Ubiquitin-mediated Processing of NF-κB Transcriptional Activator Precursor p105

RECONSTITUTION OF A CELL-FREE SYSTEM AND IDENTIFICATION OF THE UBIQUITIN-CARRIER PROTEIN, E2, AND A NOVEL UBIQUITIN-PROTEIN LIGASE, E3, INVOLVED IN CONJUGATION*

(Received for publication, April 28, 1995, and in revised form, June 22, 1995)

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In most cases, the transcriptional factor NF-κB is a heterodimer consisting of two subunits, p50 and p65, which are encoded by two distinct genes of the Rel family. p50 is translated as a precursor of 105 kDa. The C-terminal domain of the precursor is rapidly degraded, forming the mature p50 subunit, located within the N-terminal region of the molecule. The mechanism of generation of p50 is not known. It has been suggested that the ubiquitin-proteasome system is involved in this process; however, the specific enzymes involved and the mechanism of limited proteolysis in which half of the molecule is spared, have been obscure. Palombella and colleagues (Palombella, V. J., Rando, O. J., Goldberg, A. L., and Maniatis, T. (1994) Cell 78, 773-785) have shown that ubiquitin is required for the processing in a cell-free system of a truncated, artificially constructed, 60-kDa precursor. They have also shown that proteasome inhibitors block the processing both in vitro and in vivo. In this study, we demonstrate reconstitution of a cell-free processing system and demonstrate directly that: (a) the ubiquitin-proteasome system is involved in processing of the intact p105 precursor, (b) conjugation of ubiquitin to the precursor is an essential intermediate step in the processing, (c) the recently discovered novel species of the ubiquitin-carrier protein, E2-F1, that is involved in the conjugation and degradation of p53, is also required for the limited processing of the p105 precursor, and (d) a novel, ~320-kDa species of ubiquitin-protein ligase, is involved in the process. This novel enzyme is distinct from E6-AP, the p53-conjugating ligase, and from E3α, the "N-end rule" ligase.

NF-κB and other members of the Rel family of transcriptional activators have recently gained considerable attention because of their unique mechanism of activation, and their active role in cytoplasmic-nuclear signaling in response to a variety of extracellular stimuli. In most cases, NF-κB is a heterodimeric complex composed of p50 and p65 subunits. p50 is initially synthesized as an inactive 105-kDa precursor that is processed to the mature protein following removal of the C-terminal domain of the molecule. A second precursor molecule, p100, gives rise to the p52 DNA-binding subunit of NF-κB, probably via a similar mechanism. The heterodimeric complex was originally identified as an inducible B cell-specific transcription factor able to bind the κ light chain enhancer (1). NF-κB is now recognized to be a ubiquitously expressed factor that is present as an inactive ternary complex in the cytosol of most cells. In this ternary complex, a third inhibitory protein belonging to the IκB family is associated with the heterodimer (for recent reviews, see Refs. 2-4). A wide range of stimuli such as bacterial products, parasites, viruses and viral products, inflammatory cytokines, T cells, B cells, fibroblast mitogens, protein synthesis inhibitors, physical and oxidative stresses, and drugs lead to accelerated processing of the precursor, degradation of the inhibitor, and consequent translocation of the active heterodimeric factor from the cytoplasm to the nucleus, where it exerts its transcriptional activity (2).

Cellular target genes are largely involved in the acute-phase response, inflammation, lymphocyte activation, and cell growth and differentiation. These genes include, among others, immunoreceptors such as the α and β chains of the T cell receptor, major histocompatibility protein class I and II molecules, cell adhesion molecules such as ELAM-1 and VCAM-1, cytokines, hematopoietic growth factors such as granulocyte-macrophage colony-stimulating factor and interleukin-2, and acute phase proteins such as angiotensinogen and complement factor B. Interestingly, it also affects regulation of transcription of transcriptional activators, including p105, c-Rel, and IκBα (2, 5). The physiological role of NF-κB has been recently studied by using mice that lack the p50 subunit. The disruption leads to multifocal defects in a whole array of immune responses. These involve, for example, inability of B lymphocytes to respond to bacterial products, defect in basal and specific antibody production, and high susceptibility to certain pathogens (6). However, it is not clear whether the absence of other members of the Rel family, p53 or RelB for example, display a similar phenotype.

