. 2. Ubch9 is unable to form a thioester with ubiquitin. Assay for thioester formation between [^{125}I]ubiquitin and Ubch9 (0.9 μg lane 2, 1.8 μg lane 3) or GST-Ubch5 (0.7 μg lane 5, 1.4 μg lane 6) in the presence of recombinant *A. thaliana* E1 (A) or human E1 from HeLa cells (B). After 10 min at 30°C reactions were terminated and products fractionated by SDS-PAGE under non-reducing conditions. ^{125}I radioactivity in the dried gel was detected by phosphorimaging. The positions of [^{125}I]ubiquitin and thioester adducts with E1 and E2 proteins are indicated.

3. Results

3.1. Ubch9 is unable to form a thioester with ubiquitin

The NF-κB/Rel proteins are sequestered in the cytoplasm of unstimulated cells in association with IκBα. In response to external signals, the N-terminal region of IκBα is phosphorylated and ubiquitinated prior to degradation by the proteasome. Released NF-κB translocates to the nucleus and activates a large number of responsive genes. To identify proteins involved in IκBα signalling, a yeast two hybrid screen was used to isolate human cDNAs encoding proteins that could interact with the N-terminal regulatory domain of IκBα (1–74). Such a human cDNA was isolated and sequenced to reveal an open reading frame of 474 nucleotides encoding a 158 amino acid protein, identified as the human protein Ubch9, which is homologous to ubiquitin conjugating enzymes. Although this protein has been repeatedly isolated in yeast two hybrid screens evidence that the protein has ubiquitin conjugating activity has not been reported. To characterise the enzymatic activity of Ubch9 the protein was expressed as a GST fusion in bacteria and purified. A known ubiquitin conjugating E2 enzyme, Ubch5 [21], was expressed and purified in similar fashion (Fig. 1). The ability of purified Ubch9 and GST-Ubch5 to form a thioester with [^{125}I]ubiquitin in the presence of MgATP and recombinant E1 from *A. thaliana* was analysed by SDS-PAGE under non-reducing conditions. While a thioester was formed between GST-Ubch5 and

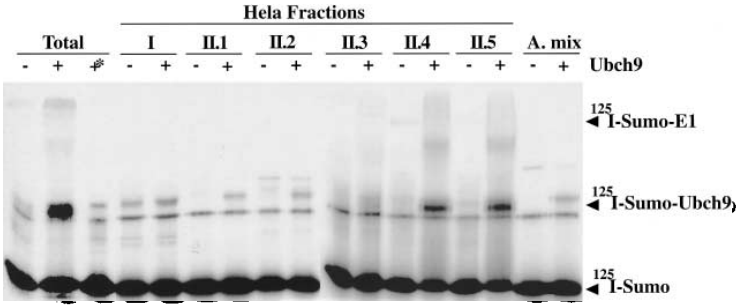

Fig. 3. Detection of a SUMO activating enzyme that transacetylates Ubch9. Unfractionated extract from HeLa cells (Total) or extract fractionated by Q-Sepharose chromatography (fractions I, II.1, II.2, II.3, II.4, II.5), was assayed for the ability to form thioester adducts with [^{125}I]SUMO either alone (−) or in the presence of 1 μg Ubch9 (+). ATP was omitted from the lane marked with an asterisk and control lanes (A.mix) did not contain any HeLa extract fraction. Reactions products were analysed as described in the legend to Fig. 2.

[^{125}I]ubiquitin none could be detected using Ubch9 (Fig. 2A). To rule out the possibility that this result was a consequence of incompatibility between plant E1 and human E2 enzymes, the human E1 enzyme was affinity purified from HeLa cells and tested in the same assay with identical results (Fig. 2B). Thus, under the conditions employed Ubch9 did not display ubiquitin conjugating activity.

