Purification and Characterization of a Novel Species of Ubiquitin-Carrier Protein, E2, That Is Involved in Degradation of Non-“N-end Rule” Protein Substrates*

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Ubiquitin-carrier proteins (E2s, ubiquitin-conjugating enzymes, UBCs) participate in proteolysis by catalyzing transfer of activated ubiquitin to the protein substrates, which can be N-end rule or non-N-end rule ubiquitin-protein ligases (E3s). Yeast UBC2 (RAD6) and the mammalian E214 kDa bind to the ligase that recognizes and is involved in the degradation of certain free amino-terminal substrates (“N-end rule” substrates). As such proteins are rather scarce, the role of these E2s in general proteolysis is probably limited. Here, we report the purification and characterization of a novel 18-kDa species of E2 from rabbit reticulocytes. Unlike most members of the E2 family, this enzyme does not adsorb to anion exchange resin in neutral pH, and it is purified from the unadsorbed material (Fraction 1). Thus, it is designated E2-F1. Like all members of the E2 family, it generates a thiol ester with ubiquitin that serves as an intermediate in the conjugation reaction. Sequence analysis revealed a significant homology to many known species of E2s. The enzyme generates multiply ubiquitinated proteins in the presence of an E3 that has not been characterized yet. Most importantly, the ubiquitination via this E2 leads to the degradation of certain non-“N-end rule” substrates such as glyceraldehyde-3-phosphate dehydrogenase (Val at the NH2 terminus) and to the ubiquitination and degradation of certain N-acetylated proteins such as histone H2A, actin, and α-crystallin. The enzyme is also involved in the conjugation and degradation of the tumor suppressor protein p53.

Ubiquitin can be transferred from E1 to several species of E2s. Pickart and Rose (1) described five different E2 enzymes in rabbit reticulocytes with molecular masses ranging from 14 to 32 kDa. All the enzymes are contained in the fraction that adsorbs to the anion exchange resin at neutral pH (Fraction 2; Ref. 2). Ten species of E2s have been described in the yeast Saccharomyces cerevisiae (UBC1–10) and their corresponding genes cloned and sequenced (3).

Structurally, all known E2s share a conserved domain of approximately 16 kDa. This domain contains the Cys residue required for the formation of ubiquitin–E2 thiol ester. Based on their structure, the E2 enzymes can be divided into three groups (3). Class I E2s consist almost exclusively of the conserved domain. Class II enzymes have COOH-terminal extensions that may contribute to substrate recognition or to cellular localization. Class III enzymes have various NH2-terminal extensions; however, their function is not known (3).

Functional studies of E2 enzymes have shown that they can be clustered in two major groups. Most E2s catalyze transfer of ubiquitin to small amines or basic proteins, such as histones, in a reaction that does not require E3. These reactions result in mono-ubiquitin derivatives that do not serve as proteolysis intermediates. Genetic studies indicate that some of these E2s play important roles in a variety of basic cellular processes. For example, UBC9-CDC34 is involved in G1-S cell cycle progression, DNA replication, and spindle pole body separation, and is essential for viability (4). The second group of E2 enzymes is involved in multiple ubiquitination that target protein substrates for degradation. Here, the first activated ubiquitin moiety is transferred from E2 to a specific Lys residue of the substrate that is bound to E3. In successive reactions, a polyubiquitin chain is synthesized by processive transfer of activated ubiquitin moieties to Lys48 of the previous (and already conjugated) ubiquitin molecule (5, 6). The binding of the substrate to E3 probably facilitates the synthesis of the polyubiquitin chain that serves, most probably, as a recognition marker for the 26 S proteasome complex that degrades these adducts.

