Identification of the Enzyme Required for Activation of the Small Ubiquitin-like Protein SUMO-1* 

(Received for publication, December 7, 1998)

Joana M. P. Desterro‡, Manuel S. Rodriguez, Graham D. Kemp, and Ronald T. Hay§

From the School of Biomedical Science, University of St. Andrews, St. Andrews, Fife KY169ST Scotland

The ubiquitin-like protein SUMO-1 is conjugated to a variety of proteins including Ran GTPase-activating protein 1 (RanGAP1), IxBα, and PML. SUMO-1-modified proteins display altered subcellular targeting and/or stability. We have purified the SUMO-1-activating enzyme from human cells and shown that it contains two subunits of 38 and 72 kDa. Isolation of cDNAs for each subunit indicates that they are homologous to ubiquitin-activating enzymes and to the Saccharomyces cerevisiae enzymes responsible for conjugation of Smt3p and Rub1p. In vitro, recombinant SAE1/SAE2 (SUMO-1-activating enzyme) was capable of catalyzing the ATP-dependent formation of a thioester linkage between SUMO-1 and SAE2. The addition of the SUMO-1-conjugating enzyme Ubc9 resulted in efficient transfer of the thioester-linked SUMO-1 from SAE2 to Ubc9. In the presence of SAE1/SAE2, Ubc9, and ATP, SUMO-1 was efficiently conjugated to the protein substrate IxBα. As SAE1/SAE2, Ubc9, SUMO-1, and IxBα are all homogenous, recombinant proteins, it appears that SUMO-1 conjugation of IxBα in vitro does not require the equivalent of an E3 ubiquitin protein ligase activity.

Covalent linkage of one protein to another represents an important means of generating protein conjugates with unique properties. Although the best characterized example of such a modification is the addition of ubiquitin to proteins destined for proteolysis, it is now recognized that a number of other small protein molecules can be linked to target proteins in a similar fashion. Ubiquitin addition is accomplished via a thioester cascade with ubiquitin first being activated by a unique E1 enzyme that utilizes ATP to adenylate the C-terminal glycine of ubiquitin. Release of AMP accompanies the formation of a thioester bond between the C terminus of ubiquitin and a cysteine residue in the E1 protein. In a transesterification reaction, the ubiquitin is transferred from the ubiquitin-activating enzyme to an E2 ubiquitin-conjugating enzyme, which may in turn transfer the ubiquitin to an E3 ubiquitin protein ligase. In many cases it is this enzyme that recognizes the protein substrate and catalyzes formation of an isopeptide bond between the C terminus of ubiquitin and the ε-amino group of lysine in the target protein. Proteins destined for degradation via the proteasome are coupled to multiple copies of ubiquitin by formation of further isopeptide bonds between additional ubiquitin molecules and lysine residues in the bound ubiquitin (1). 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), whereas ubiquitination of a plasma membrane receptor modifies ligand-stimulated endocytosis (3). Whereas addition of multiple copies of ubiquitin targets proteins for degradation, it is now widely recognized that covalent attachment of other ubiquitin-related molecules does not result in degradation of the modified protein. 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-1, 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), whereas SUMO-1 modification of PML targets the protein to PML nuclear bodies (12, 13). SUMO-1 modification of IxBα takes place on the same residues used for ubiquitination, thus rendering the protein resistant to signal-induced degradation and consequently blocking NF-κB-dependent transcriptional activation (14). SUMO-1 and Smt3p, a yeast homologue of SUMO-1, are conjugated to target proteins by a pathway that is distinct from, but analogous to, ubiquitin conjugation. A separate E1-like enzyme is responsible for SUMO-1 modification (15), and in yeast, the enzyme responsible for Smt3p activation has been shown to consist of a heterodimer of Uba2p and Aos1p (16). Also found in the complex between SUMO-1-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 (17), and mammalian homologues have been isolated repeatedly from yeast two-hybrid screens in association with a wide variety of proteins. In both yeast and human cells, the product of the Ubc9 gene acts as the SUMO-1/Smt3p E2-conjugating activity (11, 15, 18, 19). Recently an additional protein modification pathway involving the ubiquitin-like protein Rub1p has been characterized in Saccharomyces cerevisiae (21, 22). A major substrate for Rub1p is
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Cdc53cullin, which is a common component of SCF (Skp1, Cdc53, F-box) ubiquitin ligase complexes. In this system the products of the ULA1 and UBA3 genes act as a heterodimeric E1 enzyme, whereas the product of the UBC12 gene acts as the E2-conjugating enzyme. It also appears that this conjugation system is active in higher eukaryotes with the Rub1p homologue Nedd8 also being conjugated to Cdc53 (23). In all of the above cases, the protein conjugated has been similar to ubiquitin. However, a separate protein conjugation system, which is required for autophagy in S. cerevisiae, involves conjugation of a protein unrelated to ubiquitin. In this case, the C terminus of Agp12, a 186-residue protein, is conjugated to a lysine side chain in Agp5. Although Agp12 is unrelated to ubiquitin, it appears that it is activated by Agp7, which is homologous to ubiquitin E1 enzymes, and conjugated by Agp10, an E2 equivalent (24).