The NF-κB/Rel proteins are subjected to multiple regulatory influences. A major component of this regulation involves control of their intracellular localization, with the inactive proteins maintained in the cytosol. Regulation is controlled by two major pathways: (i) control of p105 and p100 processing and (ii)
interaction of the p50/p65 heterodimer with a group of inhibitor molecules, IxBs. The two pathways may respond to different stimuli, thus providing a tight control of the response. (i) The precursor molecules p105 and p100 contain in their N-terminal region the p50 and the p52 motifs, whereas the C-terminal domain contains ankyrin repeats, homologous to IxB (7, 8). The p50 subunit of NF-κB is generated by ATP-dependent processing of p105 in vivo and in vitro (Ref. 9; in vitro, the researchers used an artificially truncated form of p105 with a molecular mass of 60 kDa, p60). The processing is regulated. It is accelerated, for example, after treatment of cells with tumor necrosis factor α, double-stranded RNA, and phorbol esters (10, 11). Recent evidence suggests that the ubiquitin-proteasome pathway is involved in processing of the NF-κB precursor, p105 (12). In vitro, addition of ubiquitin to a Fraction II which does not contain the protein, stimulated processing of p60 to p50. Addition of ubiquitin-Arg48, a derivative of ubiquitin that cannot generate polyubiquitin chains, inhibited processing. However, in these two experiments the researchers have not demonstrated the formation of ubiquitin-p60 adducts, the essential intermediates in the process. In a different set of experiments, it was shown that inhibitors of the 20 S proteasome complex block processing not only of p60 in vitro, but also of p105 in intact cells (12). Inhibition of the proteasome does not necessarily indicate intermediacy of the ubiquitin system in the process. Ornithine decarboxylase, for example, is degraded in a proteasome-dependent, but ubiquitin-independent, process (13). (ii) The interaction with the inhibitor, IxB, masks a nuclear localization signal and thus leads to retention of the heterodimer in the cytosol (14). In response to a variety of extracellular stimuli, IxB is rapidly degraded, thus exposing the nuclear localization signal and allowing translocation of the p50/p65 dimer to the nucleus. Recently, it has been shown that extracellular stimuli lead to phosphorylation of the inhibitor, but that this modification is not sufficient for activation of NF-κB (15–18). It was proposed that phosphorylation signals the protein for degradation, and the protein is probably degraded while associated as a complex with the other two components of the ternary complex. Indeed, Brown and colleagues (19) have shown that phosphorylation of Ser residues 32 or 36 may serve as a proteolysis recognition signal. Furthermore, inhibitors of the 20 S proteasome stabilize specifically the phosphorylated form (20). Recent evidence from our laboratories indicate that the ubiquitin system is involved in rapid degradation of the stimulation-induced phosphorylated form of the inhibitor. The unmodified form is not recognized.1

The ubiquitin-dependent pathway plays an important role in the degradation of several key short-lived regulatory proteins (reviewed recently in Ref. 21). Degradation of a protein via the ubiquitin pathway involves two distinct steps, both requiring ATP: signaling of the protein by covalent attachment of multiple molecules of ubiquitin, and degradation of the targeted protein by a multisubunit 26 S proteasome complex. Conjugation of ubiquitin proceeds via a three-step mechanism. Initially, ubiquitin is activated to a high energy intermediate by the ubiquitin-activating enzyme, E1.2 Following activation, ubiquitin-conjugating enzyme, E2, transfers ubiquitin from E1 to a ubiquitin-protein ligase, E3, to which the target protein is bound. E3, therefore, appears to play a major role in selection of proteins for conjugation and degradation. The multiply ubiquitinated substrate is specifically recognized by the 26 S proteasome complex. The protein moiety of the adduct is degraded with the release of free and reutilizable ubiquitin (reviewed in Refs. 22, 23).

