

Molecular cloning and characterization of human AOS1 and UBA2, components of the sentrin-activating enzyme complex

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Abstract Sentrin-1/SUMO-1 is a novel ubiquitin-like protein, which can covalently modify a limited number of cellular proteins. Here we report the identification of the sentrin-activating enzyme complex, which consists of two proteins AOS1 and UBA2. Human AOS1 is homologous to the N-terminal half of *E1*, whereas human UBA2 is homologous to the C-terminal half of *E1*. The human *UBA2* gene is located on chromosome 19q12. Human UBA2 could form a β -mercaptoethanol-sensitive conjugate with members of the sentrin family, but not with ubiquitin of NEDD8, in the presence of AOS1. Identification of human UBA2 and AOS1 should allow a more detailed analysis of the enzymology of the activation of ubiquitin-like proteins.

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Key words: Ubiquitin; Sentrin; SUMO; UBC9; UBA2; AOS1

1. Introduction

Protein modification by ubiquitin has been studied extensively in the last two decades [1,2]. The ATP-dependent ubiquitination reaction is carried out by a multi-step pathway. Initially, the ubiquitin-activating enzyme (*E1*) utilizes ATP to adenylate the ubiquitin C-terminus, which is then transferred to a conserved Cys residue in the *E1*, resulting in the formation of a high-energy thiol ester bond between ubiquitin and *E1*. This thiol ester bond is subsequently transferred to a Cys residue in one of many ubiquitin-conjugating enzymes (*E2*). The final transfer of ubiquitin to an ϵ -amino group of a lysine residue in the target protein may require a ubiquitin ligase (*E3*) enzyme. During the last three years, post-translational modification of proteins by ubiquitin-like proteins has rapidly become a very active and significant area of research. We previously reported the cloning of a ubiquitin-like protein, sentrin-1, which interacts specifically with the death domains of both Fas and tumor necrosis factor receptor 1 [3]. Sentrin-1 and its yeast homologue, SMT3, have been shown to covalently modify a limited number of cellular proteins in a process analogous to protein ubiquitination [4–10]. Activation of SMT3 requires the cooperation of two yeast proteins, UBA2 and AOS1 [10] which are homologous to the C- or N-terminal portion of *E1*, respectively. It is not known whether sentrin-1 is also activated by a similar enzyme complex in mammalian cells. In this report, we describe the cloning and characterization of the human *UBA2* and *AOS1* genes from a placenta cDNA library. We show that both UBA2 and AOS1 are required for the activation of all sentrin family members. These

data strengthen the hypothesis that both sentrin and SMT3 share a similar conjugating pathway and demonstrate that the ubiquitin- and sentrin/SMT3-conjugating pathways diverged from each other at an early stage.

2. Materials and methods

2.1. cDNA cloning of the human *UBA2* and *AOS1*

To clone human UBA2, we initially performed a tBLASTn search of the TIGR Tentative Human Consensus Sequences (THCs) database using the partial sequence of human UBE1 protein (amino acids 450 to 500) containing the ATP-binding region. Seventeen positive THC fragments were identified. The nucleotide sequence of one positive THC fragment (THC206278) was used to perform another BLAST search of the TIGR THCs database. One of the positive THC fragments (THC175035) was shown to partially overlap with THC206278. A further search of TIGR THCs database using THC175035 resulted in identification of THC159422. PCR amplification was used to confirm the cDNA sequence. Two oligonucleotide primers generated according to the information from THC206278 were used to extend the cDNA sequence towards the 5' direction by RACE method. Extension of the cDNA towards the 3' direction was performed by RACE with the oligonucleotides generated from THC159422. Using a pair of redesigned 5' and 3' primers compatible with the sequence revealed in the 5' and 3' anchored PCR reactions, we then amplified a 2.7 kb cDNA fragment by PCR reaction.