To fully characterize the SUMO-1 modification reaction, we have purified the SUMO-1-activating enzyme and shown that it contains subunits of 38 and 72 kDa. CDNAs corresponding to these subunits (SUMO-1-activating enzyme (SAE)1 and SAE2) are homologous to enzymes involved in the activation of ubiquitin, Smt3p, and Rub1p. In the presence of recombinant SAE1/SAE2, Ubc9, and ATP, SUMO-1 was efficiently conjugated to the protein substrate 1xBa. Thus it appears that SUMO-1 conjugation in vitro does not require the equivalent of an E3 protein ligase activity.

EXPERIMENTAL PROCEDURES

Antibodies—The SV5 Pk tag 336 monoclonal antibody (25) was obtained from R. E. Randall, University of St. Andrews and was used to immunodetect and immunoprecipitate SAE1 containing an N-terminal SV5 epitope tag.

SUMO-1 Affinity Chromatography—Five mg of SUMOGG-1 (1–97 amino acids) purified as described (15) were coupled to a 1 ml N-hydroxysuccinimide-activated Hi-Trap column (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. 40% of the added protein was coupled to the beads. After the affinity chromatography procedure, the column was regenerated by washing with 10 column volumes of a buffer containing 50 mM Tris/HCl, pH 9.0, 1 mM DTT followed by 10-fold dilutions in 50 mM Tris/HCl, pH 7.5, 1 mM DTT. The final volume of the eluates were 10-fold-concentrated using Centricon 30 microcentrators. The final volume of the eluates was brought to 10% of the starting volume of loading solution until the bands were visible above a clear background. After elution, gel slices were washed for at least 1 h in 100 mM NH4HCO3, pH 8.0, and 30 min at 60 °C in 100 mM NH4HCO3, pH 8.0, 3 mM DTT. Iodoacetoamid was added to a final concentration of 20 μl of 0.1% trifluoroacetic acid and separated by HPLC using a microbore HPLC system. The amino acid sequence of selected peptides was determined as described for N-terminal peptide sequencing.

cDNA Cloning—Three and 8 peptides were sequenced from the 38-kDa and 72-kDa species, respectively. Each peptide sequence was searched for mammalian homologues using BLAST similarity search program and EST public data base. ATCC clones were obtained for some of the peptides (AA286737 for 38 kDa and AA 375795 for 72 kDa). Tentative of Human Consensus (THC) 183945 for the 38-kDa and THC 167372 for the 72-kDa protein were obtained when the sequences were used for search in the data base of THC. Analysis of DNA sequencing data allowed us to set up upstream and downstream primers flanking the coding sequence.

A cDNA encoding the complete open reading frame of SAE1 was obtained as a single fragment by reverse transcription followed by PCR (RT-PCR) using Boehringer Titan™ one-tube RT-PCR system. The following primer containing an EcoRI restriction site was used as the downstream primer for reverse transcription (5′-GGCGGAAAGCCGATCCCAAACCCTTTGCTGGGATTGGACTCC-3′). The upstream primer used for PCR amplification (5′-AAGCGATCTGTTGGAAGAAGAGGGTTCG-3′) contains a BamHI site at the 5′ end. PCR products were cloned as a BamHI/EcoRI insert in pGEX-2T (Amersham Pharmacia Biotech) or in pcDNA3 (Invitrogen) with an N-terminal Pk-SV5 tag that is recognized by the 336 monoclonal antibody (25). The DNA sequence encoding the SV5 Pk5 peptide (PNPULLIME) was inserted into pcDNA3 using KpnI and BamHI cloning sites and the following oligonucleotides: 5′-CATGCGAAGCCGATGACACCTTTGCGTGGAAGCTC-3′ and 5′-GATCCGGTGAGTCAATCCAGAAGGCGTGTGCGAGTGACC-3′ and 5′-GATCCGGTGAGTCAATCCAGAAGGCGTGTGCGAGTGACC-3′.

