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Complete reconstitution of conjugation and subsequent degradation of the tumor suppressor protein p53 by purified components of the ubiquitin proteolytic system

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Abstract

The wild-type tumor suppressor protein p53 is a short-lived protein that plays important roles in regulation of cell cycle, differentiation, and survival. Mutations that inactivate or alter the tumor suppressor activity of the protein seem to be the most common genetic change in human cancer and are frequently associated with changes in its stability. The ubiquitin system has been implicated in the degradation of p53 both in vivo and in vitro. A mutant cell line that harbors a thermolabile ubiquitin-activating enzyme, E1, fails to degrade p53 at the nonpermissive temperature. Studies in cell-free extracts have shown that covalent attachment of ubiquitin to the protein requires the three conjugating enzymes: E1, a novel species of ubiquitin-carrier protein (ubiquitin-conjugating enzyme; UBC),E2-F1, and an ubiquitin-protein ligase, E3. Recognition of p53 by the ligase is facilitated by formation of a complex between the protein and the human papillomavirus (HPV) oncoprotein E6. Therefore, the ligase has been designated E6-associated protein (E6-AP). However, these in vitro studies have not demonstrated that the conjugates serve as essential intermediates in the proteolytic process. In fact, in many cases, conjugation of ubiquitin to the target protein does not signal its degradation. Thus, it is essential to demonstrate that p53-ubiquitin adducts serve as essential proteolytic intermediates and are recognized and degraded by the 26S protease complex, the proteolytic arm of the ubiquitin pathway. In this study, we demonstrate that conjugates of p53 generated in the presence of purified, E1, E2, E6-AP, E6, ubiquitin and ATP, are specifically recognized by the 26S protease complex and degraded. In contrast, unconjugated p53 remains stable. The ability to reconstitute the system from purified components will enable detailed analysis of the recognition process and the structural motifs involved in targeting the protein for degradation.

Key words: p53; Degradation; Ubiquitin; Protease complex, 26S

1. Introduction

The tumor suppressor protein p53 is a short-lived protein with a $t_{1/2}$ of ~ 20 min [1]. Functionally, this instability probably reflects a requirement for rapid regulation of processes in which the protein plays a role. Structurally, it implies that the molecule contains signal(s) that target it for rapid degradation. Cumulative evidence indicates that the ubiquitin system is involved in the degradation of p53 both in vivo and in vitro. Utilizing a mutant cell line, ts20, that harbors most probably a thermolabile E1, Chowdary and colleagues have shown that wt p53 is stabilized when the cells are shifted to the restrictive temperature. Following introduction of a cDNA clone that encodes E1, degradation resumes [2]. Studies in reticulocyte lysates have demonstrated a role for the ubiquitin system in the degradation of p53 in a cell-free system. Immunodepletion of E1 resulted in a complete inhibition of proteolysis. The process resumed following addition of purified E1 to the system [3]. Werness and colleagues noted exceptionally low levels of p53 in human cervical carcinoma cell lines transformed by the 'high risk' HPVs-16 and -18. They demonstrated that the E6 proteins of the 'high risk' but not the 'low risk' HPVs associate with p53 in vitro [4]. They tested the hypothesis that, unlike other complexes that p53 forms and that lead to stabilization of the protein, HPV E6s target the protein for degradation. Such a mechanism could explain, at least in part, the tumorigenicity of these oncoproteins. Indeed, the 'high risk' E6 proteins stimulate ATP-dependent conjugation and degradation of p53 in crude reticulocyte lysates [5]. There is a clear correlation between the ability of different p53 species to associate with E6 and their sensitivity to proteolysis. For example, human p53-Thr^{232} does not associate with E6 and is relatively stable in the cell-free proteolytic system. In contrast, p53-His^{273} binds strongly to E6 and is degraded efficiently [6]. The mechanism(s) of targeting of p53 by E6 is not known. It was noted that E6 remains stable throughout the proteolytic cycle. It is possible that E6 is recognized by the ubiquitin-protein ligase, but cannot be targeted as it does not have accessible ubiquitination sites (see below). In contrast, p53 cannot bind to the ligase, but it harbors Lys residues that can be readily conjugated. Similar *trans* recognition and targeting have