It is assumed that the E2 enzymes involved in proteolysis recognize distinct species of E3s. However, they do not appear to have specific recognition sites for the substrates. This task is accomplished, most probably, by the different ligases. Of the five E2s described in reticulocytes, only the 14-kDa enzyme was shown to be involved in E3-dependent multiple ubiquitination and degradation (1). Two distinct species of E3 enzymes have been characterized: E3α, which recognizes substrates with basic and bulky hydrophobic free NH2 termini, and E3β, which recognizes proteins with small uncharged free NH2 termini (reviewed in Ref. 6). Initial analysis of the mechanism of action of E3α revealed, that in addition to the specific binding sites for ubiquitin and the substrate, it also interacts with one of the E2
enzymes with which it acts in concert (7, 8). The formation of an E2-E3 complex probably facilitates the transfer of activated ubiquitin from E2 to the protein substrate bound to the ligase. Successive studies have shown that the ability of UBC2 (RAD6) to promote polyubiquitination and degradation in vitro is dependent upon the presence of E3a (9). A similar study in yeast demonstrated that UBC2 is associated with UBR1, the yeast homolog of E3a; immunoprecipitation of UBR1 also precipitated UBC2 (10). In addition, certain UBR1 substrates ("N-end rule" substrates; Ref. 11) were stabilized in UBC2 null mutants (10).

Several lines of evidence indicate that the bulk of cellular proteins are recognized by signals that are distinct from the NH₂-terminal residue (6). (a) Approximately 80% of the cellular proteins are N-α-acetylated (12), and the majority of the residual unblocked proteins lack "destabilizing" NH₂-terminal residues (6). (b) N-α-Acetylated proteins can be degraded by the ubiquitin system, and removal of the blocking group is not dependent upon the presence of E3a (10).

Residual unblocked proteins lack "destabilizing" NH₂-terminal amino acid residue. (d) Most convincing, mutational inactivation of the N-end pathway in yeast is neither lethal, nor phenotypically conspicuous (15). Thus, if the notion that different E2 enzymes interact with specific ligases is true, a reasonable conclusion is that the N-end pathway E2s (the yeast UBC2 and the mammalian E2HAD₄) do not play an important role in general ubiquitin-mediated proteolysis. Other, still unidentified species of E2(s) probably account for the degradation of non-"N-end rule" proteins. It is also involved in ubiquitin-mediated proteolysis of the tumor suppressor protein p53.

### EXPERIMENTAL PROCEDURES

#### Materials

Sephadex G-50–150, HiLoad Superdex 75 HR (16×600 mm), and Mono Q (5×50 mm) columns were from Pharmacia LKB Biotechnology Inc. Centrifico CF-25 and Centricon 10 microconcentrators were from Amicon. Dithyrylaminothyl (DEAE)-cellulose (DE52) was from Whatman. Hexokinase was from Boehringer Mannheim. Ammonium sulfate (enzyme-grade) was from Life Technologies, Inc. Rabbit muscle G3PDH, ubiquitin, histone H2A, α-crystallin, 2-deoxyglucose, yeast inorganic pyrophosphatase, DTT, ATP, creatine phosphate, creatine kinase, Trizma (Tris base), and ovalbumin, were from Sigma. Actin was from Worthington. Acrylamide, N,N'-methylene-bisacrylamide, TEMED, ammonium persulfate, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue G-250, and unmodified molecular weight markers were from Bio-Rad. Rainbow prestained molecular weight markers were from Amersham Corp. Na⁺-DPT was from DuPont NEN. All other reagents were of high analytical grade.

#### Methods

**Preparation of Crude Reticulocyte Fractions 1 and 2—Reticulocyte-rich blood was induced in rabbits by successive injections of phenylhydrazine, and reticulocyte lysates were prepared as described (2). Lysates were resolved by ion exchange chromatography on DEAE-cellulose into unadsorbed material (Fraction 1) and high salt eluate (Fraction 2) as described (2).** For degradation experiments, Fraction 2 was not precipitated by (NH₄)₂SO₄. Instead, the high salt eluate was dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.2, 5 mM KCl, and 2 mM DTT and concentrated by Amicon Centrifico CF-25 cone (to a concentration of approximately 20 mg/ml protein).

