Enzymes Catalyzing Ubiquitination and Proteolytic Processing of the p105 Precursor of Nuclear Factor \( \alpha B1^* \)

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Nuclear factor \( \alpha B1 ^* \) (NF-\( \alpha B1 ^* \)) is a heterodimeric complex that regulates transcription of many genes involved in immune and inflammatory responses. Its 50-kDa subunit (p50) is generated by the ubiquitin-proteasome pathway from a 105-kDa precursor (p105). We have reconstituted this proteolytic process in HeLa cell extracts and purified the responsible enzymes. Ubiquitination of p105 requires E1, and either of two types of E2s, E2–25k (for which p105 is the first proven substrate) or a member of the UBC4 (UBC4) family. It also requires a new E3 of 50 kDa, which we call E3\( \alpha B \). This set of enzymes differs from the E2s and E3 reported together with 26S proteasomes, allowed formation of p50. Thus, the 26S proteasome provides all the proteolytic activities necessary for p105 processing. Interestingly, in the reconstituted system, as observed in cells, the C-terminally truncated form of p105, p97, was processed into p50 more efficiently than normal p105, even when both species were ubiquitinated to a similar extent. The ubiquitinating enzymes purified here, together with 26S proteasomes, allowed formation of p50. Therefore, some additional mechanism involving the C-terminal region of p105 influences the proteolytic processing of the ubiquitinated precursor.

NF-\( \alpha B1 ^* \) is a ubiquitous transcription factor that regulates the expression of multiple genes involved in immune and inflammatory responses (1). Greater knowledge about the mechanisms of NF-\( \alpha B1 ^* \) activation is therefore of major importance for understanding human disease and may indicate new targets for pharmacological intervention. NF-\( \alpha B1 ^* \) is a member of the Rel family of dimeric transcription factors present in many organisms (2). The prototype of this family is a heterodimer of a p50 (NF-\( \alpha B1 ^* \)) and a p65 (RelA) subunit. Its activity is regulated primarily at the posttranslational level, by two separate processes (3). The p50 subunit is generated from a relatively stable precursor, p105, which undergoes proteolytic processing in the cytoplasm (4–6). In this process, the C-terminal part of p105 is degraded, and the remaining N-terminal half of the molecule serves as the p50 subunit of NF-\( \alpha B1 ^* \). However, in uninduced cells, the p50/p65 (NF-\( \alpha B1 ^* \)) complex is maintained in an inactive form in the cytoplasm by the inhibitor \( \alpha B \), which associates with p50/p65 and prevents its migration to the nucleus (7–11). The final activation of NF-\( \alpha B1 ^* \) involves the proteolytic destruction of \( \alpha B \), which is triggered by its phosphorylation following a variety of stimuli (13, 14). The kinases \( \alpha B ^{0-16} \) (15, 16) and CHUK (17–19) have very recently been shown to be involved in this process. Previous data have shown that the processing of the p105 precursor and the degradation of \( \alpha B \) both require ubiquitin (Ub) conjugation to these polypeptides, leading to their proteolytic digestion by the 26S proteasome (20).

The Ub-proteasome system is a major pathway for degradation of intracellular proteins in eukaryotic cells (22, 23). In this pathway, substrates are marked for degradation by covalent attachment of poly-Ub chain(s) (24). In this process, the Ub-activating protein, E1, utilizes ATP to form a high energy Ub-thiol ester and then transfers the activated Ub to an E2 (Ub carrier protein (UBC)), forming an E2-Ub thiol ester. The Ub is then linked to the substrate in a reaction requiring E3, a Ub-protein ligase (24). Cells contain a large number of E2s (23), each of which acts on a limited spectrum of protein substrates (26, 27). The E3s seem to provide most of the substrate specificity of the ubiquitination process, although only a limited number have been identified.

Once ubiquitinated, proteins are usually rapidly degraded to small peptides by the 26S proteasome (22). The proteolytic core of this 2000-kDa complex is the 20S proteasome (28), which is sandwiched at each end by the 19S complex (PA700) (29, 30). The 19S complex contains multiple activities, including an isopeptidase that catalyzes the release of free Ub (31, 32) and several ATPases, the likely function of which is to facilitate the unfolding of substrates and their translocation into the 20S proteasome (33), where degradation proceeds in a processive fashion (34).