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Abbreviations: DEAE, diethylaminoethyl; DTT, dithiothreitol; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein or ubiquitin conjugating enzyme, UBC; E3, ubiquitin-protein ligase; E6, human papillomavirus 'high-risk' E6 16 oncoprotein; E6-AP, E6-associated protein; FPLC, fast protein liquid chromatography; HPV, human papillomavirus; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

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been demonstrated in the degradation of other proteins as well. A fusion protein between the N-terminal half of the HPV-16 E7 protein and the full-length HPV-16 E6, promoted the in vitro degradation of the retinoblastoma protein [7]. The E7 protein harbors the retinoblastoma binding site, whereas the E6, most probably, binds to the ubiquitin ligase and thus confers the proteolytic sensitivity. In a different case, Johnson and colleagues have shown that in a heterodimeric protein, the N-terminal residue that serves as the ligase-binding site and the Lys residue that is targeted by ubiquitin, can reside in different subunits of the protein [8]. In this case, only the ubiquitinable subunit is degraded. Recently, two additional factors involved in the conjugation of p53 have been purified and characterized. We have shown that a novel species of E2 is required for conjugation and degradation of p53 [9]. 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; see below). Thus it was designated E2-F1 [10]. The enzyme is not specific to p53 and is involved in the degradation of a variety of cellular substrates. Interestingly, it recognizes non-'N-end rule' protein substrates such a glyceraldehyde-3-phosphate dehydrogenase (Val at the N-terminal position; [10]). Scheffner and colleagues have described what appears to be a similar enzyme [11]. These researchers have also shown that the E6-p53 complex binds to a cellular protein of 100 kDa, designated E6-AP. Formation of this ternary complex leads to rapid ubiquitination and subsequent degradation of the protein. In all these studies, researchers have purified and characterized the components involved in conjugation of ubiquitin to p53. However, it has not been shown that the conjugates serve indeed as essential intermediates in the process: modification of proteins by ubiquitin can serve nonproteolytic functions as well [12]. Furthermore, it has not been shown that the 26S protease complex, the protease that recognizes and degrades ubiquitin-modified proteins, plays a role in the process. These two unresolved problems have been investigated in the present study.

2. Experimental procedures

2.1. Materials

cDNAs encoding murine wt p53 and HPV-16 E6 were subcloned into pSP64 and pGEM-2 transcription vectors as described [9]. cDNA that encodes a truncated 75 kDa form of E6-AP constructed into the bacterial expression vector pGEX-2TK vector [11] was obtained from Dr. Martin Scheffner, DKFZ, Heidelberg, Germany. A recombinant baculovirus expressing the 95 kDa form of E6-AP [13] was obtained from Drs. Jon M. Huibregtse and Peter M. Howley, Harvard Medical School, Boston, MA, USA. Wheat germ extract for in vitro translation was from Promega. All restriction enzymes used were from New England Biolabs. Centricon 30 microconcentrators were from Amicon. Rainbow prestained molecular weight markers were from Amersham. L-[^{35}S]Methionine (~ 1,200 Ci/mmol) and Enlightning enhancer were from DuPont-New England Nuclear. All other materials and reagents were as described [9].

