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# Ubiquitinylation Is Not an Absolute Requirement for Degradation of c-Jun Protein by the 26 S Proteasome\*

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**Degradation of rapidly turned over cellular proteins is commonly thought to be energy dependent, to require tagging of protein substrates by multi-ubiquitin chains, and to involve the 26 S proteasome, which is the major neutral proteolytic activity in both the cytosol and the nucleus. The c-Jun oncoprotein is very unstable *in vivo*. Using cell-free degradation assays, we show that ubiquitinylation, along with other types of tagging, is not an absolute prerequisite for ATP-dependent degradation of c-Jun by the 26 S proteasome. This indicates that a protein may bear intrinsic structural determinants allowing its selective recognition and breakdown by the 26 S proteasome. Moreover, taken together with observations by different groups, our data point to the notion of the existence of multiple degradation pathways operating on c-Jun.**

c-Jun protein is a transcription factor belonging to the AP-1 family (for a complete review, see Ref. 1). It is a basic domain-leucine zipper protein that must homodimerize or heterodimerize with other partners such as c-Fos to recognize specific DNA-binding motifs known as TRE, for 12-*O*-tetradecanoylphorbol-13-acetate-responsive element or AP-1 binding sites. Several lines of evidence points to imply this protein as a positive regulator of cell proliferation. For example, (i) it is rapidly and transiently induced in quiescent embryonic fibroblasts stimulated by growth factors (2, 3), (ii) microinjection of anti-c-Jun antibodies inhibits growth of fibroblasts (4), (iii) disruption of *c-jun* gene by homologous recombination entails retarded cell growth in culture (5), and (iv) its overexpression confers a transformed phenotype to chicken embryo fibroblasts (6, 7). Indeed, *c-jun* gene has originally been identified in the acutely transforming ASV17 chicken retrovirus (8). In this case, alterations of the protein, and particularly deletion of a 27-amino acid motif termed the  $\delta$  domain, confers increased transforming potential (8). *c-jun* is also involved in the control of certain differentiation pathways. As a matter of fact, its knock-out is responsible for embryonic death most likely linked to abnormal hepatogenesis (9). Finally, *c-jun* expression may be instrumental for triggering apoptosis (10).

c-Jun is a short-lived protein with an approximate half-life of 90 min in fibroblast cultures (11, 12). Interestingly, at least two

catabolic pathways seem to operate on this protein. First, two lines of evidence support the idea that calpains, which are abundant cytoplasmic calcium-dependent cysteine proteases, can initiate degradation of c-Jun along with that of c-Fos: (i) both proteins have been shown to constitute actual substrates for these proteases *in vitro* either under purified form or in cytoplasmic extracts (13–15) and (ii) specific modulation of calpain activity *in vivo* modifies c-Jun- and c-Fos-dependent AP-1 transcription complex activity in a transient co-transfection assay (13). In this situation, it must, however, be emphasized that other proteolytic systems must take over the action of calpains since the latter cleave their substrates only to a limited extent. Moreover, this pathway presumably concerns only cytoplasmic degradation of c-Jun and c-Fos because of the restricted intracellular distribution of calpains. Second, it has recently been demonstrated that c-Jun can be ubiquitinated *in vivo* (12). However, although the correlation between the capability to be ubiquitinated and rapid turnover is highly suggestive, the final demonstration that ubiquitinylation is actually responsible for the triggering of c-Jun protein breakdown is still missing.

Characterization of the involved enzymatic activities and reconstitution of the proteolytic machinery is necessary for full understanding of the process by which c-Jun is broken down. As a first step toward this aim, we have developed *in vitro* assays using recombinant rat c-Jun and either rat liver extracts or purified proteasome, the latter being the major soluble neutral intracellular proteolytic activity that has been characterized so far and which is putatively responsible for degradation of most cytoplasmic and nuclear proteins (for a review, see Refs. 16–18). We report here that ubiquitinylation is not an absolute requirement for *in vitro* degradation of c-Jun by the 26 S proteasome. This points to the notion that c-Jun, and possibly other proteins, does not necessarily require tagging or co-factors of any sort for degradation by the 26 S proteasome but possesses intrinsic structural motifs allowing direct recognition and subsequent breakdown by the latter (see "Discussion"). Moreover, this also suggests the existence of an additional putative degradation pathway operating on c-Jun.