The finding that the Ub-proteasome pathway is responsible for the limited processing of p105 was quite surprising, because its other known substrates undergo complete degradation. To dissect the mechanisms of this process and to define its components, we undertook to identify the enzymes necessary for p105 ubiquitination and p50 generation in HeLa cell extracts and to reconstitute this process with purified proteins.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of analytical grade. DE52 was obtained from Whatman; the Bio-Scale CHT20-I column was from Bio-Rad; the MonoQ, Superose 6, Superose 12, and Sephacryl S100 HR columns and \(^{125} \text{I}\)-labeled proteins were from Amersham Pharmacia Biotech, and iodine-125 (NE2-033A, 629 GBq/mg) from NEN Life Science Products. Cytoplasmic HeLa cell extracts were kindly provided by Dr. V. J. Palombella (ProScript, Cambridge, MA), Dr. R. Reed (Harvard Medical School) or Dr. P. A. Sharp.
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Strains, Plasmids, and Recombinant Proteins—Strains, plasmids, and recombinant proteins were generously provided by the following colleagues: E. coli strains expressing plant 6His-Ub and 6His-UbR48 by Dr. M. A. Read (ProScript). Other E. coli strains expressing the GST-yeast Ub fusion by Dr. J. M. Huibregtse (Rutgers University) (36); plasmids encoding human UBC5C (37) and human E2F1 (UBCH7) (38) by Dr. A. M. Weissman (Bethesda) and Dr. M. Scheffner (Heidelberg), respectively. UBCH5C and E2F1 were purified by cation-exchange chromatography of the flow-through of a DE52 column using an HiTrap SP column (Amersham Pharmacia Biotech). Recombinant ubiquitin E2–25K (39) was provided by Dr. C. M. Pickart (Johns Hopkins University). The C170S form of E2–25K used here has a serine in place of cysteine 170 (nonactive site Cys). Purified recombinant human UBC2 and UBCH5B (also called UBC4) (40) by Drs. W. R. King and J. M. Peters (Harvard Medical School).

Preparation of FI and FII—HeLa cell cytoplasmic extracts were centrifuged for 20 min at 10,000 × g (4 °C) to remove debris. The supernatant was dialyzed against 3 mM potassium phosphate (pH 7.0), 1 mM DTT, 10% glycerol and loaded onto a DE52 column equilibrated with the same buffer. The flow-through (fraction I) was collected, the column was washed with the equilibration buffer supplemented with 20 mM KCl, and the bound proteins (fraction II) were eluted with a buffer containing 100 mM Tris–HCl (pH 7.5), 0.5 mM KCl, 5 mM MgCl₂, 0.5 mM ATP, 1 mM DTT, 10% glycerol. FI was dialyzed overnight against the same buffer without KCl. In some experiments, FI and FII were prepared from extracts depleted of proteasomes by ultracentrifugation at 100,000 × g for 5 h or 200,000 × g for 3 h (4 °C). In those cases, after sedimentation of the proteasomes, MgCl₂ and ATP were omitted in the buffers used to prepare the FI.

Ubiquitin-affinity Chromatography—Ubiquitin was covalently bound to a CH-activated Sepharose matrix (Amersham Pharmacia Biotech), as recommended by the manufacturer. The final concentration of ubiquitin was about 20 mg/ml of gel. Fraction II was supplemented with 5 mM MgCl₂, 2 mM ATP and mixed with the Ub-Sepharose for 1 h at room temperature (with shaking). The ubiquitinating enzymes were sequentially eluted as follows (41, 42): E1 with 20 mM Tris, pH 7.5, 2 mM AMP, and 2 mM NaF; E2s with 20 mM Tris, pH 7.5, 20 mM DTT, and 100 mM KCl; other ubiquitin-binding proteins with 50 mM Tris, pH 9.0, 1 mM KCl, 2 mM DTT.

Purification of 26S Proteasomes—Pellets obtained by centrifugation at 200,000 × g for 3 h of the HeLa cytoplasmic extracts were resuspended in Buffer A (50 mM Tris, pH 7.5, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM ATP, 10% glycerol). After 10 min of centrifugation at 100,000 × g for 20 min, the supernatant was centrifuged again at 200,000 × g for 30 min to remove polyosomes. The supernatant was loaded onto a MonoQ 10/10 column equilibrated with Buffer A, and the proteins were eluted with a NaCl gradient (0–500 mM). The active fractions (containing 26S proteasomes) were eluted with a 200–500 mM NaCl gradient. Fractions active against the fluorogenic proteasome substrate Suc–LLVY–MCA were pooled and concentrated using a Centricon-50 filter (Amicon) and loaded onto a Superose 6 column equilibrated with Buffer A containing 100 mM NaCl. 20S and 26S proteasomes were identified by their activity against Suc–LLVY–MCA in the presence or absence of 0.02% SDS. At this concentration, SDS greatly activates the 20S proteasomes but inhibits the 26S proteasomes, allowing easy discrimination of the two forms (43). Fractions containing 26S proteasomes and 20S proteasomes were pooled separately and stored frozen at −70 °C.

Preparation of Substrates—The p105T, p97T, and p60Tth constructs (5) were generously provided by Dr. Tom Maniatis (Harvard University). The proteins were translated in vitro in a wheat germ extract using the coupled transcription/translation TNT system of Promega, as recommended by the manufacturer. Unless specified otherwise, the reaction mixture was diluted 3-fold after translation in the assay buffer (20 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT), and the labeled protein was separated from the free [35S]methionine using a Nick Spin column (Amersham Pharmacia Biotech) equilibrated in the same buffer. After centrifugation (WGE) containing the in vitro-translated p97 and p105 used in Figs. 7 and 8 was kindly provided by Dr. M. A. Read (ProScript).