Processing of the p105 precursor is the first example in which the ubiquitin-proteasome pathway is involved in limited proteolysis and not in complete destruction of its protein substrates. The finding that E1 (and the whole ubiquitination machinery) is required for limited processing of antigenic proteins and generation of antigenic peptides (24) has been challenged recently (25). It is not known whether the C-terminal domain is first cleaved and then degraded, or if processing proceeds via successive removal of amino acid residues from the C-terminal residue of p105. Consequently, the nature of the cleavage site, or the “stop” signal, is also obscure. Thus, because of the peculiar mechanisms involved and the central role that NF-κB plays in many basic physiological and pathological processes, dissection of the mechanism involved in ubiquitin modification and processing of p105 has broad biological implications. Analysis of the enzymatic process can provide an important insight into the regulation and the recognition signals involved.

Using a cell-free system, we have been able to reconstitute processing of the intact p105 precursor into the mature p50 protein. We demonstrate that the precursor is multiply ubiquitinated and that formation of the high molecular weight conjugates is essential for the processing to occur. We also demonstrate that the process is mediated by a recently described ubiquitin-carrier protein, E2-F1 (26) that is involved in the degradation of p53 (27). In addition, a novel, still unidentified, ~320 kDa species of ubiquitin-protein ligase, E3, appears to catalyze the final step in the conjugation reaction, transfer of ubiquitin to the substrate. The ligase is clearly distinct from E6-AP, the p53-recognizing ligase, and from E3x, the N-end rule ligase. However, like E6-AP, chemical modification of -SH groups of the new E3 enzyme inhibits its activity.

**EXPERIMENTAL PROCEDURES**

Materials—Plasmid pT7/p105 contains the entire coding sequence of human p105 fused to the 5'-untranslated region of β-globin to ensure efficient translation in vitro (7, 28). The cDNA was subcloned into pGEM2 transcription vector (Promega) that contains a T7 promoter. Vectors containing E6 and wild type p53 cDNAs were described elsewhere (27). Na[35]methionine (~1,200 Ci/mmol) was purchased from DuPont NEN. Amplify enhancer and Rainbow prestained molecular weight markers were purchased from Amersham Corp. Lysozyme, ubiquitin (bovine), ovalbumin, DTT, ATP, phosphocreatine, creatine kinase, 2-deoxyglucose, NEM, sodium iodoacetate, p-hydroxy mercurobenzoate, and Tris buffer were purchased from Sigma. Hexokinase, ribonuclease A (RNase A), and ATP-γ-S were from Boehringer Mannheim. DEAE-cellulose (DE-52) was purchased from Whatman. Acrylamide, N,N'-methylene bisacrylamide, TEMED, ammonium persulfate, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), and Coomassie Brilliant Blue were from Bio-Rad. Ammonium sulfate was obtained from Life Technologies, Inc. HEPES (N-[2-hydroxyethyl]piperazine-N'-2-ethanesulfonic acid) was purchased from Calbiochem. MG115, a 20 S proteasome inhibitor, was a kind gift from Drs. J. Adams and R. Stein, Myogenics Inc., Cambridge, MA. All tissue culture reagents were purchased from Biological Industries, Kibbutz Beit Haemek, Israel. Centricon 10 microconcentrators were from Amicon. Wheat germ and rabbit reticulocyte lysate-based TNT transcription-translation coupled kits were from Promega. HiLoad Superdex 260 HR (16 x 600 mm) column was from Pharmacia Biotech Inc. All other chemicals were of analytical grade.

Cell Line—C3.F6 B lymphocyte cell line was obtained from Dr. Osami Kanagawa, Washington University School of Medicine, St. Louis, MO. Cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

General Methods—Radio-labeled Proteins—[35S]Methionine-labeled p105 and p53 were synthesized using a TNT-wheat germ-based or TNT-rabbit reticulocyte-based transcription-translation coupled systems.
Translations were carried out according to the manufacturer's instructions in 200-μl reaction mixtures containing 100 μl of extracts and 4 μg of circular plasmid containing the appropriate cDNAs. E6 was generated in a similar manner, except that the labeled amino acid was replaced with 0.1 mM unlabeled methionine. When indicated, the translation mixture containing the labeled substrate was resolved on DEAE to remove ubiquitin and E2-F1, and other potential Fraction I-containing E2 enzymes (26, 29, 30). Alternatively, all E2 enzymes, including E2-F1, contained in the translation extract, were inactivated following translation by incubating the reaction mixture with NEM (10 mM for 10 min at room temperature). The inhibitor was neutralized by the addition of 6 mM DTT. 35S-Labeled lysozyme was prepared by the chloramine-T method as described (26).