The cDNA of SAE2 was obtained from two different RT-PCR reactions and a final PCR. An intermediate upstream and downstream primer (5′-GATATCAATCAATTTGGAAGAAC-3′ and 5′-GGTTCTAAGGATTTTGACAAC-3′) were used in each RT-PCR reaction to generate two different fragments that were later used in the final PCR reaction as template with the following primers: 5′-GAAGATTCCATCGGAATTCTCA-3′ and 5′-GGCTTCTAAGGATTTTGACAAC-3′.

The DNA sequence of all plasmids was determined by Alex Houston of the University of St. Andrews DNA sequencing facility (ABI377).

In Vitro Transcription/Translation—In vitro transcription/translation was performed using 1–2 μg of plasmid DNAs and a TNT-coupled wheat germ extract system (Promega) according to the instructions provided by the manufacturer. [35S]Methionine (Amersham Pharmacia Biotech) was used in the reactions to generate radiolabeled protein.

Expression and Purification of Recombinant Proteins—1xBa, Ubc9, and SUMOOG-1 were expressed and purified as described previously (15, 28). GST-SAE1 was expressed in Escherichia coli B834 and purified as described for glutathione S-transferase (GST)-lBe (28).

Cell Culture and Transfections—COS7 cells were maintained in exponential growth in Dulbecco’s modified Eagle’s medium containing thiglycolate to 0.1 mM in the upper electrophoresis buffer. Proteins were electrophoretically transferred to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech), and proteins were stained with 0.1% Amido Black in 40% methanol, 1% acetic acid, for 30 s before bands were excised. Excised membrane bands were extensively washed with distilled water, and the peptide sequence was determined using a Prolase microsequencer (Applied Biosystems) with on-line phenylthiohydantoin analysis.

"In-gel" Trypsin Digestion—Samples to be digested in the gel were fractionated by SDS-PAGE as described for N-terminal peptide sequencing. The gel was stained in 0.1% Coomassie Brilliant Blue R-250, dehydrated with ethanol and acetone, and air dried. After overnight digestion with trypsin, the gel slices were washed with 50% acetonitrile, 100 mM NH4HCO3, pH 8.0, for 1 h with shaking. After trypsinination, the gel slices were reswollen with 25 mM NH4HCO3, pH 8.0, containing modified trypsin (Promega) and incubated for 4 h at 37 °C. The supernatant was acidified by adding trifluoroacetic acid to a final concentration of 1%, and peptides were extracted from gel slices with water (or 60% acetonitrile in water) containing 1% trifluoroacetic acid for 20 min. All supernatants were centrifuged, and after evaporation to near dryness, peptides fragments were reconstituted in 20 μl of 0.1% trifluoroacetic acid and separated by HPLC using a microbore HPLC system. The amino acid sequence of selected peptides was determined as described for N-terminal peptide sequencing.
10% fetal calf serum. 1–2 × 10^6 293T cells were plated to subconfluent 75-cm² flasks using LipofectAMINE™ according to instructions provided by the manufacturer (Life Technologies, Inc.). After 36 h of expression, cells were washed in phosphate-buffered saline, and extracts were prepared by lysis in 20 mM sodium phosphate buffer, pH 7.5, 50 mM NaF, 2 mM EDTA, 0.5% Nonidet P-40, 5 mM tetrasodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate containing complete® protease inhibitor mixture (Boehringer Mannheim). The lysates were cleared by centrifugation, and the supernatants were used for immunoprecipitations. 10 μl of each lysate was fractionated on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Sigma). Protein expression was checked by Western blotting using the SV5 PK tag monoclonal antibody with an ECL detection system and horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech) as secondary antibody.