To identify the human AOS1 gene, we used the yeast AOS1 amino acid sequence to screen the EST databases for human ESTs encoding putative AOS1 homologue. The tBLASTn searches identified a number of matching human ESTs. AA176210 is the longest (546 bp) of these cDNAs. A search of the EST database with the nucleotide sequence from AA176210 identified two additional ESTs (AA313766 and N57252). A further search of the human EST database using N57252 identified H98166. PCR amplification was used to confirm the cDNA sequence. Nested primers were designed to the 5' and 3' end of the clone, and gene amplification using DNA from the cDNA library was utilized to recover two fragments containing more of the 5' and 3' ends.

2.2. PCR and RACE amplification of cDNA fragment

Unless otherwise specified, the PCR reaction was carried out in a volume of 50 μ l that contained 50 mM KCl, 2 mM MgCl₂, 0.2 mM dNTP, 10 mM Tris-HCl, pH 8.3, 100 ng of each primer, and 25 units of *Pfu* polymerase, which possesses proof reading activity (Clontech), on a PTC-200 programmable thermal cycler (MJ Research Inc.). The reaction mixture was denatured at 94°C for 4 min, and the amplification reaction consisted of 30 cycles of denaturation (94°C, 30 s), annealing (55°C, 50 s), and extension (72°C, 1 min) with a final extension at 72°C for 5 min. For RACE, nested primers specific to human UBA2 cDNA and nested adaptor primers were used in the primary and secondary PCRs. A thermal cycling profile of 94°C for 30 s, 65°C for 30 s, and 72°C for 90 s (25 cycles) was used in the primary PCR, with a human placenta cDNA library being used as a template. A cycling profile of 94°C for 50 s and 70°C for 3 min (30 cycles) was used for the secondary PCR, with 1 μ l of the primary PCR product functioning as a template.

2.3. DNA sequencing and analysis

The DNA sequence of the identified clones was determined on both strands using Applied Biosystems Prism dye termination DNA se-

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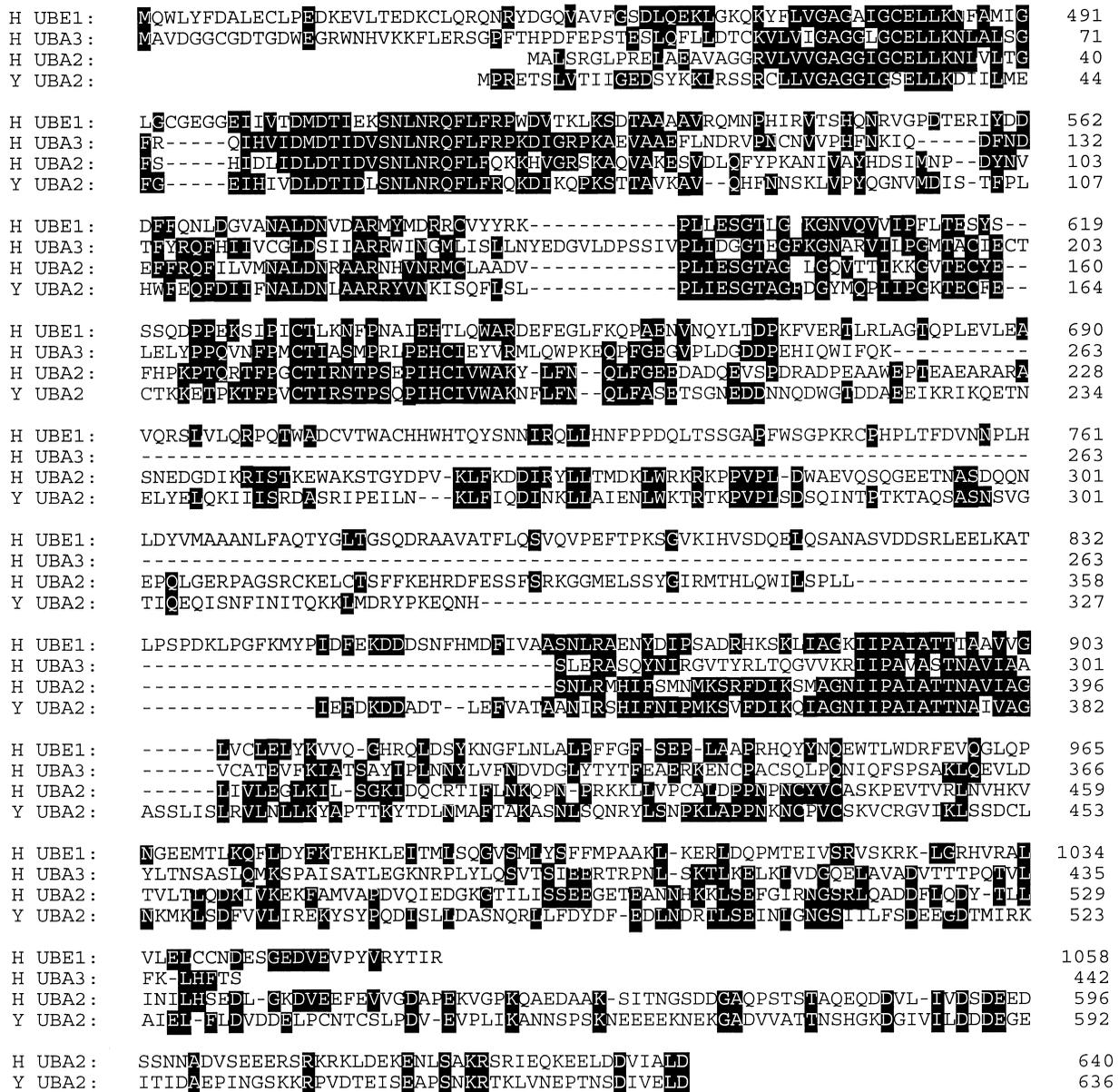