Preparation of Rabbit Reticulocyte Lysate and Conjugating Enzymes—Reticulocyte-rich blood was induced in rabbits by successive injections of phenylhydrazine, and lysate was prepared as described (31). Fractionation of the lysate into Fraction I, which contains ubiquitin and E2-F1, and Fraction II, which contains several E2 enzymes, all known E3 enzymes, and the 26 S proteasome complex, was performed as described (31). E1, E2-14-KDa, and E3v were purified from Fraction I by affinity chromatography over immobilized ubiquitin and immobi lized β-lactoglobulin as described (32). E2-F1 was purified to homogeneity from Fraction I as described (26). Recombinant Ubch5, the human homolog of E2-F1 (Ref. 30; the encoding cDNA cloned into a pET3a vector was kindly obtained from Dr. Martin Scheffner, DKFZ, Heidelberg, Germany), was purified from bacterial extracts after induction as follows. Extracts (from 1 liter of cultured cells) were chromatographed on DEAE-anion exchange resin (31), and Fraction I was resolved by ammonium sulfate precipitation into two portions, 0–38% and 42–80%. The 42–80% precipitate was dissolved in a small volume of a buffer containing 20 mM Tris-HCl, pH 7.2, and 2 mM DTT, and resolved via gel filtration chromatography on a HiLoad Superdex 75 HR (16 × 600 mm) column in a similar buffer containing also 150 mM NaCl. Fractions of 2.4 ml were collected, and Ubch5 was identified following staining with Coomassie Brilliant Blue. We obtained 1 mg of ~90% homogeneous protein.

Initial Characterization of p105 NF-κB Precursor-Ubiquitin Ligase, E3—Fraction I was fractionated by (NH4)2SO4 into Fraction IIA (0–38%) and Fraction IIB (42–80%) as described (33). 2 ml of Fraction IIA (20 mg/ml) were loaded onto a HiLoad Superdex 200 HR (16 × 600 mm) gel filtration chromatography column, and the proteins were resolved in a buffer containing 20 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 2 mM DTT (buffer A) using an FPLC system (Pharmacia). 2.4-ml fractions were collected, and salt was removed by repeated concentration-dilution cycles using Centricon 10 microconcentrators with buffer A that does not contain NaCl. Fractions were concentrated to 100 μl.

Preparation of Cell Extract—C3.F6 B Cells were washed twice in phosphate-buffered saline and incubated on ice in hypotonic lysis buffer (5 × 106 cells/2 ml of buffer) containing 20 mM HEPES, pH 7.5, 10 mM KCl, and 1.5 mM MgCl2. Lysis was completed using a Dounce homogenizer. Nuclei were removed and cytosolic extract was prepared as described (34). Extracts contained ~8-10 mg of protein/ml. Fraction I and Fraction II from cytosol were prepared as described for the preparation of the similar fractions from reticulocyte lysate (see above).

Processing of p105—[35S]Methionine-labeled p105 was processed to p50 in a cell-free system containing 25 μl of reaction mixture, the following processing extracts: crude reticulocyte lysate (10 μl; ~1 mg of protein) or C3.F6 cytosolic extract, crude reticulocyte Fraction I, or wheat germ extract (~100 μg) as protein of each). In addition, the reaction mixture contained 40 mM Tris-HCl, pH 7.6, 5 mM KCl, 5 mM MgCl2, 2 mM DTT, ubiquitin (4 μg unless otherwise indicated), and ~40,000 cpm of the labeled substrate. Purified p105 was labeled with MeUb via gel filtration chromatography on a HiLoad Superdex 200 HR (16 × 600 mm) column in a similar buffer containing also 150 mM NaCl. Fractions of 2.4 ml were collected, and MeUb was identified following staining with Coomassie Brilliant Blue. We obtained 1 mg of ~90% homogeneous protein.