Immunoprecipitations—Extracts from COS 7-transfected cells or in vitro transcribed/translated SAE proteins were incubated for 1 h at 4 °C with 336 anti-SV5 antibody either in lysis buffer or 50 mM Tris, pH 7.5, 10 mM MgCl₂, 1 mg/ml bovine serum albumin containing complete® protease inhibitor mixture and 0.5% Nonidet P-40. Washing buffer was removed, and beads, after resuspension in 3× disruption buffer (5% SDS, 0.15 M Tris/HCl, pH 6.7, 30% glycerol, 0.72 M β-mercaptoethanol) were fractionated in a 10% polyacrylamide gel. Dried gels were exposed to a phosphorimaging screen to detect 35S radioactivity. In vitro transcribed/translated SAE proteins were incubated for 1 h at 4 °C with 336 anti-SV5 in 50 mM Tris, pH 7.5, 10 mM MgCl₂ containing complete® protease inhibitor mixture, antigen-antibody complexes were used as the source of SUMO-1-activating enzyme in thioester or conjugation assays.

Protein Interaction Assays—GST or GST-SAE1 beads were incubated with [35S]methionine-labeled in vitro transcribed/translated SAE2 for 1 h at room temperature. Beads were collected by centrifugation and washed 4 times with 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂ buffer containing 1 mM ATP and complete® protease inhibitor mixture, respectively. Antigen-antibody complexes were collected by adding 20 μl of protein A-Sepharose beads, and incubation was continued for an additional 2 h on a rotating shaker. The beads were collected by brief centrifugation, and after extensive washing with ice-cold 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂ containing complete® protease inhibitor mixture, antigen-antibody complexes were used as the source of SUMO-1-activating enzyme in thioester or conjugation assays.

Thioester Assay—Recombinant SUMO-GG-1 was acetylated and radiolabeled with carrier-free Na[125I] (Amersham Pharmacia Biotech) by the chloramine T method as described (15). To detect the activity of SUMO-1-activating enzyme, formation of thioester adducts between either immunoprecipitated or affinity-purified SAE and SUMO-GG-1 was determined essentially as described (29). Immunoprecipitated protein A beads or 200 ng of affinity-purified SAE were incubated with 1 unit of inorganic pyrophosphatase (Sigma), 0.5 μg of 125I-SUMO-GG-1 in the presence or absence of 0.6 μg of recombinant Ubch9 in a final volume of 20 μl of 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP. Reactions were incubated at 30 °C for 10 min and terminated either by boiling for 5 min in the presence of 2% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, and 50 mM DTT (± DTT) or by incubating the samples at 30 °C for 20 min in the same buffer containing 4 M urea instead of DTT and mercaptoethanol. Samples were subjected to SDS-PAGE (10%), and dried gels were analyzed by phosphorimaging (Fujix BAS1500, MacBAS software).

In Vitro SUMO-1 Conjugation Assay—SUMO-1 conjugation using recombinant proteins was accomplished in a 20-μl reaction containing 50 mM Tris/HCl, pH 7.6, 5 mM MgCl₂, 2 mM ATP, 10 mM creatine phosphate, 3.5 units/ml creatine kinase, and 0.6 units/ml inorganic pyrophosphatase and 0.6 μg of human recombinant IκBα (or mutants), 0.5 μg of 125I-SUMO-GG-1, 0.6 μg of Ubch9, and either recombinant SAE-immunoprecipitated protein A beads (rSAE) or 90 ng of affinity-purified SAE. Reactions were incubated at 37 °C for 2 h and terminated by boiling in 3× disruption buffer. Samples were subjected to SDS-PAGE (10%), and dried gels were analyzed by phosphorimaging.

RESULTS

Purification of SUMO-1-Activating Enzyme—The purification procedure for the SAE used an affinity procedure previously described (27, 30) for isolation of ubiquitin-activating enzymes based on covalent binding of the enzyme to ubiquitin-Sepharose beads in the presence of ATP. FrII.4 containing SUMO-1-activating activity from HeLa cells (15) was thus applied to SUMO-1-Sepharose beads in the presence of ATP and inorganic pyrophosphatase to suppress the reverse reaction. Under these conditions, SAE activates the immobilized SUMO-1, forming a thioester bond that retains the SAE on the column. The affinity column was washed sequentially with buffers containing ATP, 1 M KCl, Tris buffer alone, and AMP + pyrophosphate. Column eluates were assayed for their ability to form thioester adducts with 125I-SUMO-1 either alone (−) or in the presence of recombinant Ubch9. After 10 min at 30 °C, reactions were stopped, and products were subjected to SDS-PAGE (12.5%) under nonreducing conditions. Dried gels were analyzed by phosphorimaging. The positions of 125I-SUMO-1 and thioester adducts with SAE and Ubch9 are indicated. B, fractions indicated in A were fractionated by SDS-PAGE (10%) and stained with Coomassie Blue. Before concentration, the AMP eluate was supplemented with bovine serum albumin, which was also loaded on lane 6 as a control. The molecular weight of protein markers (M) and SAE components are indicated.
to the fractions to prevent nonspecific adsorption to surfaces during concentration (Fig. 1B).