Ubiquitination and Processing Reaction—Unless specified otherwise, reactions were carried out in the assay buffer (20 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT) supplemented with other components as indicated. When the processing of p105 was studied, an ATP-regenerating system (0.1 mg/ml creatine phosphokinase, 10 mM creatine phosphate) was added. The products were separated by SDS-PAGE (44), and the gels were dried and analyzed using a PhosphorImager (Molecular Dynamics) or a Fujix Bas 1000 (Fuji) and autoradiography.

Analysis of Ub Thiol Ester Formation—Reactions were carried out for 5 min at 37 °C in 10 mM Tris, pH 7.6, 10 mM MgCl₂, 2 mM ATP, 0.1 mM DTT in the presence of [125I]labeled Ub (50–100 μg/ml) and 50 units/ml of inorganic pyrophosphatase. Reactions were stopped by addition of 2× sample buffer (120 mM Tris, pH 6.8, 4% SDS, 4 mM urea, 20% glycerol) containing or not containing 100 mM DTT. The products were analyzed by SDS-PAGE as described above.

RESULTS

Ubiquitin Conjugation Is Essential for NF-κB1 Processing—Prior studies have shown that p105 and its C-terminally truncated forms p60 and p97 can be converted into the NF-κB subunit p50, when expressed in cells or added to crude cell extracts (5, 6). To learn more about this process, [35S]-labeled p60, translated in WGE, was added to a cytoplasmic extract (S100) of HeLa cells, and its fate was analyzed at different times by SDS-PAGE and autoradiography (Fig. 1). Within minutes, very high molecular weight forms of p60 appeared, which entered the resolving gel only slightly. This heterogeneous [35S]-labeled material then disappeared concomitantly with an increase in the mature, processed p50 (although some p50 also appeared within 5 min of p60 addition). Thus the high molecular weight forms behave like intermediates in the proteolytic processing of p60 and probably correspond to ubiquitinated forms of p60.

To verify this conclusion, the extract was supplemented with various recombinant species of Ub: wild-type Ub; UbR48, a mutated form of Ub that has a defect in Ub chain formation due to the replacement of lysine 48 by an arginine (45) (both with a His₆ tag); and GST-Ub, a hybrid molecule in which the enzyme glutathione S-transferase has been fused to the N-terminal end of normal Ub (36). Addition of the modified Ub species significantly altered the events from those seen upon addition of normal Ub. With UbR48, we found p60 conjugates of reduced size (data not shown), probably because of the premature termination of the Ub-chain due to the K48R mutation. Moreover, addition of UbR48 to the crude extract inhibited the production of p50, although this inhibition was not complete, probably due to the presence of endogenous normal Ub in this extract. These observations are consistent with previous work showing that UbR48 inhibits p105 processing (6). It is noteworthy that the effect of UbR48 on p105 ubiquitination and processing rules out a possible explanation of how p105 may be converted to p50: that p105 is modified by addition of an atypical type of poly-Ub residue, which directs the protein toward limited processing instead of complete degradation. Indeed, lysine 48 of Ub is the residue commonly used for complete degradation of proteins by the proteasome (45).
The ubiquitin-proteasome pathway of the cell degrades intracellular proteins by the proteasome. The slow degradation of some proteins was proposed to be the result of their association with longer-lived ubiquitin-protein adducts (10). These adducts, which were most easily observed in vitro, were isolated from a cell-free translation system with the use of a Sepharose 4B column, thereby enriching for long-lived species (10). The column was eluted with increasing concentrations of KCl, and the fractions were assayed for their ability to support degradations of p60 and p105. The finding that all three reactions appeared to be enriched in distinct component(s) of the ubiquitination pathway. These components were then further purified by anion-exchange chromatography of FIIA and FIIIB, using a MonoQ column. To assay their ability to support ubiquitination of p60, each fraction derived from FIIA was combined with FIIIB, and each from FIIIB was combined with FIIA, in the presence of E1 and GST-Ub. We used GST-Ub rather than normal Ub because the larger conjugates formed with GST-Ub accumulate at the top of the acrylamide gel and are easily detected.

After anion-exchange chromatography of FIIA and FIIIB, two protein fractions were obtained: A (from FIIA), which eluted at about 250 mM NaCl, and B (from FIIIB), which eluted at about 100 mM NaCl. These two fractions, when mixed, supported efficient formation of Ub conjugates when p60 or p105 was used as the substrate (Fig. 3A). Interestingly, the decrease in the p60 and p105 bands (which accounted for 30% or less of the substrate added, as analyzed with a PhosphorImager) could not account for the amount of labeled protein accumulating as conjugates. Therefore, the bands of lower molecular weight (most likely degradation products or products of premature termination of translation) apparently can also be ubiquitinated by these enzymes.