RESULTS

p50 is Synthesized from p105 in an ATP-dependent Process—As can be seen in Fig. 1, in vitro processing of p105 requires ATP. Similar results in a cell-free system were published by Fan and Maniatis (9) using p60, a truncated form of p105. We also demonstrated that p50 is generated from p105 in a time-dependent manner. Following a short translation (pulse), the predominant form generated is p105. When translation is inhibited (by the addition of excess unlabeled methionine and RNase A) and p105 is further incubated (chase), an increasing amount of p50 is generated with time at the expense of the precursor molecule (not shown).

p105 is Processed and Conjugated in Mammalian but Not in Wheat Germ Extract—Since only a small amount of p105 protein is synthesized in the in vitro cell-free translation system, it is virtually impossible to isolate it from the many components of the ubiquitin proteolytic system. Therefore, it was important to attempt to generate a substrate that will not be conjugated or processed co-translationally. Scheffner and colleagues (37) reported that p53 is conjugated by a specific E3 enzyme, designated E6-AP, that is present in mammalian cell but not in wheat germ extract. We tested the hypothesis that the p105-ubiquitin ligase and/or a different component of the conjugating machinery demonstrates a similar plant- animal kingdom distribution. As can be seen in Fig. 2, reticulocyte lysate and C3.F6 B cell cytosol can process p105 to p50. In striking contrast, wheat germ extract is inactive. In correlation with this finding, conjugation of ubiquitin to p105 also occurs in the mammalian cell extracts but not in the plant extract (Fig. 3).

We noted that both processing and conjugation are more efficient in the lymphocyte cytosol (compare Fig. 2, lanes 3 and 4, and Fig. 3, lanes 3 and 5). It is possible that an essential
Multiple Ubiquitination of p105 Is Essential for Processing—In order to demonstrate that ubiquitination is an essential intermediate step in processing of p105, it was necessary to show that inhibition of one process leads to suppression of the other as well. We demonstrated that conjugation in crude extract is extremely inefficient and can be stimulated significantly only with the addition of free ubiquitin (Fig. 4). This may not be surprising, as most of the ubiquitin in the extract may be “trapped” in conjugates, and the level of free ubiquitin is rather low (38). Addition of MeUb to a system that contains also exogenously added free ubiquitin inhibited conjugation (Fig. 5) and, consequently, processing of p105 (Fig. 6). MeUb is an analogue of ubiquitin in which all the free -NH₂ groups have been modified. It acts as a chain terminator and inhibits the formation of polyubiquitin chains that are essential for recognition of the adduct by the 26 S proteasome complex. Consequently, introduction of this derivative leads to inhibition of degradation (36, 39). Addition of excess free ubiquitin alleviated inhibition of both conjugation and processing (Figs. 5 and 6). Thus, it is clear that conjugation of ubiquitin is an essential intermediate step in processing of p105. In the processing reaction we noted the appearance of an additional processed form of a slightly higher molecular mass (Fig. 6). This form may be a processing intermediate or a product that was processed in a different site. We noted the appearance of this product, although in lower amounts, in most of the processing reactions (see, for example, Figs. 1 and 2; it should be noted that in the pulse-chase experiment demonstrating precursor-product relationships between p105 and p50 (see above), this upper form diminished with time, supporting the notion that it may well be a processing intermediate; not shown).