cDNA Cloning of Human SUMO-1-activating Enzyme—Proteins present in the AMP eluate were fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membrane, and the location of bound species was detected by Amido Black staining. Both the 90-kDa and 40-kDa species were excised and subjected to direct N-terminal sequencing. Although the N terminus of the 90-kDa species appeared to be blocked, a unique sequence was obtained from the N terminus of the 40-kDa species. To obtain sequences from the 90-kDa species and additional sequences from the 40-kDa species, peptides generated by in-gel trypsin digestion were fractionated by microbore HPLC. Unambiguous sequence was obtained from 3 40-kDa- and 8 90-kDa-derived peptides (Fig. 2A, underlined sequences). BLAST searching of the EST public data base allowed the identification of ATCC clones, which were used in combination with RT-PCR to construct full-length cDNAs for each species. The 40-kDa species was designated as SAE1 and has a predicted molecular mass of 38 kDa, whereas the 90-kDa species was designated as SAE2 and has a predicted molecular mass of 72 kDa (Fig. 2A).

Amino acid sequence alignments of different E1s demonstrate that SAE is similar to Smt3p (Aos1p/Uba2p) and Rub1p (Ula1p/Uba3p)-activating enzymes (16, 22). Although SAE1 displays significant similarities to the N terminus of human S. cerevisiae Uba1, the SAE2 protein contains the putative active-Cys (Cys-173) and is homologous to the C terminus of Uba1. The apparent molecular mass of the 125I-SUMO-SAE thioester conjugate of 100 kDa (Fig. 2A) confirms the presence of the active-site cysteine in SAE2. Most of the sequence similarity (Fig. 2B) is concentrated in a limited number of previously identified domains (16, 22).

**SUMO-1 Is Activated by an E1-like Enzyme Containing Two Subunits**—cDNA corresponding to SAE1 and SAE2 obtained by RT-PCR was cloned into a eukaryotic expression vector permitting proteins to be produced and 35S-labeled by coupled in vitro transcription and translation (Fig. 3A). SAE1 was fused at the N terminus to an epitope from simian virus 5 that is recognized by the previously characterized SV5 Pk Tag (336) monoclonal antibody (25). Although SAE1 and SAE2 copurified in equilibrium amounts (Fig. 1B) during SUMO-1 affinity chro-
matography, it was not clear that they were associated. To obtain direct evidence for such an interaction, different mixtures of [35S]-labeled in vitro transcribed-translated proteins were immunoprecipitated with a specific (336) and a nonspecific (214) monoclonal antibody. In the presence of SV5-tagged SAE1, SAE2 was co-immunoprecipitated with anti-SV5 antibody (336). Labeled SAE2 protein was not immunoprecipitated with either the control antibody (214) or when SAE2 was immunoprecipitated using anti-SV5tag monoclonal antibody or with an unrelated antibody (214). After extensive washing with 50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 0.5% Nonidet P-40 immunoprecipitates were analyzed as described in panel B. The IVTT protein present and antibody used for the immunoprecipitation are shown for each reaction. [35S]Methionine-labeled SAE1tag and SAE2 proteins are indicated.

To demonstrate that SAE1 and SAE2 are required for SUMO-1 activation in vitro, its ability to form a stable adduct with radioactively labeled SUMO-1 in an ATP-dependent reaction was tested. SAE proteins were either in vitro transcribed-translated or expressed in transfected COS7 cells. In both situations, the activity was immunoprecipitated using anti-SV5 tag protein A beads. The ability of recombinant SAE to form a thioester with [125I]-SUMO-1 in presence of ATP was analyzed by SDS-PAGE fractionation. This immunoprecipitated activity was capable of catalyzing formation of a Ubch9-[125I]-SUMO-1 thioester when recombinant Ubch9 was added to the reaction (Fig. 4, A and B). The linkages formed between SAE2 and [125I]-SUMO-1 or Ubch9 and [125I]-SUMO-1 were labile to reducing agents such as DTT (Fig. 4A), indicating that they are likely to be thioester bonds. Neither SAE1 nor SAE2 alone were capable of forming a thioester complex with SUMO-1. Some activity is detected when SAE1 is immunoprecipitated from COS7 SAE1-transfected extracts, probably because of endogenous SAE2 activity (Fig. 4B). These results together suggest that the SUMO-1-activating enzyme is composed of SAE1/SAE2.