**Ammonium Sulfate Fractionation of Fraction 2—Fraction 2 was further resolved by (NH₄)₂SO₄ into Fraction 2A (0–38% saturation) and Fraction 2B (42–80% saturation) as described (17).** Fraction 2A contains the E3 enzymes, while Fraction 2B contains the E1 and E2 enzymes.2

**Purification of the Conjugating Enzyme E2—** Purification from crude reticulocyte Fraction 2 by affinity chromatography over immobilized ubiquitin as described (2). E2 purified from Fraction 2 was designated E2-F2.

**Iodination of Proteins—** Proteins were radiolabeled with iodine using the chloramine T method as described (18). Iodination of ubiquitin was determined by measuring the radioactivity released into triiodoacetic acid-soluble fraction as described (18). All assays were carried out in a final volume of 50 μl and contained 35 μl of whole reticulocyte lysate or 200 μg of protein of Fraction 2, 5 μg of ubiquitin, and additional fractions as indicated in the description of the specific experiments. All reactions were incubated for 2 h at 37 °C. In all cases, two sets of reaction mixtures were incubated; one set contained ATP and ATP-regenerating system, whereas the other set contained hexokinase and 2-deoxyglucose. Values of ATP-independent degradation did not exceed 20% of energy-dependent breakdown. Results reflect ATP-dependent degradation.

**Purification of E2-F1—** Chromatographic separations were carried out at 4 °C and dialysis of samples at 0 °C. Relevant fractions from the different purification steps were subjected to quantitative activity analysis (see above and below), protein concentration (19), and SDS-PAGE (20). A unit of enzyme is defined as the amount of enzyme that stimulates the degradation of 125I-labeled G3PDH at 1% in a standard assay as described above under "Degradation of 125I-Labeled Proteins."
but not 2-mercaptoethanol was added to one portion, and a standard sample buffer was added to the other portion. The samples were then resolved via SDS-PAGE (12.5% acrylamide) at 4 °C using a buffer in which the SDS concentration was reduced to 0.5%. Separation was at 5 mA for 12 h. The gels were fixed, dried, and exposed to XAR-5 film (Kodak) as described (2, 21).

Formation of [125I]Ubiquitin-Protein Conjugates—Ubiquitin-protein conjugates were generated essentially as described (2, 21). Briefly, the reaction mixture contained (in a final volume of 25 μl) 50 μM Tris-HCl, pH 7.6, 5 μM MgCl2, 2 mm DTT, 2 mm ATP, 1.0 μg of [125I]-labeled ubiquitin (~150,000 cpm), and Fraction 2 (100 μg). E1 (1.25 μg), E2 enzymes (0.25 μg), and Fraction 2A (80 μg), as indicated. Following incubation for 20 min at 37 °C, the reactions were resolved via SDS-PAGE (12.5% acrylamide) and the gels were fixed, dried, and exposed as described (2, 21).

Sequencing of E2-F1—7.2 μg (approximately 400 pmol) of E2-F1 (derived from the Mono Q purification step, see above) were blotted from a 10–20% SDS-polyacrylamide gel (Enprotech, Natick, MA) onto a Trans-Blot transfer PVDF membrane (Bio-Rad) using the protocol of Matsudaira (22) and sequence analysis was attempted in an NH2-terminal fashion. Once failure to derive sequence information suggested NH2-terminal blockage, in situ cyanogen bromide digestion was performed in the following manner. The PVDF pieces were removed from the sequencer and immersed in 100 μl of 3% CNBr and 70% formic acid. The reaction was incubated overnight in the dark, and the pieces were reloaded onto the sequencer with a Polybrene filter overlay to which the CNBr supernatant was applied. Automated sequencing was reintiated.

In order to obtain internal sequence information, peptide fragments were obtained as follows: 18 pg (approximately 1,000 pmol) of E2-F1 were blotted onto PVDF membrane as described above. An in situ trypsinolysis was performed as described by Fernandez and colleagues (23). Tryptic peptides released from the PVDF membrane were separated by RP-HPLC using a Brownlee RP-300 Aquapore C8 column (Applied Biosystems model 120A PTH Analyzer fitted with a Brownlee 2.1-mm (inner diameter) PTH-C18 column.)