Fig. 1. Sequence alignment of human UBE1, UBA3, UBA2 and yeast UBA2. Amino acids that are identical to the corresponding position are shaded black. The human UBA2 sequence is deposited in the GenBank database with the accession number AF079566.

quencing reagents and an automated ABI 377 sequencer (Applied Biosystems).

2.4. Expression and purification of GST fusion proteins

The plasmids pGEX-UB, pGEX-NEDD8, and pGEX-sentrin-1, -2, and -3 were used for expression of GST fusion proteins. The GEX expression vectors were introduced into competent BL21 bacteria, and expression of GST fusion proteins was induced with isopropyl-β-D-thiogalactopyranoside and purified as described previously [12].

2.5. In vitro translation

For in vitro translation of UBA2 and AOS1, we constructed pcDNA3-UBA2 and pcDNA3-AOS1 plasmids by inserting full length UBA2 and AOS1, respectively, into the pcDNA3 vector. The resulting plasmids were used as templates for in vitro transcription and translation using T7 RNA polymerase (Promega) under conditions recommended by the supplier. Translation reaction were performed in TNT T7-coupled rabbit reticulocyte lysates (Promega) in a final volume of 50 μl. Reaction mixtures contained 25 μl of lysate, 40 μCi of [³⁵S]methionine (1000 Ci/mmol), 15 units of RNasin (Promega),

50 μM amino acid mixture (minus methionine), and 1 μg of DNA. The reactions were incubated for 90 min at 30°C and stopped with the addition of three volumes of Laemmli buffer. Translation products were analyzed using 15% SDS-polyacrylamide gel electrophoresis. Gels were fixed, treated in amplify solution (Amersham), dried, and processed for autoradiography.