## MATERIALS AND METHODS

**Preparation of F2 Rat Liver Extracts: Proteasome Purification**—For preparation of F2 extracts, rat livers were homogenized in 1 volume of 50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol (DTT),<sup>1</sup> 0.1 mM soybean trypsin inhibitor, 0.1 mM phenylmethylsulfonyl fluoride using a Waring blender. Nuclei and tissue debris were eliminated through two steps of centrifugation at 4 °C ( $6,000 \times g$  for 30 min and  $70,000 \times g$  for 2 h). Crude extracts were incubated in the presence of 20 mM deoxyglucose and 2 mM dinitrophenol for 1 h at room temperature to deplete ATP. DEAE-cellulose chromatography and concentration of proteins were

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<sup>1</sup> The abbreviations used are: DTT, dithiothreitol; PBS, phosphate-buffered saline.

carried out as described in Hershko *et al.* (19). F2 extracts (20 mg/ml) were aliquoted and kept at  $-20^{\circ}\text{C}$  in 50 mM Tris-HCl, pH 7.5, 2 mM DTT, 20% glycerol until use. Their activity was stable over months. Purification of 20 and 26 S proteasome was conducted as described by Sawada *et al.* (20) through successive chromatography steps using Mono Q and Superose 6 columns (20). Purified particles were kept at  $-20^{\circ}\text{C}$  in 20 mM Tris-HCl, pH 7.5, 2 mM ATP, and 50% glycerol. 26 S proteasome was used within a few days after purification because of rapid loss of activity. On the contrary, 20 S proteasome was stable at least for weeks.

**Electrophoresis, Western Blotting, and Immunodetection, Immunoprecipitations and Antibodies**—Proteins were fractionated through 15% polyacrylamide gels containing SDS according to Laemmli (21) using the mini-Protein II system from Bio-Rad. Proteins were electrotransferred onto nitrocellulose membranes (0.2- $\mu\text{m}$  pores; Schleicher and Schuell) for 45 min at 0.8 mA/cm<sup>2</sup> using the semidry transfer apparatus from LKB. Membranes were blocked by incubation for 1 h at room temperature in 10% skimmed milk into PBS (0.15 M NaCl, 0.01 M sodium phosphate pH 7). Anti-protein antibodies were added for 2 h at room temperature at a concentration of 0.1  $\mu\text{g/ml}$ . Membranes were washed 3 times for 10 min in PBS. A 1:1000 dilution of a peroxidase-conjugated sheep anti-mouse or anti-rabbit IgG (Amersham Corp.) as a second antibody was then added and incubated for 2 h at room temperature. Membranes were washed 3 times in PBS, 0.1% Tween 20 and 3 times with PBS. Immunodetected proteins were revealed using the ECL chemoluminescent system from DuPont NEN according to the specification of the supplier. c-Jun was detected using the SC45 anti-c-Jun from Santa-Cruz. Ubiquitin was detected using the Z148 rabbit antiserum from Dakko. Rabbit anti-p53 antibodies were a gift from Dr. L. Debüssche (Rhône-Poulenc-Rohrer, France). Anti-E1 antibodies were obtained by immunization of rabbits with E1 enzyme purified from *Xenopus* oocytes. Anti-whole proteasome and proteasome subunits were gifts from Drs. K. Tanaka and K. Scherrer, respectively.

Immunoprecipitations were performed using protein A-Sepharose (Sigma) essentially as described in Harlow and Lane (22). In a typical experiment, 100  $\mu\text{l}$  of immune antiserum-purified immunoglobulins (approximately 10 mg/ml) were added to the same volume of F2 extract. Incubation was carried out for 2–4 h at  $0-4^{\circ}\text{C}$  under agitation. 100  $\mu\text{l}$  of protein A-Sepharose resuspended in 20 mM Tris-HCl, pH 7.5 (immunoglobulin binding capacity of 5 mg/ml), were added, and the reaction was allowed to proceed for several hours at  $4^{\circ}\text{C}$ . Protein A-Sepharose was eliminated through a centrifugation step (10,000  $\times g$  for 5 min). The process was repeated once and immunodepleted extracts were adjusted to 20% glycerol before being aliquoted and frozen until use.