Similarities between the obtained sequences and known protein sequences were investigated through computer based searches of the National Biomedical Research Foundation protein sequence data bases, the Genpept, SwissProt, PIR, SPUpdate, tfd, palu, and GPUpdate protein data bases (25–27), and NCBI (National Center for Biotechnology Information), using the BLAST Network Service.

RESULTS

Crude Reticulocyte Fraction 1 Contains a Factor That Stimulates the Degradation of the Non-N-end Rule Protein Glyceraldehyde-3-phosphate Dehydrogenase in Ubiquitin-supplemented Fraction 2

As can be seen in Table I, the addition of crude reticulocyte Fraction 1 to a ubiquitin-supplemented Fraction 2 increases significantly the degradation of rabbit muscle GA3PDH. Recently, we showed that Fraction 1 contains a factor (designated FH) that is necessary for the degradation of N-a-acetylated proteins (28). This factor, which is a homodimer with molecular mass of approximately 96 kDa, is required for the degradation of already conjugated proteins. It is not necessary for their ubiquitin tagging. As rabbit muscle GA3PDH has a free NH2 terminus (Val; the NH2-terminal region is Val1-Lys-Val-Gly5...; Ref. 29; confirmed by direct sequencing of the prepara- tion used in this study), we assumed that the factor necessary for degradation of the dehydrogenase is not FH. A direct experiment showed that this is indeed the case; addition of partially purified FH to ubiquitin-supplemented Fraction 2 did not affect the degradation of GA3PDH (data not shown). Therefore, we decided to purify and characterize this novel factor.

Purification of E2-F1

In an attempt to remove hemoglobin (which is the major protein in Fraction 1), crude reticulocyte Fraction 1 was resolved by gel filtration chromatography on a Sephadex G-50 column. As can be seen in Fig. 1, most of the hemoglobin was eluted in fractions 4–8, whereas the stimulatory activity was...
recovered in fractions 9–14. This experiment further corroborated the notion that the factor that stimulates the degradation of GA3PDH is different from FH as its native molecular mass is significantly smaller (it is smaller than hemoglobin).

To further characterize the enzyme and to remove residual hemoglobin, fractions 9–14 were pooled, concentrated by ammonium sulfate precipitation, and subjected to gel filtration chromatography over a Superdex 75 HR FPLC column. The active fractions eluted (33–37) were virtually free from hemoglobin. As can be seen in Fig. 2, three peaks of protein could be detected: A, B, and C. Peak A contains mostly hemoglobin, whereas Peak B contains unidentified proteins. Neither peak contains any E2-F1 activity. All the activity is resolved with Peak C. At this stage it also became clear that the degradation of GA3PDH is mediated by ubiquitin, as the protein was not degraded in reaction mixtures that did not contain exogenously added ubiquitin. This was not clear prior to the FPLC-gel filtration purification step, as Fraction 1 contains ubiquitin and the first gel filtration purification step over Sephadex G-50 did not remove ubiquitin completely from the active fractions. To further corroborate the notion that the degradation of our model substrate is indeed mediated by the ubiquitin pathway, we used neutralizing antibodies against E1 (13). The antibody blocked completely the degradation of GA3PDH in crude reticulocyte lysate. Degradation resumed following removal of the antibody and addition of purified E1 (not shown). To determine the molecular mass of E2-F1, molecular weight markers were resolved on the Superdex 75 column under identical conditions to those utilized for the separation of E2-F1. The native molecular mass of the enzyme is approximately 18 kDa (not shown).