2.6. GST-pulled down assay

To demonstrate interaction between ubiquitin, NEDD8, or sentrin-1 and UBA2, 10 μl of in vitro translated, [³⁵S]-labeled UBA2 were incubated with 50 μl of glutathione-Sepharose beads containing approximately 1 μg of GST, GST-UB, GST-NEDD8 or GST-sentrin-1 for 30 min at room temperature in a solution of 20 mM Tris-HCl (pH 7.52), 50 mM NaCl, 10 mM MgCl₂, 0.1 mM DTT, 4 mM ATP and 5 U/ml inorganic pyrophosphatase. After washing five times with the same buffer, the samples were treated with SDS loading buffer and loaded onto a 15% SDS-polyacrylamide gel and visualized by autoradiography. In order to demonstrate the formation of UBA2-sentrin conjugates, the above reaction was terminated following a 10 min incubation time in the presence of AOS1. Briefly, 10 μl of

[³⁵S]methionine-labeled UBA2 and 40 μl of AOS1 were mixed with 50 μl of glutathione-Sepharose beads containing approximately 1 μg of GST, GST-UB, GST-NEDD8 or GST-sentrin-1 for 10 min at room temperature in a solution of 20 mM Tris-HCl (pH 7.52), 50 mM NaCl, 10 mM MgCl₂, 0.1 mM DTT, 4 mM ATP and 5 U/ml inorganic pyrophosphatase. The reactions were terminated with the addition of SDS-containing loading buffer.

3. Results and discussion

3.1. Cloning of the human UBA2 cDNA

In an effort to identify the activating enzyme for sentrin, we searched the Tentative Human Consensus Sequences (THCs) database generated by the Institute for Genomic Research (TIGR). Using an approach described in Section 2, a 2683 bp cDNA fragment was cloned from a human placenta cDNA library. The cloned cDNA contains an open reading frame encoding a protein of 640 amino acid residues. The amino acid sequence was compared with sequences in several databases using the BLAST network service at the National Center for Biotechnology Information. As shown in Fig. 1, the predicted amino acid sequence is more closely related to yeast UBA2 (33% identity) than human UBA3 (22% identity) [11] or the C-terminal portion of human E1 (24% identity). We named this protein UBA2 because of its close homology to yeast UBA2 and its ability to form a thiol ester bond with

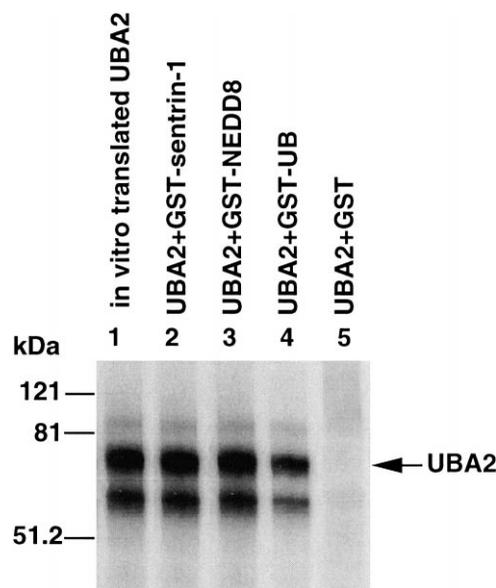


Fig. 2. In vitro interaction of ubiquitin, NEDD8, and sentrin-1 with UBA2. In vitro translated UBA2 (lane 1) was precipitated by GST-sentrin-1 (lane 2), GST-NEDD8 (lane 3), GST-ubiquitin (lane 4) and GST (lane 5) in the presence of ATP.