2.2. Methods

Subcloning of cDNAs and synthesis of mRNAs and labeled proteins. All manipulations of plasmids (such as transformations, purification, and restriction analyses) were carried out as described [14]. Synthesis of mRNAs of p53 and E6 from the corresponding cDNAs was carried out as described [9,14] using the appropriate RNA polymerases. mRNA was translated in vitro in the presence of [^{35}S]methionine (p53) or unlabeled methionine (E6) except that wheat germ extract was used instead of rabbit reticulocyte lysate. Unlike the mammalian cells, the plant cells do not contain E6-AP [11,13]. This selective development enables a relatively easy purification of the labeled protein and monitoring its conjugation and degradation following addition of exogenous purified enzyme. Following synthesis, the proteins were further purified using anion exchange chromatography as described [3,9].

Preparation of E1, E2-F1, E6-AP, and 26S protease complex. Reticulocyte-rich blood was induced in rabbits by successive injections of phenylhydrazine, and reticulocyte lysates were prepared as described [15]. Following depletion of ATP, lysates were resolved by anion exchange chromatography on DEAE-cellulose into unadsorbed material (Fraction 1) and high salt eluate (Fraction 2) as described [15]. E1 was purified to homogeneity from Fraction 2 following 'covalent' affinity chromatography over immobilized ubiquitin as described [15]. E2-F1 was purified to homogeneity from Fraction 1 as described [10]. E6-AP was purified from three independent sources: (a) glutathione-transferase-E6-AP fusion protein was purified using affinity chromatography over immobilized glutathione [11]; (b) using anion exchange chromatography over Mono-Q FPLC column, E6-AP was partially purified from Sf9 cells following infection with a recombinant virus expressing the protein; (c) the enzyme was partially purified from reticulocyte Fraction 2 by $(NH_4)_2SO_4$ precipitation (0–38%) and gel filtration chromatography over Superdex 200 HR (16 × 600 mm) column. 8 mg of protein were loaded onto the column and the proteins were resolved in a buffer that contained 20 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 1 mM DTT. Fractions of 2.4 ml were collected. The samples were concentrated to 200 μl and the buffer was rapidly changed to a similar buffer that does not contain salt by repeated concentration–dilution cycles using Centricon 30 microconcentrators. Although all three forms of the ligase are active in conjugation of ubiquitin to p53, the bacterially expressed truncated enzyme has a relatively low specific activity. Therefore, in order to generate sufficiently large amount of degradable adducts, we used either the baculovirus expressed or the reticulocyte-based enzyme. The reticulocyte enzyme was used in most assays as it is more readily available. The 26S protease complex was purified to homogeneity as described [16].

Reconstitution of proteolysis of p53 by purified components of the ubiquitin system. A complete reaction mixture contained in a final volume of 25 μl: 0.5 μg E1, 0.15 μg E2-F1, 3.0 μg E6-AP, ~ 20,000 cpm p53 (~ 1 ng; 9), 0.5 μl E6 (~ 1.8 ng; 6-fold the molar amount of p53; 9), 1.5 μg 26S protease complex, 1.0 μg ubiquitin, 2 mM ATP, 5 mM $MgCl_2$, 2.5 mM NH_4COOH, 2 mM DTT, and 40 mM Tris-HCl, pH 7.6. Degradation in crude reticulocyte lysate was monitored in a similar reaction mixture in which the enzymes (E1, E2-F1, E6-AP and the 26S protease) were substituted for 10 μl of crude reticulocyte lysate. In addition, the mixture also contained creatine kinase and phosphocreatine (for regeneration of ATP) as described [9]. Following incubation at 30°C for 3 h, degradation of the [^{35}S]methionine-labeled protein was determined by monitoring the radioactivity released into trichloroacetic acid (TCA)-soluble fraction as described [3,9]. In all cases, two sets of reactions were incubated, one contained ATP and the other contained hexokinase (0.5 μg) and 2-deoxyglucose (10 mM). Degradation values in systems from which ATP was depleted did not exceed 20% of the values obtained in the presence of ATP. These values were subtracted and results are presented as net ATP-dependent degradation (% of total protein degraded).