To further purify E2-F1, fractions 33–37 from the Superdex 75 gel filtration step were pooled, dialyzed against a buffer at pH 8.5, and loaded onto a Mono Q ion exchange column. E2-F1 did not adsorb to the resin even at this mildly alkaline pH: it was recovered in fractions 2–4 in the unadsorbed material with a peak of activity in fraction 3 (not shown). SDS-PAGE analysis of these fractions (Fig. 3) revealed a major protein with a molecular mass of 18 kDa that was slightly contaminated with carbonic anhydrase (31 kDa), an abundant protein in reticulocytes. All the other proteins that accompanied E2-F1 in the Superdex 75 gel filtration chromatography were adsorbed to the column, eluted at high salt (not shown), and did not contain any E2-F1 activity. This preparation was used as a source of purified enzyme for both functional assays and sequence analysis. The purification procedure is summarized in Table II.

As can be seen, loss of activity occurred mostly in two steps, the Sephadex G-50 gel filtration chromatography and the Mono Q ion exchange chromatography. The reason for these losses is not known. It is not due to loss of other species of E2 or factors necessary for degradation of the test substrate during the purification of E2-F1. Such factor(s) could not be found in the remaining fractions (not shown).

E2-F1 Is a Novel Species of Ubiquitin-Carrier Protein

E2-F1 Is Sensitive to -SH Agents—As the molecular mass of E2-F1 is relatively small, we suspected that the enzyme may be a novel species of E2; many of these enzymes are small proteins with a molecular mass range of 14–32 kDa (1). As all E2 enzymes contain a functional Cys residue in the active site, we...
tested the sensitivity of the E2-F1 to the -SH blocking agent, N-ethylmaleimide. 50 units of the enzyme were incubated in the presence of 10 mM inhibitor for 10 min at room temperature. The inhibitor was neutralized by the addition of DTT to a final concentration of 5.5 mM, and the activity of the enzyme in stimulating the degradation of $^{125}$I-labeled GA3PDH in ubiquitin-supplemented Fraction 2 was monitored. The enzyme that was incubated in the presence of the inhibitor was completely inactive (not shown). An enzyme preparation to which DTT was added prior to the addition of the inhibitor, or a preparation that was treated only with DTT, retained most of
its activity. Thus, E2-F1 contains a functionally essential -SH group.

E2-F1 Accepts Activated Ubiquitin Moiety from E1 and Generates an E2-F1-Thiol Ester—The mechanism of activation of ubiquitin involves formation of a high energy thiol ester intermediate between ubiquitin and E2. The activated ubiquitin moiety is donated to E2 from the ubiquitin-activating enzyme, E1 (1, 2, 21). To test whether E2-F1 can also generate such a thiol ester, we first tested the Sephadex G-50-resolved fractions. As can be seen in Fig. 4, fractions 9–14 contain activity that generates a thiol ester in the presence of E1 and that coincides with the activity of E2-F1. The molecular mass of the thiol ester is approximately 25 kDa, corresponding to an adduct that is composed of a single molecule each of E2-F1 and ubiquitin. The thiol ester disappeared completely following boiling of the samples in the presence of 2-mercaptoethanol (not shown). Also, it did not form if E1 was not present (not shown). Interestingly, fractions 5–8 contained an additional form of E2 that also generated a thiol ester with E1 (Fig. 4; marked as E2-F1(3)-S-UB). This species of E2 is clearly larger than E2-F1, and its function is not known. The fact that the thiol ester it generates with ubiquitin is smaller than the one formed by E2-F1 suggests that in its native form this E2 can be a multimeric protein; separation of the thiol ester in a buffer that contains SDS separates the monomers from one another. Characterization of this enzyme is currently under way.

Next, we studied the ability of the purified E2-F1 to generate a thiol ester with ubiquitin. As can be seen in Fig. 5, Mono Q-purified E2-F1 generates a thiol ester with ubiquitin (lane 3). Again, the molecular mass of the thiol ester corresponds to a single adduct between ubiquitin and E2. The reaction is dependent on the presence of E1 (lanes 2 and 5). The ester formed is sensitive to 2-mercaptoethanol (lanes 8–14). Also, the ester was not formed when excess unlabeled ubiquitin was added simultaneously with the labeled ubiquitin and E1, suggesting that only the E1-bound ubiquitin is donated to E2 (lanes 4 and 7).