**Proteins**—Purified ubiquitin and rabbit immunoglobulins are from Sigma. The recombinant wild-type human p53 protein was a kind gift from Dr. L. Debüssche. It has been affinity-purified from a *Escherichia coli* protein-soluble fraction using the pAb421 monoclonal antibody as described in Ref. 23. Recombinant c-Jun (24) was purified from *E. coli* as follows. 400 ml of mid-exponential phase bacteria were induced for 3 h in the presence of 1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside, centrifuged down, resuspended in 3 ml of 50 mM Tris-HCl, pH 8, 1 mM EDTA, 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 0.3 mg/ml lysozyme and let on ice for 20 min. 4 mg of nadeoxycholate and 200 units of Benzon nuclease (Merck) were added, and the mixture was incubated for 30 min at  $37^{\circ}\text{C}$ . Inclusion bodies, which contain the bulk of c-Jun, were pelleted (7,000  $\times g$  for 15 min), resuspended in 10 ml of 50 mM Tris-HCl, pH 8, 10 mM EDTA, 100 mM NaCl, 0.5% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride and washed under agitation for 5 min at room temperature. The washing procedure was repeated twice, and the pellet was dissolved in 3 ml of denaturing buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, 8 M urea) for 1 h at  $37^{\circ}\text{C}$ . The solution was centrifuged for 1 h at 15,000  $\times g$  at room temperature, and the supernatant was either frozen or used for further processing. c-Jun, which represent more than 95% of proteins of inclusion body proteins as determined by electrophoretic analysis, was purified to homogeneity by electrophoresis through SDS-containing 12% polyacrylamide gels using the Prep Cell apparatus from Bio-Rad. The renaturation protocol described is an adaptation of that of Chang *et al.* (25) and proved reproducible. Electrophoresis-purified c-Jun, in a volume of approximately 50 ml of 150 mM Tris-HCl, pH 8.6, 0.1% SDS was concentrated to 1–2 ml using Macrosep filters (Filtron) at room temperature. The solution was then filtered through a 0.2- $\mu\text{m}$  Minisart filter (Sartorius) and dialyzed for 16 h against 50 ml of 50 mM Tris-HCl, pH 7, 50 mM DTT, 2 mM EDTA, 7 M guanidinium chloride in order to quantitatively precipitate c-Jun. Progressive redissolution was obtained through 5 cycles of 2-h dialysis against 100 ml of 50 mM Tris-HCl, pH 7, 100 mM NaCl, 2 mM DTT, 2 mM EDTA, 0.1% Nonidet

P-40 at  $20-25^{\circ}\text{C}$ . The solution was adjusted to 50% glycerol, and c-Jun was kept frozen at  $-20^{\circ}\text{C}$  at a concentration of 0.5 mg/ml until use.

**Ubiquitinylation and Degradation Assays**—<sup>125</sup>I iodination of ubiquitin was performed using the chloramin T method exactly as described in Hershko *et al.* (19). Ubiquitinylation reactions were incubated for 30 min at  $37^{\circ}\text{C}$  in a volume of 50  $\mu\text{l}$  in the presence of 20 mM Tris-HCl, pH 7.5, 2 mM ATP, 5 mM MgCl<sub>2</sub>, 10 mM phosphocreatine (Sigma), 1 mg/ml creatine kinase (Sigma), 2 mM DTT, 5  $\mu\text{g}$  of <sup>125</sup>I-labeled ubiquitin, and 2 mg/ml protein extract. Samples were electrophoresed through a 15% polyacrylamide gel and then autoradiographed.