The poly-ubiquitination of a substrate involves the successive thiol ester linkage of Ub to E1 and then to E2 and, in some cases, to E3 (24, 25). Using 125I-Ub, such a thiol ester adduct can be detected after SDS-PAGE, provided that the sample is not exposed to a reducing agent. We analyzed fractions A and B for their content of enzymes capable of forming a thiol ester linkage with 125I-Ub. As shown in Fig. 3B, one DTT-sensitive band of about 110 kDa was detected in fraction A after electrophoresis. This band comigrated with the band formed when Ub was incubated with E1 purified from rabbit reticulocytes, and thus it corresponded to the human E1. No E2 could be detected in fraction A. In fraction B, no Ub-thiol ester could be detected, unless reticulocyte E1 was added. With E1 present, two Ub-protein adducts of about 27.5 and 33 kDa were evident under nonreducing conditions, but they disappeared if the sample was boiled in the presence of DTT. These two bands therefore must correspond to distinct ubiquitin carrier proteins (E2s) linked to Ub by a thiol ester. Finally, mixing fractions A and B allowed formation of the same two Ub-thiol esters, without exogenous E1 addition, and did not reveal any additional E2 (Fig. 3B). In addition, some high molecular weight radiolabeled bands (Fig. 3B, vertical bar) were formed that were DTT-resistant and therefore corresponded to Ub conjugates of proteins in fractions A and B.

Neither fraction A, which contains E1, nor fraction B, which contains two E2s, was able by itself to conjugate Ub to p60 or p105, even if reticulocyte E1 was added (Fig. 3A). Significant conjugation to p60 or p105 occurred only when fractions A and B were mixed. Thus, in addition to E1, fraction A must contain another activity, presumably an E3, that is required for the ubiquitination of p60 and p105. The finding that all three activities were present in fractions A and B is further evidence that FII is fully competent in p105 ubiquitination and that no essential ubiquitinating enzyme was derived from the WGE, as had been suggested by Orian et al. (47). The activities present in fractions A and B were then purified further using a Superose 12 column (Amersham Pharmacia Biotech). Surprisingly, the E3 activity from fraction A, which we propose to call E3kB,
was eluted with an apparent molecular mass of ~60 kDa (data not shown), which is appreciably smaller than known E3s (see below).

Several E2s Can Function in NF-κB1 Ubiquitination—Following the gel filtration step, the active fraction B', derived from fraction B, still contained two E2s able to form thiol esters with radioactive Ub. To identify which E2 is active in p105 ubiquitination, we tested whether fraction B' could be replaced in the reaction by individual E2s isolated from rabbit reticulocytes FII by Ub-affinity and MonoQ chromatography (41). Only one of the reticulocyte E2s, E2–25K, was able to promote p60 ubiquitination in this screen (data not shown). This E2, when incubated with ^125I-Ub in presence of E1, formed a thiol ester adduct that migrated upon electrophoresis at the same position as the slowest-migrating E2-Ub thiol ester formed from fraction B' (Fig. 4A). This finding strongly suggested that E2–25K functions in p105 ubiquitination.

To investigate this possibility further, we tested whether recombinant bovine E2–25K (39) can function in this process in a manner similar to the E2 purified from HeLa cells. As shown in Fig. 4B, recombinant E2–25K supported the ubiquitination of p60 and p105 when mixed with E1, E3κB, and GST-Ub. These findings provide strong evidence that E2–25K is the E2 supporting p105 ubiquitination and processing in FII. The other E2 present in fraction B' corresponds to a lower molecular mass E2 of about 18 kDa, which was eluted from the MonoQ column at a slightly lower salt concentration than E2–25K. In subsequent experiments, better separation of this 18 kDa E2 from E2–25K allowed us to show that it is not involved in p105 ubiquitination (data not shown).

Although E2–25K by itself appears to account for the E2 activity supporting p105 ubiquitination in fraction II, we tested whether certain other recombinant E2s could also function in this reaction with E3κB. Human UBC2 (Rad6) (40) was not active in this process (data not shown). However, recombinant human UBC5B (also called UBC4) (37, 40) supported p105 ubiquitination...
ubiquitination (Fig. 4B), as could UBCH5C, an E2 closely related to UBCH5B (Ref. 37 and see below). This result is consistent with the observation of Orian et al. (47) that E2s from the UBCH5 subfamily can support p105 ubiquitination, although they used the UBCH5A isoform (37, 48). In view of the extensive similarity of the different E2s of the UBCH5 subfamily (37), it is likely that UBCH5A can also function in our p105 ubiquitination assay. We did not find any E2 of the UBCH5 subfamily in our fractionation of the HeLa extract because these proteins are found in fraction I (36). In conclusion, in the presence of E1 and E3xB, we found that at least two different types of E2s can efficiently catalyze the ubiquitination of p105: one from FII, E2–25K, and a subfamily of E2s from FI, UBCH5.