Role of SAE in Conjugation of SUMO-1 to IκBα—To demonstrate that the cloned SAE is functional in an Ubch9/ATP-dependent SUMO-1 conjugation reaction, its ability to mediate SUMO-1 conjugation to recombinant IκBα was investigated. Although SUMO-1 conjugation of IκBα is dependent on Ubch9, ATP, and FrI.4 (14), a complete description of the biochemical activities present in the HeLa cell fraction or in the in vitro transcribed-translated substrate was lacking. The SAE was affinity-purified from FrI.4, but by analogy with ubiquitin modification, it is not clear if other activities such as an E3 equivalent would be required. In the presence of recombinant, immunoprecipitated SAE, Ubch9, and ATP, [125I]-SUMO-1 was efficiently conjugated to recombinant IκBα (Fig. 5A). This reaction was dependent on SAE, Ubch9, ATP, and IκBα substrate (Fig. 5B). The enzymatic properties of rSAE were identical to those of the protein isolated from HeLa FrI.4. rSAE formed a thioester adduct with [125I]-SUMO-1 (Fig. 4) and produced sim-
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FIG. 5. SUMO-1 modification of IxBα with purified proteins. SAEl/SAE2 and Ubch9 are necessary and sufficient for conjugation of SUMO-1 to IxBα. Recombinant IxBα was incubated at 37 °C for 2 h with 125I-SUMO-1 in the presence (+) or absence (−) of either recombinant immunoprecipitated SAEl/SAE2 (rSAE, panel A) or affinity-purified SAEl (panel B), ATP, and Ubch9. Reactions products were fractionated by SDS-PAGE and analyzed by phosphorimaging. C, substrate specificity in the purified SUMO-1 conjugation system. Purified recombinant IxBα, an N-terminal-deleted IxBα (ΔN IxBα) and a C-terminal-deleted IxBα (IXBαC) were assayed for conjugation to 125I-SUMO-1 in a reaction containing ATP, Ubch9, and SAEl/SAE2. Recombinant GST protein was analyzed as negative control. Reactions were incubated at 37 °C for 2 h, and the products were analyzed by SDS-PAGE and phosphorimaging as before.

SUMO-1 conjugates under identical reactions conditions (Fig. 5). Because SUMO-1, Ubch9, and IxBα are all homogeneous recombinant proteins expressed in bacteria and the SAEl is either highly purified (Fig. 5B) or recombinant anti-SV5-immunoprecipitated (Fig. 5A), it appears that SUMO-1 conjugation of IxBα in vitro does not require an equivalent E3 activity and that after SUMO-1 activation by SAEl, the SUMO-1-conjugating enzyme Ubch9 is capable of recognizing, then transferring, SUMO-1 onto IxBα. To demonstrate specificity in vitro, two deleted forms of IxBα and GST were used as substrates in reactions also containing purified SAEl, Ubch9, 125I-SUMO-1, and ATP. SUMO-1 is efficiently conjugated to IxBα and IXBαC (lacks the C-terminal 61 amino acids) but is not conjugated to either ΔN IxBα (lacks the N-terminal 70 amino acids) or GST (Fig. 5C). That the lysine involved in SUMO-1 modification is present in wild type IxBα and IXBαC but absent in ΔN IxBα indicates that the purified conjugation system is displaying the expected specificity.

DISCUSSION

Targeting of proteins for ubiquitin-mediated proteolysis is an irrevocable decision, and as such, the process needs to be highly specific and tightly regulated. This specificity appears to be accomplished by a combination of E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. In many cases, the E3 appears to consist of a multiprotein complex that recognizes the substrate and brings it to intimate contact with the E2, which catalyzes the addition of ubiquitin to the substrate. Because the E1, ubiquitin-activating enzyme is unique, it does not appear to play a role in selecting protein substrates for ubiquitination. However, ubiquitin co-exists with a number of ubiquitin-like molecules, and the E1 enzymes must distinguish between these molecules. Because distinct E1 activities have been described for ubiquitin (31), Smt3p (16), and Rub1p (21, 22), we undertook the isolation and characterization of the SAEl.