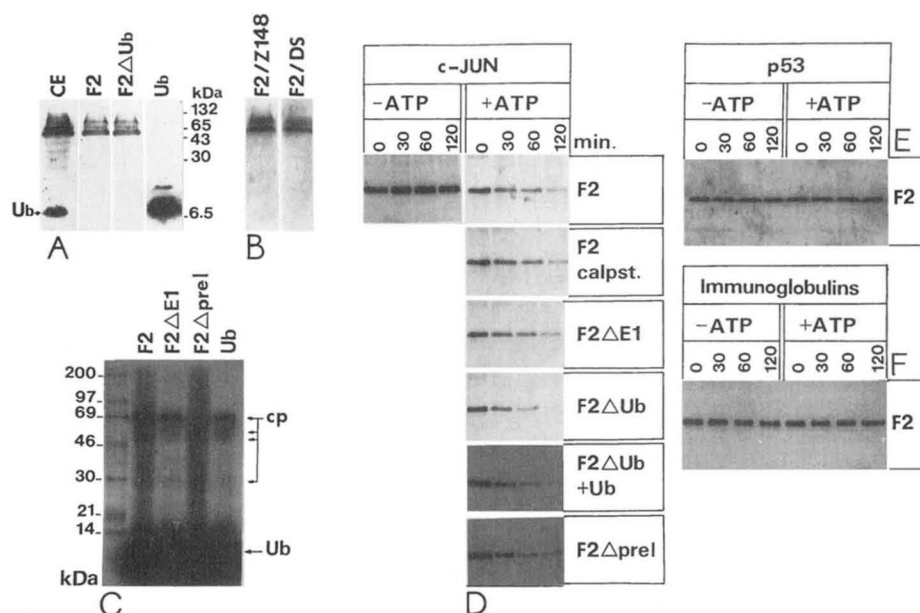
Degradation experiments were performed at  $37^{\circ}\text{C}$  in a volume of 100  $\mu\text{l}$  in the presence of 20 mM Tris-HCl, pH 7.5, 350 ng of recombinant rat c-Jun (or other proteins), and usually 40  $\mu\text{g}$  of proteins from the different extracts (similar results were obtained with 10-fold less proteins). When needed, ATP and MgCl<sub>2</sub> (5 mM each), ubiquitin (500  $\mu\text{g/ml}$ ), and the calpastatin peptide (160 ng/ml) were added. Aliquot fractions were sampled at various time points and immediately boiled for 10 min in Laemmli sample buffer. Proteins were fractionated through 15% polyacrylamide gels and electrotransferred onto nitrocellulose. Immunodetection was carried out as described above.

## RESULTS

**Multi-ubiquitinylation of c-Jun Is Not Necessary for rapid and Specific ATP-dependent Breakdown in F2 Rat Liver Extracts**—The multiubiquitinylation of proteins involves multiple steps (18, 26, 27). Free ubiquitin is first bound by an ubiquitin-activating enzyme, E1. E2 ubiquitin-carrier proteins then catalyze the transfer of activated ubiquitin from E1 to protein substrates eventually bound to specific E3 ubiquitin-protein ligases. To determine whether ubiquitinylation is necessary for the rapid breakdown of c-Jun, ubiquitinylation competent and incompetent rat liver-derived cell-free degradation assays were developed.

Preparation of protein extracts was carried out as follows. In a first step, a protein fraction was purified by anion-exchange chromatography (F2) from a crude rat liver cytosolic extract according to Hershko *et al.* (19). Here, two points are worth being emphasized. First, ATP was depleted from the cytosolic extract to allow deconjugation of ubiquitin, which, in its free form, is quantitatively eliminated during the chromatography step. Second, F2 still contains E1, as well as most, but not all, of E2s and E3s together with major soluble proteolytic enzymes. To ensure the total absence of ubiquitinylation in further degradation assays, either of two additional treatments were applied separately to F2. In one case, residual ubiquitin, if any, was eliminated using an antibody-specific for both free and conjugated ubiquitin (F2 $\Delta$ Ub extract). In the other case, the ubiquitinylation pathway was inactivated by E1 immunodepletion (F2 $\Delta$ E1 extract). Western blotting analysis (Fig. 1A) shows that free ubiquitin and intermediate molecular weight ubiquitin-protein conjugates were quantitatively eliminated from F2 and F2 $\Delta$ Ub. However, high molecular weight bands due to the detection system used (Fig. 1B) are found in all types of extracts and may mask residual ubiquitin-protein conjugates in F2 and F2 $\Delta$ Ub extracts. The possibility of ubiquitin recycling during the time course of degradation experiments was, however, ruled out since, on one hand, incubation of F2 extracts for 2 h under degradation experiment conditions (see below) did not lead to any detectable modification in the pattern of bands and, on the other hand, in a comparable experiment carried out in the presence of anti-ubiquitin antibodies, we detected no release of free ubiquitin as assessed by Western blotting (not shown). F2 $\Delta$ E1 extract does not allow ubiquitinylation, either, as tested by lack of conjugation of exogenous <sup>125</sup>I-labeled ubiquitin to proteins of the extract in contrast to both F2 and F2 processed with a preimmune serum (F2 $\Delta$ PreI), which by the same test are shown to be competent for ubiquitinylation (Fig. 1C).