The fact that fraction II contains all the enzymes necessary for p105 ubiquitination (E1, E2–25K, and E3xB), as well as the proteasome, confirms our observation that FII is active in p105 processing (Fig. 2). However, Orian et al. (47) reported that FII from rabbit reticulocytes was unable to conjugate Ub to p105 unless supplemented by E2s present in FI (E2F1 or UBCH5), which could be provided by the WGE in which the substrate was synthesized. They reported that inactivation of the E2s in the WGE by treatment with N-ethylmaleimide prevented p105 ubiquitination in FII and that addition of FI or of E2F1 (or UBCH5) alone could restore this reaction (47). These authors therefore concluded that E2F1 (or UBCH5) was essential for p105 processing. However, Fig. 5 shows that under the same conditions, recombinant E2–25K (lane 6) can stimulate p60 ubiquitination to the same extent as FI or UBCH5C (lanes 4 and 5), even though this E2 is already present in FII. Similar stimulatory effects of addition of FI, UBCH5C or E2–25K were observed with p105 as substrate and when the WGE was not pretreated with N-ethylmaleimide (data not shown). Therefore, the conclusion that E2F1 or UBCH5 is essential for p105 ubiquitination seems unwarranted, especially because Orian et al. (47) did not test E2–25K in their experiments. Interestingly, because the addition of exogenous E2–25K stimulated NF-κB ubiquitination in FII, the content of E2–25K appears to be rate-limiting for p105 ubiquitination in this fraction.

There is an apparent contradiction concerning the effect of addition of FI to FII between Fig. 2 (no effect) and Fig. 5 (stimulation). However, direct comparison of the two experiments is difficult, because the conditions used were quite different. In addition, in Fig. 2, showing a processing assay, only a limited amount of FI protein was added to prove that FI did not contain any factor absolutely required for the processing reaction, whereas in Fig. 5, showing a shorter ubiquitination assay, similar amounts of protein from FI and FII were compared.

A New E3, E3xB, Is Required for p105 Ubiquitination—To further characterize the E3 (E3xB) responsible for p105 ubiquitination, we purified this activity from crude extracts by a combination of chromatographic methods. The following steps were employed: removal of proteasomes by prolonged ultracentrifugation; preparation of FII using DE52 chromatography; and removal of Ub-conjugating enzymes (E1 and E2s) and other Ub-binding proteins by Ub-affinity chromatography, hydroxyapatite chromatography, ion-exchange chromatography, and gel filtration. E3xB activity was assayed by its ability to promote p105 ubiquitination in the presence of E1, recombinant E2s, and GST-Ub (Fig. 6). Several findings are noteworthy. 1) When FII was loaded onto a Ub-affinity column, the E3 activity was found in the flow-through. Thus, this activity does not have a strong affinity for free Ub. 2) When loaded onto the hydroxyapatite column (CHT20, Bio-Rad), the activity was eluted over a broad range of phosphate concentrations (Fig. 6A). Although most bound weakly and was eluted between 20 and 30 mM phosphate, some activity was eluted continuously until the phosphate concentration reached ~200 mM. The activities eluted at high and low phosphate concentrations behaved similarly upon subsequent chromatography on MonoQ and Sephacryl S100 and therefore seemed to correspond to the same molecule. This unusual behavior upon hydroxyapatite chromatography most likely reflects some heterogeneity in the enzyme, possibly due to posttranslational modification or interactions with other proteins. 3) Upon gel filtration on a Sephacryl S100 column, E3xB was eluted with an apparent molecular mass of ~50 kDa (Fig. 6C), which is consistent with the 60-kDa value obtained earlier using a Superose 12 column (data not shown). Because its molecular mass is significantly lower than that of other E3s, E3xB is apparently a new E3. After the gel filtration step, several protein bands could be detected upon SDS-PAGE in the fractions containing E3xB activity, but no protein of about 50 kDa (or potential smaller subunits) was found reproducibly with an elution profile matching that of the E3xB activity.

Reconstitution of the Proteolytic Process Generating p50—A critical issue in this project was to determine whether the E2s and E3 we purified could actually support the conversion of p105 or p97 to p50. Initial experiments used the truncated form p97, which lacks the 89 C-terminal amino acids of p105 and is more efficiently processed in vivo into p50 than p105 (5). In the absence of 26S proteasomes, p97 was efficiently ubiquitinated in vitro if pure E1, E2s, and E3xB were present (Fig. 7A), as expected from our previous results with p105 and p60. The addition of pure 26S proteasomes to this mixture led to the disappearance of the ubiquitinated form of p97 and to the concomitant appearance of p50 (Fig. 7A). This new band was identified as p50 because it migrated similarly to the p50 band formed upon incubation of p97 with the crude extract (Fig. 7A, lanes labeled S100). Thus, the purified E3xB can catalyze the ubiquitination reaction, and the 26S proteasome can provide all of the proteolytic activities necessary for p50 formation.