By taking advantage of the mechanism of ubiquitin-activating and -conjugating enzymes, which involves formation of a thioester intermediate with ubiquitin, we have used SUMO-1 affinity chromatography to isolate a novel enzyme that catalyzes the ATP-dependent activation of SUMO-1, the first step in the conjugation pathway. Furthermore this enzyme could also transfer activated SUMO-1 to Ubch9, the conjugating enzyme involved in this process (11, 15, 18, 19) (Fig. 4). Although the E1 activity for ubiquitin is contained within a single large polypeptide, the E1 activity of SUMO-1, like that of Smt3p and Rub1p, is partitioned between two smaller polypeptides, SAEl and SAE2. Sequence comparisons between the E1 enzymes indicates that SAEl is homologous to Aos1p, Ula1p, and the N terminus of the ubiquitin-activating enzymes (Fig. 2), whereas SAE2 is homologous to Uba2p, Uba3p, and the C terminus of the ubiquitin-activating enzymes. The association between SAEl and SAE2, confirmed by immunoprecipitations and GST pull-downs (Fig. 3), brings together conserved domains present in each subunit. Because purified SAEl contains equimolar amounts of SAEl and SAE2 and the two proteins associate in vitro, it is probable that, like the Smt3p E1, the activating enzyme is a heterodimer. Each SAEl subunit contains a conserved nucleotide binding motif, GXGXXG (positions 24–29 in SAEl) and positions 43–48 in SAE2, and the putative cysteine (Cys-175), which forms a thioester bond with the C-terminal glycine of SUMO-1, is in an active-site consensus sequence (KXXPZCTXIXX) found in conserved domain III. Conserved domain II is present in SAEl, whereas conserved domain IV is found in SAE2. The function of conserved domains II and IV has yet to be determined. The C-terminal extension of SAE2 contains a region that matches with two consensus sequences for nuclear localization signals (KKRR, 610–613, and KRRKLDHENL4SR, 610–626), which are also present in the C-terminal region of Uba2p (32).

To further investigate the enzymatic properties of the SUMO-1-activating enzyme, recombinant SAEl protein was tested for the ability to activate SUMO-1 in a purified in vitro SUMO-1 conjugation system together with an ATP regenerating system, Ubch9, and a recombinant IxBα substrate. Under the conditions employed in this assay, SUMO-1 was efficiently conjugated to IxBα, indicating that conjugation does not require the presence of an E3-like protein ligase activity. However, we cannot rule out the possibility that in vivo, such proteins may increase the efficiency of the conjugation process. Because our initial yeast two-hybrid screen demonstrated a protein-protein interaction between Ubch9 and IxBα (15), it is likely that substrate specificity is achieved by Ubch9.
range of proteins have been shown to interact with UbC9 in yeast two-hybrid experiments, and this may be a direct consequence of substrate recognition by UbC9. Again it is not possible to rule out the participation of yeast proteins in these interactions.

Within the cell it appears that virtually all of the SUMO-1 is present in protein conjugates, and there is a very low concentration of free SUMO-1(7). Thus it is likely that the availability of free SUMO-1 is tightly controlled by a dynamic equilibrium between SAE/Ubc9-mediated conjugation of SUMO-1 and deconjugation mediated by the highly active but as yet uncharacterized SUMO-1-deconjugating and processing enzymes. However the cellular signals that regulate this process have yet to be defined.

The large number of ubiquitin-specific proteases and ubiquitin C-terminal hydrolases (UCH) already identified (33) suggest that they may be involved in the recognition of different types of ubiquitin conjugates, but little is known about their biological roles. It is likely that some of the known ubiquitin-specific proteases and ubiquitin C-terminal hydrolases will be responsible for processing of ubiquitin-like proteins. UCH-L3, a putative ubiquitin C-terminal hydrolase, was recently identified as a NEDD8 (23)-interacting protein, able to cleave the C terminus of NEDD8 but not bind to sentrin-1, sentrin-2, or sentrin-3 (34). The availability of the genes for the SUMO-1-activating and -conjugating enzymes, SAE1/SAE2 and Ubc9, will facilitate further biochemical and cell biological studies aimed at defining the role of these proteins in vivo.

Acknowledgments—We would like to thank Ellis Jaffray for large scale growth of HeLa cells, Paul Talbot for protein sequencing, and Alex Houston for DNA sequencing.