The Ubiquitin-Carrier Protein E2-F1 Is Essential for Conjugation of p105—In order to identify the enzymatic components involved in conjugation of p105, we gradually reconstituted the conjugation system from several essential components. As can be seen in Fig. 7 (panel A-I), the addition of ubiquitin to crude reticulocyte Fraction II was sufficient to promote efficient conjugation (compare lane 1 to lane 2). According to this result, one may conclude that the E2 is contained in Fraction II. It can be any of the many E2 enzymes contained in this fraction, including E2–14 kDa and E2–25 kDa (22, 23). Not surprisingly, addition of E2–14 kDa (that as noted is present in Fraction II) did not affect conjugation (lane 3). However, we noted a slight increase in conjugation following the addition of the E2-F1 enzyme that is derived from Fraction I (lane 4). Similarly, Palombella and colleagues (12) also reported that the addition of ubiquitin to Fraction II is sufficient to promote processing. Thus, their results obtained in crude Fraction II, using p60 as a substrate, are in accordance with the results of the experiment reported here. However, the slight increase in conjugation observed following the addition of E2-F1 (panel A-I, compare lane 4 to lanes 2 and 3), raised the suspicion that this enzyme (alone or with its Fraction I homologs) is involved in conjugation and is contained in the translation extract added with the labeled substrate. Thus, it is carried un-noticed into the reaction mixture and catalyzes conjugation of p105. Palombella and colleagues (12) used a similar translation extract to generate their substrate. To corroborate the possible involvement of E2-F1 in the conjugation of p105, we treated the translation extract (following completion of the reaction) with NEM, followed by neutralization of the reagent with DTT (Fig. 7, panel A-II; see “Experimental Procedures”). As all known E2 enzymes contain an essential -SH group necessary for their activity (23), the treatment inactivates the E2 enzymes contained in the extract. Indeed, when the substrate-containing extract is first treated with NEM, conjugation becomes completely dependent upon the addition of E2-F1. As can be seen in Fig. 7 (panel A-II), addition of ubiquitin (lane 2) and E2–14 kDa (lane 3) was not sufficient to promote conjugation. Only addition of purified E2-F1 (lane 4) restored conjugation. Using a different approach, we resolved the translation mixture on DEAE-cellulose to Fraction I and Fraction II. The substrate is contained in Fraction II, whereas E2-F1 is resolved with Fraction I (not shown). Addition of the resolved substrate to Fra-
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Fig. 4. Ubiquitin-dependent conjugation of p105. Conjugation of ubiquitin to p105 was monitored in reaction mixtures containing C3.F6 cytosolic extract as described under “Experimental Procedures.” Ubiquitin and ATPγS were added as indicated. Conj. denotes ubiquitin conjugates. Notes and molecular size markers are as described in the legend to Fig. 1.

Fig. 5. MeUb inhibits conjugation of p105, and suppression is alleviated by the addition of excess free ubiquitin. Conjugation of ubiquitin to p105 was monitored in reaction mixtures containing C3.F6 cytosolic extract as described under “Experimental Procedures.” Reaction mixtures contained the indicated amounts of ubiquitin and MeUb. ATPγS was added or omitted as indicated. Conj. denotes ubiquitin conjugates. Notes and molecular size markers are as described in the legend to Fig. 1.

Fig. 6. MeUb inhibits processing of p105, and inhibition is alleviated by excess free ubiquitin. Processing of p105 was monitored in reaction mixtures containing C3.F6 cytosolic extract as described under “Experimental Procedures.” Reaction mixtures contained the displayed amounts of ubiquitin and MeUb. ATP was added or omitted as indicated. Reaction mixtures were incubated in ice or at 37 °C as indicated. Notes and molecular size markers are as described in the legend to Fig. 1.

It should be noted that the partial peptide sequences obtained from E2-F1 that is derived from rabbit (26) are not identical to homologous areas contained in UbcH5 (30), which is a human enzyme. Both enzymes, however, are contained in Fraction I and are involved in p53 conjugation and degradation (27, 30, 37). Thus, either the E2-F1 enzyme is indeed the rabbit homolog of UbcH5 or, alternatively, two catalytically similar enzymes exist in Fraction I.

A Novel Ubiquitin-Protein Ligase, E3, Is Involved in Conjugation of p105—Identification of the E2 involved in conjugation of p105 allowed initial characterization of the ligase, E3. We had previously found that all the conjugation activity is contained in Fraction IIA (not shown), which is devoid of E1 and known E2 enzymes (26). Thus, it was appropriate to use this fraction as an initial source for purification of the ligase. Indeed, as can be seen in Fig. 7 (panel A), Fraction IIA contains an activity, that along with E1 and E2-F1 (or recombinant UbcH5, not shown) is necessary for conjugation of p105 (compare lane B to lane A). Resolution of the activity by gel filtration chromatography reveals that the activity has an apparent molecular mass of ~320 kDa. An identical result was obtained when the column fractions were screened in the presence of recombinant UbcH5 (not shown). This activity is clearly distinct from that of E6-AP, the ligase involved in conjugation of p53 (Fig. 8, panel B). This enzyme (40), which has a molecular
mass of ~100 kDa, migrates in the gel filtration column described in Fig. 8 as a ~120-kDa protein. The reason for the slight "deviation" from the authentic molecular mass is due to the presence of an ATP-independent protease with a molecular mass of ~80 kDa that digests both the substrate and conjugates (see for example Fig. 8, panels A and B, lanes 30). The disappearance of the labeled products leads to a slight deviation in the calculation of the molecular mass of E6-AP under the conditions employed. It is clear, however, that the two ligases are distinct enzymes: the p105 conjugating activity peaks in Fraction 24, whereas E6-AP peaks at Fraction 29. As can be seen in Fig. 9, the novel ligase is also distinct from E3x1, the N-end rule ligase. Addition of E3x1 to either E2-14 kDa or E2-F1 does not lead to the formation of conjugates (lanes A3 and A4). As expected, E3x1 promotes conjugation of lysozyme, a bona fide N-end rule substrate, when incubated in the presence of E1 and E2-14 kDa (Fig. 9, panel B).