We then tested whether E2–25K and UBCH5C were individually able to support p50 formation (Fig. 7B), using as a source of E3xB FII depleted of E1 and E2s by Ub-affinity chromatography. In the presence of 26S proteasomes (Fig. 7B, right panel, lane 2), this mixture allowed a little p50 formation, probably due to some E2s in the WGE in which p97 was synthesized. Despite this background activity, addition of either E2–25K or
UBCH5C (lanes 4 and 5) markedly stimulated the formation of p50, in accord with our earlier findings. We also tested whether E2F1, an E2 present in fraction I that was reported to support p105 processing (47), could also function in this reconstituted system. In contrast to the other E2s tested, little or no stimulation of p97 processing was found with purified recombinant E2F1 (Fig. 7, lane 6), although we cannot conclude that E2F1 has no activity in this assay because of the high background in this reaction.

**Differences in Rates of p97 and p105 Processing**—One surprising finding was that although these extracts and reconstituted preparations efficiently converted p97 to p50, they were consistently less active in forming p50 from the full-length UCH-{\textsuperscript{5}}C. The ubiquitination and processing of p97 requires E3x3B and proteasomes. The ubiquitination and processing of p97 were monitored in a 20 μl reaction for 2 h at 37 °C, in the presence of 2 mM ATP and 0.5 mg/ml Ub, using 1 μl of the undiluted translation mixture containing p97 in each assay. The reaction mixtures were supplemented with either 3 or 5 μl of crude extract (S100) or with purified human E1 (0.4 μg), a mixture of three recombinant E2s (bovine E2–25K, human UBCH5C, and E2F1, 2 μM each) and with the fractions and volumes indicated at the top. Lane F corresponds to the FII depleted of ubiquitinating enzymes by Ub-affinity chromatography and depleted of proteasomes by ultracentrifugation. E3x3B was purified as shown in Fig. 6. As indicated, either buffer (−26S) or 2 μg of purified HeLa cell 26S proteasome (+26S) was added. The vertical bar indicates the position of ubiquitinated p97. The experiment was performed as in A, using 6 μl of the fraction II depleted of ubiquitinating enzymes and proteasomes as a source of E3x3B. Lane 1, no E2 and no incubation; lane 2, no E2; lane 3, E2–25K, UBCH5C, and E2F1 (2 μM each); lane 4, E2–25K alone; lane 5, UBCH5C alone; lanes 6, E2F1 alone. The assays were all performed at 30 °C in the presence of 1 mM dithiothreitol.

FIG. 7. E3x3B, E2–25K, and UBCH5C support the ubiquitin-proteasome-dependent processing of p97. A, the processing of p97 requires E3x3B and proteasomes. The ubiquitination and processing of p97 were monitored in a 20 μl reaction for 2 h at 37 °C, in the presence of 2 mM ATP and 0.5 mg/ml Ub, using 1 μl of the undiluted translation mixture containing p97 in each assay. The reaction mixtures were supplemented with either 3 or 5 μl of crude extract (S100) or with purified human E1 (0.4 μg), a mixture of three recombinant E2s (bovine E2–25K, human UBCH5C, and E2F1, 2 μM each) and with the fractions and volumes indicated at the top. Lane F corresponds to the FII depleted of ubiquitinating enzymes by Ub-affinity chromatography and depleted of proteasomes by ultracentrifugation. E3x3B was purified as shown in Fig. 6. As indicated, either buffer (−26S) or 2 μg of purified HeLa cell 26S proteasome (+26S) was added. The vertical bar indicates the position of ubiquitinated p97.
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Efficient processing of p97 was due to differences in rates of ubiquitination or to differences in some other step. Surprisingly, ubiquitination did not appear to be the rate-limiting step in the processing of p105 into p50. In several experiments, p97 seemed to be a better substrate for ubiquitination than p105 (Fig. 8A), but in others (Fig. 8B), p97 and p105 were ubiquitinated to a similar extent. In these cases, addition of pure 26S proteasomes caused a significant increase in p50 production above background levels only when p97 was used as the substrate. Thus, the failure of p105 to be efficiently processed into p50 does not seem to be due to a deficiency in its rate of ubiquitination. This observation suggests that the processing of p105 by the 26S proteasome is inhibited by its C-terminal region after the precursor has been ubiquitinated.

**DISCUSSION**

Enzymes Catalyzing p105 Ubiquitination in HeLa Cells—

These studies demonstrate that E2–25K, as well as the members of the UBC5 family of E2s, can support the ubiquitination of p105. Although E2–25K was described and its cDNA was cloned several years ago (39), p105 is the first protein directly shown to be a substrate for E2–25K. However, Huntingtonin, the product of the gene altered in Huntington’s disease, is another likely substrate of E2–25K (50). E2–25K has the ability to form chains of poly-Ub in the absence of a protein substrate and of an E3 (51). However, this property does not appear to be important in p105 ubiquitination, which strictly requires the presence of an E3.