E2-F1 Generates Ubiquitin-Protein Conjugates with E3—Several species of E2 enzymes generate mono-ubiquitin conjugates with certain protein substrates. Other species of E2s, along with E3 enzymes, mediate the generation of multiply ubiquitinated proteins that are destined for degradation (see Introduction). To examine the role of E2-F1 in the conjugation process, we incubated the enzyme with E1 and E3. As can be seen in Fig. 6, E2-F1 generates a high molecular mass conjugates only in the presence of E1 and E3. Quantitative analysis reveals that E2-F1 is at least 5-fold more "efficient" than its Fraction 2 counterpart(s); it generated 6-fold more conjugates when equal amounts of the two enzymes were used (compare lane 9 to lanes 10 and 12; radioactivity in the lanes was determined quantitatively). This efficiency probably reflects availability of appropriate substrates in crude Fraction A rather than higher affinity or smaller $K_d$ for the same substrates. We assume that non-N-end rule substrates that are conjugated/degaded via the E2-F1/E3(?) pathway are more abundant than N-end rule substrates degraded via the E2-F2/E3a/E3b pathway (see "Discussion").

While it is clear that the degradation of GA3PDH proceeds in a ubiquitin-dependent manner, we were not able, despite many efforts, to demonstrate ubiquitin conjugates of the enzyme, and therefore, the participation of E2-F1 in their formation. Introduction of ubiquitin aldehyde, a specific inhibitor of several isopeptidase (30), and ATP-$\gamma$S, an ATP analog that promotes conjugation of ubiquitin but not degradation of conjugates (31), did not promote formation of conjugates. Other "classical" substrates of the ubiquitin system, including the N-end rule substrate BSA, demonstrate similar behavior (18). It is possible that the overall equilibrium in the degradation pathway of certain substrates favors degradation of the conjugates (via the 26 S protease and isopeptidases) over their formation. We are convinced, however, that the degradation of a substrate that requires E1, E2, ubiquitin, and ATP, is mediated via the ubiquitin pathway.

Sequence Analysis of Purified E2-F1—Tryptic hydrolysis generated several internal fragments. Three of these fragments (fragments 7, 17, and 20) were subjected to Edman degradation, and their sequences revealed homologies to several known species of E2 enzymes. The homologies are presented in Fig. 7.

DISCUSSION

We have purified and characterized a novel species of ubiquitin carrier protein, E2, from mammalian cells. Unlike many E2 enzymes that adsorb to anion exchange resins at neutral pH, the new species is probably more basic and does not adsorb even at pH 8.5. Therefore, we designated the enzyme E2-F1, as it is eluted with Fraction 1. Like many other species of E2s, E2-F1 is relatively small; it is composed of a single polypeptide chain of 18 kDa. Also, like all other known E2 enzymes, the novel carrier protein contains an essential -SH group; modification of this group inhibits completely the activity of the enzyme. This -SH group is involved in the formation of ubiquitin-E2-F1 thiol ester that donates the activated ubiquitin moiety to the target substrate (Figs. 4–6). Functional analysis reveals that the enzyme is not involved in E1-mediated mono-ubiquitination and direct recognition of substrates. Instead, it acts with E3 to generate multiply ubiquitinated proteins that are destined for degradation (Fig. 6 and Table I). Furthermore, it appears that E2-F1 is more active in conjugation than its 14-kDa Fraction 1-derived counterpart that was initially shown to participate in proteolysis (Fig. 6; Ref. 1). This is probably due to the availability of appropriate substrates recognized by this novel E2 enzyme and its cognate E3 (see below). Sequence analysis of the purified protein further support the notion that E2-F1 is indeed a novel species of E2; analysis of three internal peptides reveals strong homology to many known species of E2s, including viral, yeast, and mammalian enzymes. Though the sequenced residues represent only a small part of the molecule (46 residues sequenced out of approximately 160 residues), it is interesting to note that E2-F1 resembles most closely yeast UBC1, UBC4, and UBC5, and the fly enzyme UBCD1. Genetic analysis shows that these enzymes are involved in multiple ubiquitination and proteolysis (3). Some of the other enzymes (analyzed in Fig. 7) are involved in mono-ubiquitination, whereas the function of many of the remaining enzymes is not known.