Formation and degradation of ubiquitin-p53 conjugates. Ubiquitin-p53 conjugates were generated in a 200 μl reaction mixture that contained: 4.0 μg E1, 1.2 μg E2-F1, 24.0 μg E6-AP, ~ 200,000 cpm p53, 4.0 μl E6, 10.0 μg ubiquitin, 2 mM ATP, 5 mM $MgCl_2$, 2.5 mM NH_4COOH, 2 mM DTT, and 40 mM Tris-HCl, pH 7.6. Following incubation for 1 h at 30°C, 10 μl of 2 N NaOH was added and the incubation continued for additional 10 min at 30°C. The base was neutralized by the addition of 9 μl of 2 N HCl (to a final pH of ~ 8–9) and the conjugates were stored at 4°C. The mixture contained conjugated p53 as well as unreacted protein. However, it did not contain any active conjugating

enzyme: the enzymes were inactivated by NaOH. To study the effect of the 26S protease complex on unconjugated p53, we incubated p53 in a similar reaction mixture that did not contain the conjugating enzymes, E6, ubiquitin and ATP. Quantitative analysis revealed that 37% of the labeled protein was shifted to high molecular mass ubiquitin adducts. To follow the partial purification of E6-AP, we also monitored formation of conjugates. In this case, the reaction mixture contained in a final volume of 25 μl: 0.5 μg E1, 0.15 μg E2-F1, 2.0 μl of the Superdex 200 HR column (as a source for E6-AP; see above), ~ 10,000 cpm p53, 0.5 μl E6, 2.0 μg ubiquitin, 1 μg ubiquitin aldehyde, a specific inhibitor of certain isopeptidases [17], 5 mM ATPγS, 5 mM $MgCl_2$, 2.5 mM NH_4COOH, 2 mM DTT, and 40 mM Tris-HCl, pH 7.6. Degradation of conjugates or free p53 by the purified 26S protease was monitored in a reaction mixture that contained in a final volume of 25 μl: ~ 10,000 cpm p53-ubiquitin conjugates-free p53 mixture or free p53, 1.5 μg 26S protease complex, 5 mM ATP (or a system without ATP), 5 mM $MgCl_2$, 2.5 mM NH_4COOH, 2 mM DTT, and 40 mM Tris-HCl, pH 7.6. Following incubation at 30°C for 3 h, degradation of the [^{35}S]methionine-labeled protein was determined by measuring the radioactivity released into TCA-soluble fraction as described above. Formation and degradation of conjugates were also monitored by SDS-PAGE (10%) [18] followed by fluorography as described [9].

3. Results

3.1. Purification of E6-AP, the ubiquitin-protein ligase involved in conjugation of p53

Whereas E1 and E2-F1, the two first enzymes in the conjugation process, have been purified to homogeneity, the E3 involved in the process, E6-AP, is not available in large enough amounts to enable one to carry out reconstitution of the complete proteolytic system. Initially, we utilized a truncated recombinant form that lacks the N-terminal domain of the enzyme [11]. The enzyme is expressed as a fusion protein with glutathione transferase and has been purified via affinity chromatography over immobilized glutathione. The enzyme is active; however, the specific activity is extremely low and does not allow formation of large amounts of conjugates that can serve as substrates to the 26S protease complex (not shown). The low activity is probably due to the fact that the protein is not complete. Trials to express the protein from full-length cDNA have failed (J.M. Huibregtse, M. Scheffner, and P.M. Howley, unpublished results). Therefore, we have partially purified an intact enzyme from either Sf9 cells that express a recombinant virus containing the enzyme, or from a native source, reticulocyte lysates. Both enzymes were similarly active, and the assays presented in this study have been performed using the reticulocyte enzyme. Fig. 1 demonstrates the last purification step, gel filtration chromatography over Superdex 200 HR FPLC column. Fractions 27–29 were pooled and served a source for the enzyme.