The UBC5 family in yeast includes two closely related heat-shock proteins, UBC4 and UBC5 (52), and in higher species it contains several isoforms (37, 38, 53). In humans, three members of this family are known (UBCH5A, UBC5B, and UBC5C), which are co-expressed in most cells and tissues (37, 40, 48). In our study, both recombinant UBCH5B and recombinant UBCH5C were found to support p105 ubiquitination. Presumably, therefore, all E2s from the UBC5 family can function in this process. In mammalian cells, this family of E2s is involved in the ubiquitination of a broad range of substrates, including p53 (40, 48) and the cyclins (26).

In addition to E1 and E2s, ubiquitination of p105 requires a novel E3, E3XB. Unlike certain E3s, such as E3a (24), E3XB does not bind to an Ub-affinity column. Also, we could not detect formation of a thiol ester with radioactive Ub when E3XB was incubated with E1 and E2–25K or UBCH5B (Fig. 3B). In this respect, E3XB seems to behave similarly to E3a and to differ from E6AP and other members of the Hect family of E3s (54), which form thiol ester intermediates while transferring Ub from E2s to the substrate (25). However, our inability to detect a Ub-thiol ester with E3XB may simply mean that the amount (or the stability) of this intermediate is too low for it to be detected.

A surprising property of E3XB is its small size, 50 kDa, which is much smaller than that of other E3s. Most of the known E3s have native molecular masses greater than 100 kDa (23); the closest in size to E3XB is the Pub1 protein (85 kDa), a yeast protein involved in cdc25 degradation (55). Our several purifications of E3XB, using different E2s or combinations of E2s for screening, all yielded an enzyme of 50 kDa. Because this small size was found consistently, it is probably not due to proteolytic cleavage during purification. Thus, E3XB appears to be a new E3, although definitive proof will require further characterization.

As noted above, the p105 ubiquitination enzymes characterized here from HeLa cells apparently differ from those reported to catalyze this process in reticulocytes (47). Orian et al. (47) reported that no active E2 is found in FII and that the crucial E2s, either E2F1 or UBC5A, are in FII. They also described a

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**Fig. 8. Comparison of the ubiquitination and processing of the truncated version p97 and the full-length p105.**

A, the processing of p97 is more efficient than the processing of p105. The ubiquitination and conversion of p97 or p105 to p50 were monitored at 37 °C in an 80-μl reaction. At the times indicated, 20 μl were removed and analyzed by SDS-PAGE and autoradiography. The substrates were incubated in the presence of 2 mM ATP, 0.5 mg/ml Ub, 10 μg/ml human E1, 2 μM of each recombinant E2–25K and UBCH5C, and an ATP-regenerating system. The source of E3XB was the FII depleted of ubiquitinating enzymes by Ub-affinity chromatography and of proteasomes by ultracentrifugation (5 μl (about 20 μg of protein) per 20 μl of reaction mixture). The translation mixtures containing p97 or p105 were diluted 20-fold. As indicated at the bottom of the figure, either buffer (−26S) or 2 μM of purified HeLa cell 26S proteasome (+26S) was added. B, although ubiquitination of p105 and p97 was similar, processing was less efficient for ubiquitinated p105. The ubiquitination and processing of p97 (1 μl of the undiluted translation mixture) and p105 (0.5 μl of the undiluted translation mixture) were monitored as in Fig. 7A. The following enzymes were added: E2s, 2 μM final concentration of E2–25K and UBCH5C; E3, 4 μl of purified E3XB; 26S, 2 μg of purified HeLa cell 26S proteasomes. The bottom two panels show quantification using a Fujix Bas 1000 (Fuji) of the radioactivity that accumulated in each lane as p50 or as ubiquitinated proteins (brackets). In each case, the values obtained in the absence of E3XB and of proteasomes were subtracted.

p105 precursor (Fig. 8A). This finding is in accord with previous observations that truncated forms of p105, including p97, are more efficiently processed into p50 than the full-length protein in intact cells (5, 49). We therefore tested whether the more...
new E3 of 320 kDa that catalyzes p105 ubiquitination (47). Their conclusion that the active E2s are in FI is contradicted by our observation that E2–25K can support p105 ubiquitination and processing. Moreover, in our experiments, recombinant E2F1 showed little activity, if any, in p105 ubiquitination and processing, even in crude fractions (see Fig. 7B). However, Orian et al. (47) used an E2F1 isolated from rabbit reticulocyte FI (and not a recombinant protein), which may be contaminated by other E2s. To avoid this potential problem, they used the recombinant human UBC5A, which they assumed to be the human homolog of the rabbit E2F1. However, it is now clear that E2F1 and UBC5A are distinct enzymes (38). Because both E2s are small proteins with neutral pI, present in FI, it seems possible that their E2F1 preparation was in fact contaminated by UBC5A, which accounted for the stimulation of p105 ubiquitination.