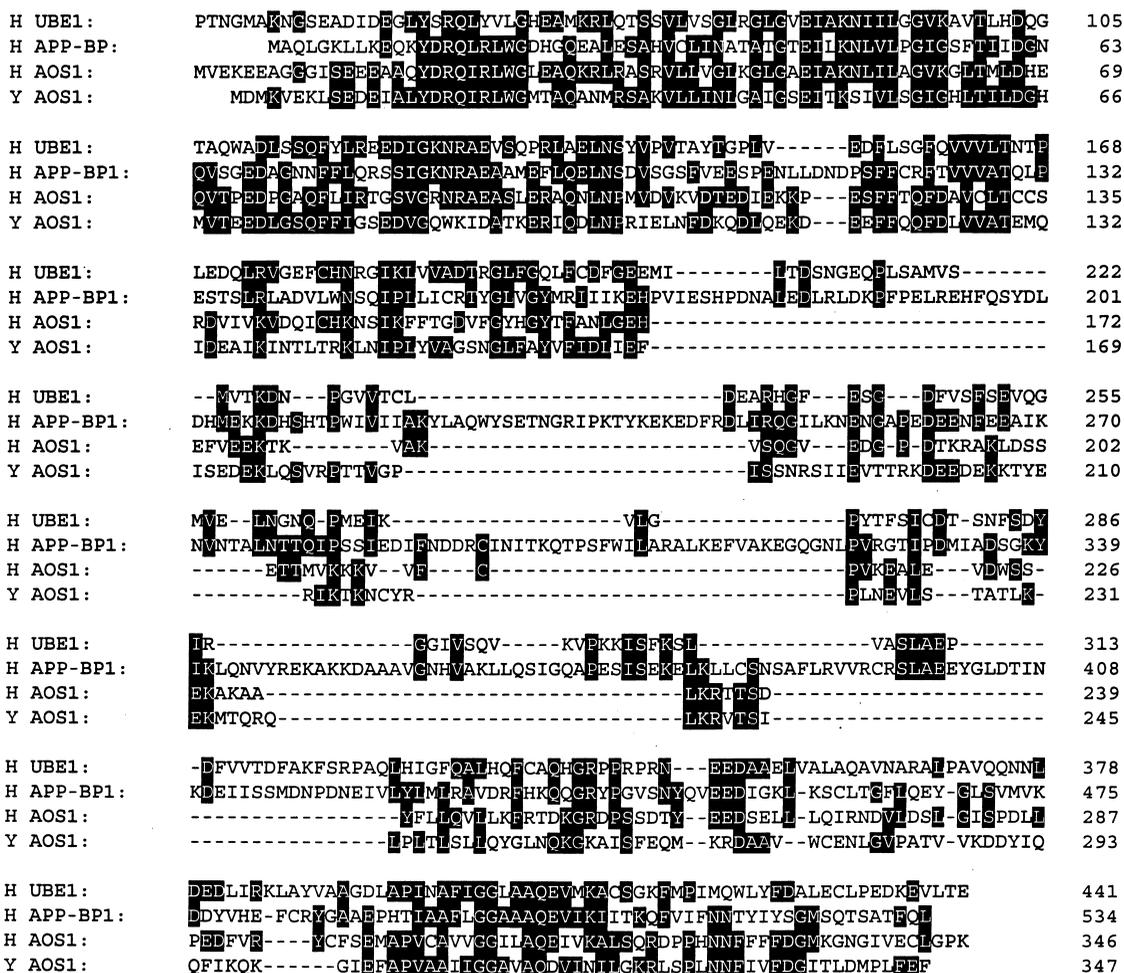


Fig. 3. Sequence alignment of human UBE1, APP-BP1, AOS1 and yeast AOS1. Amino acids that are identical to the corresponding position are shaded black. The human AOS1 nucleotide sequence is deposited in the GenBank database with the accession number AF046025.