3.2. Reconstitution of degradation of p53 from purified components of the ubiquitin proteolytic system

Following purification of all the enzymatic components involved in the proteolytic process, it was impor-

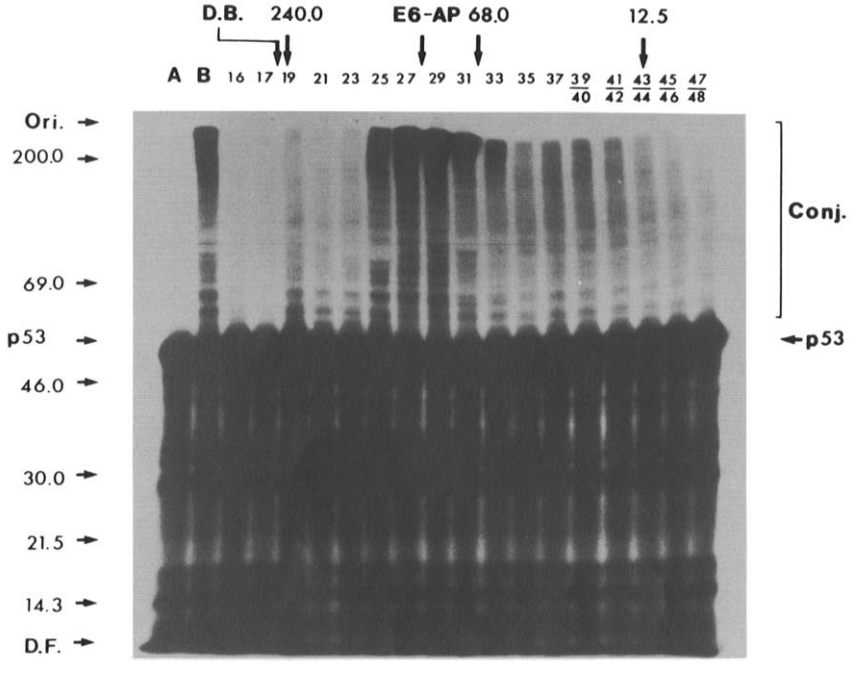

Fig. 1. E6-AP activity: gel filtration chromatography of ammonium sulfate-fractionated Fraction 2. Monitoring of E6-AP activity was carried out in the presence of purified E1, E2-F1, E6, ubiquitin, and ATP in fractions resolved on a Superdex 200 FPLC column as described in section 2. Lane A = p53 substrate; lane B = reaction carried out in the presence of 10 μg unresolved extract; Lanes 16–37 = activity in fractions 16–37; lanes 39/40–47/48 = activity in the corresponding paired pooled fractions. D.B. = elution of dextran blue; 240, 68 and 12.5 = elution of catalase, bovine serum albumin, and cytochrome c, respectively. Molecular weight markers are: 200.0 = myosin; 69.0 = bovine serum albumin; 46.0 = ovalbumin; 30.0 = carbonic anhydrase; 21.5 = soybean trypsin inhibitor; 14.3 = lysozyme. Ori. denotes origin of gel; Conj. denote conjugates; D.F. denotes dye front. Labeled p53 is marked.