REFERENCES
1. Herskoe, A., and Ciechanover, A. (1992) Annu. Rev. Biochem. 61, 761–807
2. Bradbury, E. M. (1992) Bioessay 14, 9–16
3. Hicke, L., and Riezman, H. (1996) Cell 84, 277–287
4. Haas, A. L., Ahrens, P., Bright, P. M., and Ankel, H. (1987) J. Biol. Chem. 262, 11315–11323
5. Narasimhan, J., Potter, J. L., and Haas, A. L. (1996) J. Biol. Chem. 271, 324–330
6. Kamitani, T., Nguyen, H. P., and Yeh, E. T. H. (1997) J. Biol. Chem. 272, 14001–14004
7. Matunis, M. J., Coutavas, E., and Blobel, G. (1996) J. Cell Biol. 133, 1457–1470
8. Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997) Cell 88, 97–107
9. Shen, Z., Pandittington-Purtymun, P. E., Comeaux, J. C., Moyzis, R. K., and Chen, D. J. (1996) Genomics 36, 271–279
10. Boddy, M. N., Howe, K., Elkin, L. D., Solomon, E., and Freemont, P. S. (1996) Oncogene 13, 971–982
11. Saitoh, H., Sparrow, D. B., Shiomi, T., Pu, R. T., Nishimoto, T., Mohun, T. J., and Dasso, M. (1998) Curr. Biol. 8, 121–124
12. Muller, S., Matunis, M. J., and Dejean, A. (1998) EMBO J. 17, 61–70
13. Sterner, T., Jensen, K., and Will, H. (1997) J. Cell Biol. 139, 1621–1634
14. Desterro, J. M. P., Rodriguez, M. S., and Hay, R. T. (1998) Mol. Cell 2, 253–259
15. Desterro, J. M. P., Thomson, J., and Hay, R. T. (1997) FEBS Lett. 417, 297–300
16. Johnson, E. S., Schwienhorst, I., Dohmen, R. J., and Blobel, G. (1997) EMBO J. 16, 5509–5519
17. Seufert, W., Futcher, B., and Jentsch, S. (1995) Nature 373, 78–81
18. Johnson, E. S., and Blobel, G. (1997) J. Biol. Chem. 272, 26799–26802
19. Schwarz, S. E., Matuschewski, K., Liakopoulos, D., Scheffner, M., and Jentsch, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 560–564
20. McGrath, J. P., Jentsch, S., and Varshavsky, A. (1991) EMBO J. 10, 227–236
21. Lammer, D., Mathias, N., Laplaza, J. M., Jiang, W., Liu, Y., Callis, J., Goebi, M., and Estelle, M. (1998) Genes Dev. 12, 914–926
22. Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998) EMBO J. 17, 2208–2214
23. Kamitani, K., Maeda, E., and Yeh, E. T. H. (1997) J. Biol. Chem. 272, 28557–28562
24. Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M. D., Klionsky, D. J., Ohsumi, M., and Ohsumi, Y. (1998) Nature 395, 395–398
25. Hanke, T., Szawlowski, P., and Randall, R. E. (1992) J. Gen. Virol. 73, 655–660
26. Kroll, M., Conconi, M., Desterro, M. J., Marín, A., Thomas, D., Friguet, B., Hay, R. T., Virleizier, J. L., Arezana-Seisdedos, F., and Rodrigues, M. S. (1997) Oncogene 15, 1841–1850
27. Ciechanover, A., Elias, S., Heller, H., and Herskho, A. (1982) J. Biol. Chem. 257, 2537–2542
28. Jaffray, E., Wood, K. M., and Hay, R. T. (1995) Mol. Cell. Biol. 15, 2166–2172
29. Haas, A. L., Waums, J. B., Herskho, A., and Rose, I. A. (1982) J. Biol. Chem. 257, 2543–2548
30. Herskho, A., Heller, H., Elias, S., and Ciechanover, A. (1983) J. Biol. Chem. 258, 8206–8214
31. Handley, P. M., Mueckler, M., Siegel, N. R., Ciechanover, A., and Schwartz, A. L. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 258–262
32. Dohmen, R. J., Stappen, R., McGrath, J. P., Forrova, H., Kolarov, J., Goffeau, A., and Varshavsky, A. (1995) J. Biol. Chem. 270, 18099–18109
33. Wilkinson, K. D. (1997) Oncogene 15, 1841–1850
34. Wada, H., Kito, K., Caskey, L. S., Yeh, E. T. H., and Kamitani, T. (1998) Biochem. Biophys. Res. Commun. 251, 688–692