To further characterize the novel E3, we investigated the possibility that, like E6-AP (41), it also contains an active -SH group. As can be seen in Fig. 10, the activity of the enzyme is inhibited by a variety of alkylating agents. It should be noted that since we did not use a purified enzyme, the possibility always exists that the sensitive protein is not the ligase, but a factor that is necessary for its activity. In this case, the fractions that contain the ligase activity represent an overlapping region between the ligase and the putative factor. If this is indeed the case, it is expected that combination of aliquots from the margins of the peak (that represent the individual peaks of the ligase and the factor) should give a synergistic effect in conjugate formation. This is clearly not the case (not shown). Thus, it is likely that the alkylating agents affected the E3 directly.

The 20 S Proteasome Is Involved in Processing of p105—The 20 S proteasome complex is the core catalytic subunit of the 26 S proteasome complex involved in degradation of ubiquitin-tagged proteins and recycling of ubiquitin. The 20 S enzyme can be inhibited by a variety of C-terminal aldehyde derivatives of short peptides. As expected, the agents inhibit degradation of substrates of the ubiquitin system (see for example Refs. 12, 20, and 42) but potentially also that of substrates that are degraded by the 26 S proteasome in a ubiquitin-independent manner. As can be seen in Fig. 11, addition of MG115 (12, 42) leads to inhibition of processing. Thus, it is clear that the 20 S proteasome, most probably as part of the 26 S complex, is involved in processing of p105 in vitro. Using a similar inhibitor, Palombella and colleagues described similar results for processing of p105 in vivo.

DISCUSSION

We have shown that the ubiquitin-proteasome pathway is involved in processing of intact p105 in vitro. In a previous study, Palombella and colleagues (12) utilized a truncated form of p105, p60, to demonstrate the involvement of ubiquitin in processing. They have not demonstrated, however, formation of conjugates as essential intermediates in the process. While analysis of mutant/truncated proteins can be powerful, one should be cautious. The ubiquitin system may recognize abnormal/truncated/ misfolded proteins, but not their normal counterparts. For example, a truncated form of E1A adenovirus protein (amino acid residues 1–85) is much more sensitive to degradation then the wild type (1–289) protein (29, 43). The case of p53 is even more illustrative, although the direction appears to be "opposite." A single mutation can render the wild type protein resistant to conjugation and degradation because of a significant crucial alteration in the three-dimensional structure (27, 44, 45). Processing of p105 to p50 occurs posttranslationally. It requires ATP and occurs in a time-dependent manner. The process occurs only in mammalian cell extracts and not in plant. Our analysis revealed that the plant extract, most probably, does not contain the required ubiquitin-protein ligase, E3 (see below). Further investigation revealed that conjugation of ubiquitin precedes processing and is an essential intermediate step in the process; MeUb inhibits both processes, and the inhibition is alleviated by the addition of...
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Fig. 9. Ubiquitin-p105 ligase is a novel species of E3 distinct from E3α. Ubiquitin conjugates of p105 (panel A) and 125I-labeled lysozyme (panel B) were generated essentially as described under "Experimental Procedures." The experiment described in panel A was analyzed by PhosphorImager. Lane A1, incubation in the presence of E1 and E2-F1. Lane A2, same as lane A1, but with Fraction IIA (7.5 μg). Lane A3, same as lane A1, but with E3α (0.6 microunit; 1 unit of enzyme catalyzes the incorporation of ubiquitin into conjugates at a rate of 1 μmol/min; Ref. 31). Lane A4, reaction mixture contained E1, E2–14 kDa (0.15 μg), and E3α. Lane B1, with E1 and E2–14 kDa. Lane B2, same as lane B1, but with E3α. E1, and E2-F1 were added in the same amounts described in the legend to Fig. 8. Conj. denote ubiquitin conjugates. Notes and molecular size markers are as described in the legend to Fig. 1.