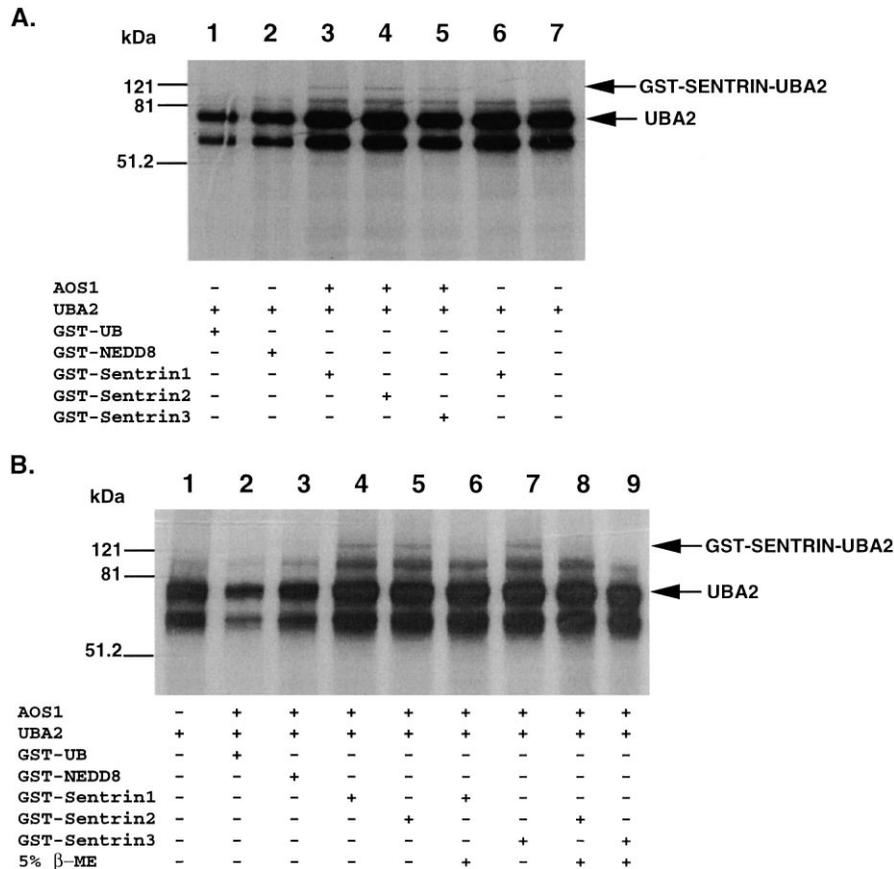


Fig. 4. Formation of UBA2-sentrin conjugates. A: Formation of a 120 kDa conjugate of UBA2 and sentrin-1 (lane 3), sentrin-2 (lane 4), and sentrin-3 (lane 5) in the presence of AOS1. B: The conjugates between UBA2 and sentrin family members (lanes 4, 5, and 7) were sensitive to β-mercaptoethanol treatment (lanes 6, 8, and 9).

sentrin family members (see below). The calculated molecular weight, based on the cDNA derived amino acid sequence of human UBA2, is 71.5 kDa, identical to the kDa value estimated by SDS-PAGE analysis (see below).

3.2. Chromosomal localization of the human UBA2 gene by EST mapping

To determine the chromosomal localization of the human UBA2, we employed an EST mapping strategy. The human UBA2 cDNA sequence was used to search the GenBank EST database using the BLAST program, resulting in the positive identification of 79 EST clones. All EST clones were subsequently used to check the Human Gene Map database. Eight clones, namely AA017441 (nt position 2216–2682), AA029770 (nt position 2199–2682), AA088220 (nt position 2199–2682), AA169833 (nt position 2265–2682), AA132238 (nt position 2275–2682), H09723 (nt position 2211–2676), N50800 (nt position 2366–2682), and D57425 (nt position 2295–2550), have been mapped to chromosome 19 between the D19S222 and D19S425 microsatellite markers at 49–58 centimorgans (National Center for Biotechnology Information). Thus, the UBA2 gene is located on chromosome 19q12. Both E1-like genes UBE1L and UBA3 are located in chromosome 3, whereas E1 (UBE1) is located in chromosome X.

3.3. Human UBA2 could bind to sentrin-1, ubiquitin, and NEDD8 in vitro

To determine whether UBA2 might directly associate with

sentrin-1, we employed a modified GST-pulled down assay. GST fusion proteins of ubiquitin, NEDD8, and sentrin-1, bound to GSH beads, were incubated with in vitro translated UBA2 at room temperature for various periods of time in the presence of ATP. The GST beads were extensively washed and the precipitated proteins were analyzed with SDS-PAGE. As shown in Fig. 2, UBA2 translated in vitro results in two major bands (72 and 62 kDa) (Fig. 2, lane 1). This is most likely due to differential usage of the two ATG codons (91–93, 379–381) in the UBA2 sequence. Both UBA2 bands could be precipitated by GST-ubiquitin (Fig. 2, lane 2), GST-NEDD8 (Fig. 2, lane 3), and GST-sentrin-1 (Fig. 2, lane 4), but not with GST (Fig. 2, lane 5). Similar binding pattern could be observed in the absence of ATP (data not shown). No higher molecular weight conjugate was observed in any of these GST-pulled down assays, suggesting that UBA2 alone cannot form a thiol ester conjugate with sentrin-1.