In examining potential substrates that are recognized by E2-F1, we found that the enzyme is necessary for the degradation of GA3PDH. The dehydrogenase is a homotetramer composed of four 35-kDa chains with a Val residue in their NH2-terminal position (29). Val is a "stabilizing" residue according to its position in bacteria, yeast, and mammalian cells (11). It was clear, therefore, that this protein is not recognized by E3z and E3b, the two ubiquitin-protein ligases that recognize certain proteins via their free destabilizing NH2-terminal residues. The dehydrogenase, as the vast majority of cellular proteins that have either blocked or stabilizing NH2 termini, is probably recognized by novel species of E3(s) that have not been identified yet. It was not clear, however, whether conjugation (and subsequent degradation) of these substrates requires also novel species of E2(s), or whether the existing N-end rule E2s (E2-14 kDa from reticulocytes and the yeast UBC2:RAD6; Refs. 1 and 8–10) can also mediate their degradation via interaction with non-N-end rule ubiquitin protein ligases. Our data suggest, albeit indirectly, that E2 enzymes act in concert with specific ligases. The N-end rule E2-14 kDa from
reticulocytes could not whether the E2s that are involved in E3-mediated degradation also recognize specific structural motifs in their protein substrates. This question is still open and the present study does not attempt to resolve it. It is our hypothesis, however, that "proteolytic" E2s interact with their cognate E3s and not with the proteolytic substrates.

We were not able to generate ubiquitin conjugates of GAG3PDH and, thus, to demonstrate directly a role for E2-F1 in the conjugation of this protein. However, insufficient evidence indicates that GAG3PDH is degraded via the ubiquitin system; its degradation requires ubiquitin, E1, E2, and ATP (see "Results"). Other classical substrates of the ubiquitin system, such as BSA (that is degraded via the N-end rule pathway), also do not form detectable conjugates. It is assumed that the equilibrium of the multiple reactions involved in the degradation of these substrates favors rapid degradation of the conjugates.

Initial characterization of additional E2-F1-related substrates showed that the enzyme stimulates significantly the degradation of actin, histone H2A, and α-crystallin, three N-acetylated proteins, in a reconstituted system (not shown). Furthermore, it increases dramatically the conjugation of ubiquitin to histone H2A in a reconstituted system (not shown). Thus, it seems that the new E2 plays an important role in targeting other non-N-end rule substrates for degradation. The finding that E2-F1 from Fraction 2 can also generate conjugates of histone H2A along with E3s (28) suggests that specific E2-F1 pairs overlap to a certain extent. It is not clear whether the pair E2-F1 - E3a is the preferable ligase complex in this process (28). It is likely that the combination E1, E2-F1, and a specific, yet unidentified ligase, will be more efficient in conjugating NH2-terminal modified proteins than the combination of E1, E2-F2, and E3a.

The E3 that interacts with E2-F1 has not been identified. Girod and Vierstra (16) have shown that the E2 they purified from wheat germ (it is probably the plant homolog of E2-F1) can cooperate with several distinct, though uncharacterized, species of E3a. As the test substrate used by these investigators was lysosome, a classical N-end rule substrate, it is difficult to determine at this stage the characteristics of these ligases.

In the accompanying paper (40), we show that E2-F1 is involved in the conjugation and degradation of the tumor suppressor protein p53 in vitro. While it is not clear whether E2-F1 fulfills a similar function in the intact cell as well, a correlation between the in vitro and in vivo recognition of wt and several mutant species of p53 suggests that this may well be the case.

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