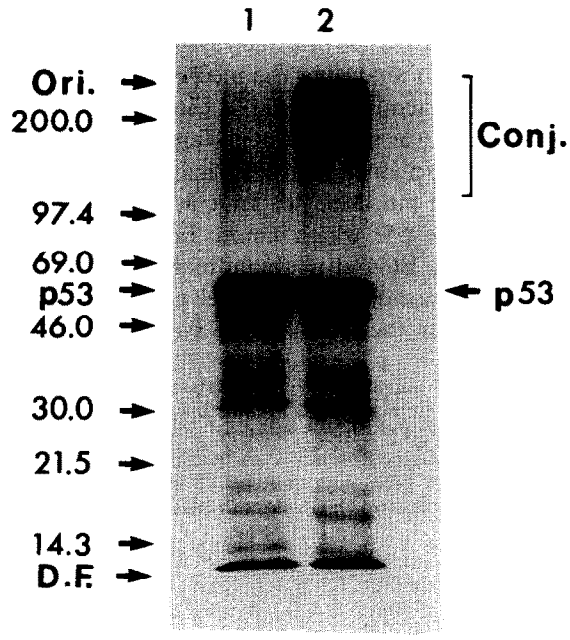

Fig. 2. Formation of p53-ubiquitin conjugates. Conjugates were generated as described in section 2. Lane 1 = reaction mixture was incubated without the conjugating enzymes, E6, ubiquitin and ATP; lane 2 = complete reaction mixture. Molecular weight markers are as described in the legend to Fig. 1, except that 97.4 is phosphorylase *b*. Other marks are as described in the legend to Fig. 1.

tant to demonstrate that degradation can be reconstituted following addition of all the components to the reaction mixture. As can be seen from Table 1, this is indeed the case. It should be noted that omission of E1 does not inhibit the reaction completely. This is probably due to the fact that the purified preparations of p53 and E6, that are derived from wheat germ extract, contain a small amount of the enzyme. E1 is an extremely active enzyme and small amounts that have not been removed can explain activity in such extracts. Also noted is the activity obtained in the absence of E6. We have noted already [3,9] that ATP- and ubiquitin-dependent degradation proceeds also in the absence of the viral oncoprotein, though at a lower rate. This is not surprising, as wt p53 is short-lived in all cells studied, most of them do not

Table 1
Reconstitution of degradation of p53 from purified components of the ubiquitin proteolytic pathway

Components of system	Degradation (%)
Complete system	9.3
Complete system – E1	3.2
Complete system – E2-F1	0.0
Complete system – E6-AP	0.0
Complete system – E6	2.6
Complete system – 26S protease	0.0
Reticulocyte lysate	21.3

Degradation in the various reconstituted systems and in crude reticulocyte lysate was monitored as described in section 2.

contain E6. An important finding relates to the role of the 26S protease complex in the proteolytic process: in this experiment it has been demonstrated clearly that the protease serves an essential role.

3.3. *The 26S protease complex degrades preformed p53-ubiquitin conjugates but not free p53*

To demonstrate the intermediacy of p53-ubiquitin adducts in the proteolytic process, we have first generated large enough amounts of high molecular mass adducts. These are demonstrated in Fig. 2. It is clear that the adducts are generated from p53, as loading of equal amounts of radioactivity on the gel demonstrates reduction in the amount of free p53 with a concomitant increase in the amount of the conjugates formed (Fig. 2, compare lane 1 to lane 2). These conjugates were subjected to proteolysis by purified 26S protease complex. As can be clearly seen from Table 2, the conjugates are degraded in an ATP-dependent mode. This finding rules out the possibility that the 20S protease can also recognize the adducts, as the activity of this enzyme does not