3.4. Cloning of human AOS1

The fact that human UBA2 is similar to the C-terminal region of E1, but lacks a region homologous to the N-terminus of E1, and the failure of UBA2 to form a thiol ester conjugate with sentrin-1 suggests that an unidentified cofactor might be required for sentrin-1 activation. During our study of human UBA2, Johnson et al. [10] reported that the yeast homologue of sentrin-1, SMT3, is activated by an enzyme complex composed of UBA2 and AOS1. Since SMT3 and sentrin-1 both utilized UBC9 as a specific conjugating enzyme

[12–14], it is likely that another component, similar to yeast AOS1, is required for sentrin-1 activation. In order to clone the human AOS1 gene, we used the yeast AOS1 amino acid sequence to screen the EST database for human ESTs encoding putative AOS1 homologues. Using a strategy similar to the one used to clone UBA2, we cloned a 2119 bp cDNA. The cloned fragment encodes a 346 amino acid polypeptide, AOS1. A comparison of the deduced amino acid sequence of the AOS1 protein with that of yeast AOS1 shows 34% identity and 54% similarity (Fig. 3). Further analysis revealed a highly conserved region in the N-termini of AOS1 (AA position 17–57), *E1*, and APP-BP1 (a subunit of the NEDD8-activating complex [11]). This conserved region has revealed quite a few interesting amino acid substitutions, which may provide some insight into the manner in which these different enzymes function biochemically, as well as a basis for their substrate specificity. First of all, AOS1 and APP-BP1 have a negatively charged aspartic acid at position 20 (in human AOS1), whereas *E1* has an uncharged, polar serine residue at the corresponding position. Another interesting substitution occurs at position 24 (in human AOS1) where both AOS1 and APP-BP1 have a positively charged arginine residue and *E1* has an uncharged, polar tyrosine residue. Lastly, both AOS1 and APP-BP1 have an uncharged, polar glutamine residue at position 31 (AOS1), whereas *E1* has a non-polar methionine residue at that position.

3.5. Both UBA2 and AOS1 are required for sentrin activation

To confirm that sentrin-1 activation requires both UBA2 and AOS1, [³⁵S]met-labeled UBA2 and in vitro translated AOS1 were mixed and incubated with GST-sentrin-1 and ATP. As shown in Fig. 4A, a GST-sentrin-1-UBA2 conjugate of ~120 kDa was visualized (lane 3). In contrast, neither ubiquitin (lane 1) nor NEDD8 (lane 2) can form this 120 kDa conjugate. The formation of the GST-sentrin-1-UBA2 conjugate requires the presence of AOS1 (compare lane 3 and lane 6). We were unable to observe any UBA2-ubiquitin conjugation product following longer exposure of the gel. Furthermore, ubiquitin could not be conjugated to UBA2 in the presence of ATP and AOS1 with a range of incubation time from 10 min to 2 h. Importantly, this 120 kDa band could be destroyed by β -mercaptoethanol treatment, suggesting that it is a thiol ester linked conjugate (Fig. 4B). It is important to note that these thiol ester conjugate occurs with-

in 10 min of incubation. However, we were unable to detect any thiol ester conjugate beyond 15 min, raising the possibility that sentrin, once activated, is quickly transferred to UBC9 present in our translation mix. There are three sentrin family members [8,9,15–17] that have overlapping substrate specificity. We also demonstrated that sentrin-2 or sentrin-3 could form a β -mercaptoethanol-sensitive conjugate with UBA2 (Fig. 4B, lanes 4 and 5). Thus, all of the sentrin family members can be activated by the UBA2/AOS1 enzyme complex.

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