3.2. *Ubch9 forms a thioester with iodinated Sumo*

Recently a small ubiquitin-like protein known variously as UBL1, SUMO, Sentrin and PIC1 has been found covalently linked to RanGAP1 and associated with Ubc9 [6–11]. We therefore investigated whether Ubch9 might be a SUMO conjugating enzyme rather than a ubiquitin conjugating enzyme. Full length SUMO or a modified form lacking four amino acids from the C-terminus, which is thought to be the active form of SUMO [6], were produced in bacteria and purified. To determine if ubiquitin activating enzyme could activate SUMO, human and *A. thaliana* E1 were used in a thioester assay with Ubch9 but were unable to promote thioester formation between Ubch9 and SUMO (data not shown), although these E1 enzymes were active for the formation of a thioester between ubiquitin and GST-Ubch5 (Fig. 2). Thus it seemed likely that SUMO activation would require a distinct E1 activity, distinct from that involved in ubiquitin activation. To explore this possibility a cytoplasmic extract from HeLa cells was incubated with [^{125}I]SUMO in the presence or absence of Ubch9. An activity capable of catalysing formation of a Ubch9-[^{125}I]SUMO thioester in an ATP dependent fashion was detected with the 97 amino acid form of SUMO (Fig. 3) but not with full length SUMO (data not shown). Fractionation of the HeLa extract by anion exchange chromatography revealed that the SUMO E1 activity was eluted with 0.4 and 0.5 M KCl (Fig. 3) and was distinct from the ubiquitin E1 activity which elutes with 0.3 M KCl (data not shown). An apparent background activity detected in the absence of ATP could be explained by formation of a disulphide linkage between C52 in SUMO and Ubch9. This activity was eliminated by iodoacetamide mediated acetylation of SUMO. The ability of Ubch9 to form a thioester with acetylated [^{125}I]SUMO in the presence of the SUMO E1 activity contained in Fr II.4 was determined. In presence of ATP Ubch9 forms a conjugate with [^{125}I]SUMO that is labile to reducing agents, such as 2-mercaptoethanol (Fig. 4), indicating that the two molecules are linked by a thioester bond. Under the same conditions GST-Ubch5 was unable to form conjugates with [^{125}I]SUMO. Thus Ubch9 has the properties of a SUMO conjugating enzyme that can act in concert with a unique SUMO activating enzyme.

4. Discussion

Ubch9 was isolated in a yeast two hybrid screen as a protein that could interact with the N-terminal region of IκBα. As IκBα undergoes signal induced ubiquitination within the N-terminal domain our expectation was that Ubch9 could participate in the ubiquitination of IκBα. Using an in vitro system for the phosphorylation and ubiquitination of IκBα [20,22] we were unable to demonstrate a role for Ubch9 (data not shown). Furthermore Ubch9 was unable to form a thioester with ubiquitin, in the presence of the E1 ubiquitin activating enzyme, under conditions where this activity could be clearly demonstrated with Ubch5 (Fig. 2).

As it has been reported [9] that the ubiquitin-like protein SUMO is found in complexes containing Ubc9 it has been suggested that Ubc9 might be involved in SUMO conjugation rather than ubiquitination [11]. Here we demonstrate that this is indeed the case as Ubch9 can form a thioester with SUMO (Figs. 3 and 4) provided that an, as yet, unidentified SUMO activating enzyme is present. A possible candidate for such an activity is the yeast protein Uba2p which is homologous to Uba1, the *Saccharomyces cerevisiae* E1 ubiquitin activating enzyme. The UBA2 gene is essential for viability in yeast and overexpression of the UBA1 gene does not complement

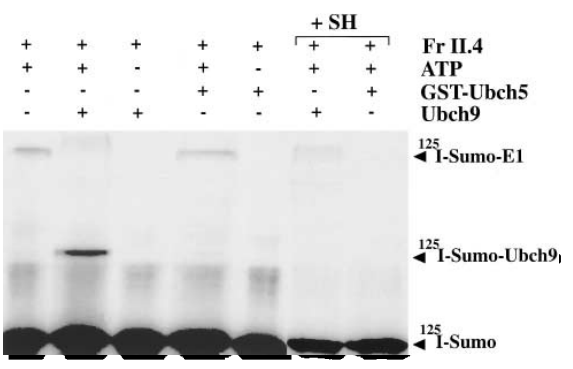

Fig. 4. Ubch9 is a SUMO conjugating enzyme. Ubch9 (1 μg) and GST-Ubch5 (1 μg) were assayed for their ability to be transacetylated by [^{125}I]SUMO (treated with iodoacetamide) in the presence of the SUMO activating enzyme present in HeLa fraction II.4. Assays were conducted either in the absence (−) or presence (+) of ATP and reaction products analysed as described in the legend to Fig. 2. The indicated samples (+SH) were analysed under reducing conditions after treatment with 2-mercaptoethanol.

this defect. Like Uba1, Uba2p contains an essential cysteine but is unable to activate or form a thioester with ubiquitin [23]. It seems likely that SUMO conjugation is carried out by a series of transacetylation reactions that is similar but distinct from the ubiquitination pathway. Others have reported an interaction between Ubch9 and IκBα in yeast [24] and interpreted this as an involvement in IκBα ubiquitination and degradation. Given the inability of Ubch9 to conjugate ubiquitin in vitro it seems unlikely that Ubc9 is involved in IκBα degradation, but a role for SUMO modification in other aspects of IκBα metabolism, such as nuclear import [25], cannot be ruled out.

A remaining puzzle is why Ubc9 is isolated from yeast two hybrid screens by such a wide variety of proteins. One possible explanation is that 'bait' proteins are modified by SUMO in yeast and it is the association between SUMO and Ubc9 that mediates the apparent interaction between Ubc9 and bait proteins.

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