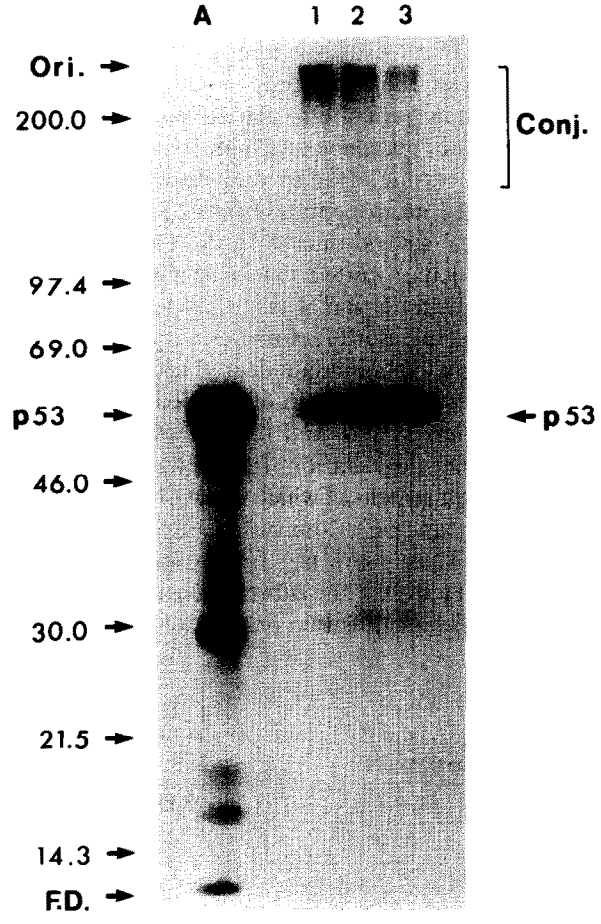

Fig. 3. Degradation of p53-ubiquitin conjugates by purified 26S protease complex. Degradation of conjugates was monitored as described in section 2. Lane A = unreacted p53; lane 1 = reaction mixture without protease complex; lane 2 = with 0.75 μg 26S complex; lane 3 = with 1.5 μg 26S complex. All other marks are as described in the legend to Fig. 1. F.D. denotes front of dye.

require energy. In contrast, unconjugated p53 is not recognized and degraded. Fig. 3 demonstrates similar findings. Interestingly, certain amount of the intact protein is regenerated (compare lane 1 to lanes 2 and 3). This is probably due to the activity of isopeptidase(s) that is an integral part of the complex. Such an isopeptidase has been described [19], though, the p53-regenerating activity is not necessarily the same as the one described.

4. Discussion

We have shown that degradation of p53 can be reconstituted from purified components of the ubiquitin proteolytic system. The study clearly demonstrates that ubiquitin protein conjugates serve as essential intermediates in the proteolytic process and that the free protein cannot be degraded. Furthermore, it shows, for the first time, that the 26S protease is involved in recognition of the conjugates and in their degradation. It should be noted, however, that degradation in crude reticulocyte lysate is more efficient. It may be that in the crude extract the different components act more efficiently. For example, they may associate with one another to form multienzyme complexes that can process the substrate in a processive manner. An alternative explanation is that the system is still not complete and additional auxiliary factors are still missing. Hadari and colleagues have recently described an isopeptidase (Iso-T) that stimulates degradation of several factors [20]. Gonen and colleagues have demonstrated that a specific factor is required for the degradation of several N-terminal blocked proteins (FH) [16]. Both factors act along with the 26S protease complex to stimulate degradation of ubiquitin-modified substrates. Addition of purified Iso-T and FH to both complete system and the 26S protease did not stimulate further degradation. It may well be that other factors, the requirement of which is not obvious from our current understanding of the system, also play a role in the process. These can be, for example, molecular chaperones that present the substrate in a proper conformation for either conjugation or degradation. Establishment of a cell-free proteolytic system can promote isolation of such putative factors. Such systems can also serve as useful tools in analyzing the mechanism of action of the ubiquitin system. For example, the mechanistic basis for the role of E6 as a *trans* recognition element can now be studied. As explained above, E6 may harbor the ligase binding domain but does not have a ubiquitinable Lys residue, whereas p53 which cannot bind directly to the ligase, binds via E6 and is ubiquitinated at specific Lys residues and degraded. This model can now be tested experimentally in the reconstituted system.

Table 2
Degradation of ubiquitin-p53 adducts and free unconjugated p53 by purified 26S protease complex

Substrate	System	Degradation (%)
p53	−ATP	0.9
	+ATP	2.7
p53-Ub	−ATP	4.1
	+ATP	35.6

Degradation of free p53 and ubiquitin-p53 conjugates was monitored following release of radioactivity to the TCA-soluble fraction as described in section 2. As the preparation of conjugates contain also free unreacted p53, results were corrected to reflect the proportion of radioactivity in the conjugates.

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