It is possible that distinct pathways for p105 ubiquitination function in different cell types, with some cells using the enzymes described here and others using the activities found in reticulocytes. However, it is also possible that the 50-kDa E3kB is actually a component of the 320-kDa activity found by Orian et al. (47), which was isolated only by gel filtration of FIIA, a purification step that might preserve a possible complex between E3kB and other proteins. In fact, upon gel filtration of FII, we could detect p105 ubiquitination over a broad range of fractions (data not shown), perhaps indicating the presence of large complexes containing E3kB that are dissociated during the extensive purification, which always yielded E3kB.

The physiologic relevance of the ubiquitination enzymes that we isolated is suggested by their ability to support the generation of p50. No evidence was presented as to whether the reticulocyte enzymes can also function in this process. Rigorous conclusions about the relative importance in vivo of these different enzymes in p105 ubiquitination will require information on their relative concentrations and kinetic properties or their genetic inactivation.

Reconstitution and Control of p105 Processing—The present data provide the first direct demonstration that the processing of p105 and its truncated form p97 requires their ubiquitination, as proposed previously (6, 47). In the absence of E3kB, no ubiquitination occurred, and no p50 was formed. We also show that the 26S proteasome provides all the proteolytic activities necessary for this process, because once p97 was ubiquitinated, it was processed only if 26S proteasomes were added (Figs. 7 and 8).

It remains unclear how the 26S proteasome produces p50 from the ubiquitinated p105 (p97). Proteolysis in this complex occurs within the 20S particle, the active sites of which are isolated inside its central chamber (22, 28). Substrate entrance and product exit are restricted by a small opening (28) into which only unfolded proteins can enter (56). Within the 20S particle, protein degradation is highly processive and generally converts polypeptides to small peptides without substrate release (34). It is therefore likely that the N-terminal part of p105, p50, is spared because it never reaches the central chamber of the 20S proteasome. Either degradation of p105 occurs from its C-terminal half with much of the protein still outside the 20S chamber, and stops before p50 enters, or an endoproteolytic cleavage first releases p50 from the C-terminal half of p105, which is subsequently degraded. A recent study presented evidence for such an endoproteolytic cleavage that is determined by an upstream glycine-rich region present in p50 (57). If such an endoproteolytic activity actually exists, it must be associated with the 26S proteasome, presumably in the 19S component, because the 26S proteasome clearly can provide all proteolytic activities necessary for p50 generation.

An important observation was the unexpected finding that the processing of the C-terminally truncated forms of p105, in the reconstituted system, was much more efficient than that of the full-length protein (Fig. 8), as had been observed previously in cultured cells. (5, 49). Thus, both in vivo and in vitro, the C-terminal 89 residues of p105 seem to inhibit the conversion of p105 to p50. Recently, Mackichan et al. (49) reported an enhancement of p105 processing upon treatment of cells with phorbol esters and ionomycin. This effect required the phosphorylation of a PEST sequence within the C-terminal region of p105. Moreover, after deletion of this region, the truncated forms were more susceptible to constitutive processing (49). These results confirm the inhibitory effect of the C-terminal region of p105 and suggest that phosphorylation relieves this effect. Therefore, it seems likely that in our purified system, p105 was not efficiently processed because the appropriate kinase was missing.

It is noteworthy that p105 ubiquitination, although necessary for further processing, does not per se ensure conversion to p50 (Fig. 8B). Although the ubiquitinated p105 was not efficiently processed to p50, it disappeared upon addition of the 26S proteasome (Fig. 8). Therefore, the ubiquitinated p105 may have two fates: 1) it may be recognized as a typical substrate by the 26S proteasome and degraded completely, or 2) alternatively, it may be de-ubiquitinated by the isopeptidase activity of the 26S proteasome (31, 32). Either process could constitute an important type of regulation of NF-kB production in vivo. Our findings indicate that p105 ubiquitination is not rate-limiting for formation of p50 under these in vitro conditions (Fig. 8B). Moreover, in vivo, the unprocessed form of p105 accumulates as the full-length p105 and not as larger, ubiquitinated species (5, 49, 58). These observations suggest that after ubiquitination, p105 molecules that are not been processed into p50 are de-ubiquitinated by the 26S proteasome rather than totally degraded. A stimulation of p105 processing could thus be achieved by modifying the kinetic partitioning between the isopeptidase and the “processing” activities of the 26S proteasome. Indeed, recent studies have also suggested regulatory functions for the isopeptidase activity of the 26S proteasome (32). Future experiments involving measurements of the different possible fates of ubiquitinated p105 in extracts and in vivo should allow us to test this suggestion.

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