Fig. 10. The ubiquitin-p105 ligase has an active-SH group. Conjugation of ubiquitin to p105 was monitored essentially as described under "Experimental Procedures." All reaction mixtures contained E1, E2-F1, and C3-F6 cytosolic extract as a source for E3. The amounts of the enzymes added are as described under "Experimental Procedures" and in the legend to Fig. 8. Lane 1, complete reaction mixture containing all three conjugating enzymes, but without ATP. Lane 2, same as lane 1, but with ATP. Lane 3, same as lane 2, but the cytotoxic extract was treated first with sodium iodoacetate and neutralized with DTT. Lane 4, same as lane 2, but the extract was treated first with NEM and neutralized with DTT. Lane 5, same as lane 2, but the extract was treated first with p-hydroxymercuribenzoate and neutralized with DTT. Lane 6, same as lane 2, but NEM was added after the addition of DTT. In all cases the inhibitor was added at 10 μm and the extract incubated for 10 min at room temperature. DTT was then added to a final concentration of 6 μm. Conj. denotes ubiquitin conjugates. Notes and molecular size markers are as described in the legend to Fig. 1.

excess free ubiquitin. To study the conjugating enzymes involved, we reconstituted a cell-free system and demonstrated that E2-F1, a recently characterized ubiquitin-carrier protein isolated from crude reticulocyte Fraction I (26), is required for the process. We have also demonstrated that the human homolog of E2-F1, UbcH5 (30), also catalyzes conjugation of p105. Interestingly, E2-F1 is also involved in the conjugation and degradation of glyceraldehyde-3-phosphate dehydrogenase (26), p53 (27), and c-Fos,3 and therefore, does not appear to be specific. However, another species of ubiquitin-carrier protein, E2–14 kDa, is not able to promote conjugation. We have shown that a novel, still unidentified, species of E3 is involved in the process. This ligase, which is not present in wheat germ, is distinct from E6-AP, the ligase involved in E6-dependent tagging of p53 (37), and from E3α, the "N-end rule" ligase. Thus, it appears that E2-F1 can act in concert with several species of E3s, and the E3 enzymes are responsible for the specific recognition of the substrates. The novel E3 appears to contain an -SH group essential for its activity. As expected, processing is inhibited by inhibitors of the 20 S proteasome complex.

An important problem involves the mechanism of processing. Unlike all other substrates of the ubiquitin system, p105 is only partially degraded. It is not known whether the C-terminal domain is first cleaved and then degraded, or whether digestion starts in the C-terminal residue of p105 and stops at certain point. The cleavage site, or the "stop" signal, has not been identified, although it is clear that it may reside in the region of amino acid residue 420 (46). Another problem concerns regulation of processing. It has been suggested that stimulation-induced phosphorylation of the precursor may accelerate its processing (47). Also, it is possible that activation of one or more specific components of the ubiquitin system occurs following stimulation. Interestingly, phosphorylation of the inhibitor IκBα leads to its rapid degradation (18–20). This process also appears to be mediated by the ubiquitin system and to involve E2-F1.3 Therefore, it is clear that reconstitution of the cell-free proteolytic system and identification of the enzymes involved are essential for further understanding of the mechanisms of recognition and regulation of the components of the transcriptional activator complex by the proteolytic system.

Acknowledgments—We acknowledge Drs. Ross Stein and Julian Adams (MyoGenics, Inc., Cambridge, MA) for the kind gift of the 20 S proteasome inhibitor, MG115, and Dr. Martin Scheffner (DKFZ, Heidelberg, Germany) for the kind gift of the UbcH5 clone. We are also grateful for the generous help and advice of Dr. Hedva Gonen (Technion, Haifa, Israel).

3I. Stancovski and A. Ciechanover, unpublished results.
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