For degradation experiments, *E. coli*-produced rat c-Jun (24), *E. coli*-produced human wild-type p53 (23), and rabbit immu-



**FIG. 1. Ubiquitin is not necessary for degradation of c-Jun protein in rat liver F2 fraction.** A, presence of ubiquitin in various extracts. Ubiquitin immunodepletion from F2 was performed as described under "Materials and Methods." For Western analysis, 25  $\mu$ g of crude cytosolic extract (CE), F2, and F2 $\Delta$ Ub protein samples and 5  $\mu$ g of ubiquitin, as a control, were electrophoresed through a 20% polyacrylamide gel. Immunodetection was performed using the Z148 antibody from Dako. B, 25  $\mu$ g of F2 extracts were processed as in A except that detection was carried out in the presence (Z148+DS) or in the absence (DS) of anti-ubiquitin antibodies. C, ubiquitinylation of F2 endogenous proteins. E1 immunodepletion and ubiquitinylation reactions were conducted as described under "Materials and Methods." Samples were electrophoresed through a 15% polyacrylamide gel and autoradiographed. Arrows indicate proteins contaminating ubiquitin. (F2 $\Delta$ E1, E1-depleted F2; F2 $\Delta$ preI, F2 treated with a preimmune serum). D, degradation of c-Jun. Degradation experiments were performed at 37  $^{\circ}$ C in a volume of 100  $\mu$ l in the presence of 350 ng of recombinant rat c-Jun and 40  $\mu$ g of proteins from the different extracts as described under "Materials and Methods." However, similar results were obtained with 10-fold less protein extract. When needed ATP and MgCl<sub>2</sub> (5 mM each), ubiquitin (500  $\mu$ g/ml), and the calpastatin peptide (160 ng/ml) were added. E and F, Degradation of p53 and rabbit immunoglobulins, respectively. Degradation experiments were carried out as in C with human wild-type p53 or rabbit immunoglobulins instead of c-Jun, and detection was conducted with rabbit anti-p53 antibodies and goat anti-rabbit immunoglobulins, respectively.

noglobulins were added in parallel experiments to the different extracts in the presence, or in the absence, of ATP, and aliquot fractions of the reaction mix were sampled at different time points for immunoelectroblotting analysis. Importantly, appropriate folding of c-Jun was controlled both by testing its ability to homodimerize and to bind specifically to AP-1 DNA motifs and to stimulate transcription in an *in vitro* assay and by analysis of calpain-generated cleavage products since this protease revealed a good topological probe due to both its specificity for tertiary structural determinants and its limited action on c-Jun (not shown) (13–15). c-Jun turned out to be degraded at a similar rate in all extracts in an ATP-dependent manner (Fig. 1D). On the contrary, immunoglobulins (Fig. 1F) and p53 (Fig. 1E) remained unaffected, in keeping with the observations that antibodies are very stable when expressed intracellularly, and, although p53 is very unstable and degraded through the ubiquitin pathway, the E2 enzyme specific for ubiquitinylation is absent from F2 (28). Interestingly, the addition of high amounts of exogenous ubiquitin to F2 did not accelerate c-Jun decay (Fig. 1D). Moreover, calpains, which, as mentioned previously, are capable of cleaving c-Jun (13–15), did not interfere in degradation experiments since a highly specific inhibitor peptide (29) derived from calpastatin, which is the physiological inhibitor of calpains, does not stabilize c-Jun.

Thus, selective degradation of c-Jun in F2 rat liver extracts is neither dependent on ubiquitinylation nor stimulated by the presence of ubiquitin.

**ATP-dependent Degradation of c-Jun in Rat F2 Extracts Is Dependent upon the Presence of Proteasome**—The 26 S proteinase complex(es) (26 S proteasome(s)) is the major soluble neutral proteolytic activity in the cell. Its proteolytic core, the 20 S proteasome, is perhaps never found free in cells and, in con-

trast to the 26 S particle, neither requires ATP for proteolysis nor usually degrades large proteins (16–18). To determine whether the proteasome is responsible for the ATP-dependent degradation of c-Jun in rat liver extracts, F2 was proteasome-immunodepleted (F2 $\Delta$ prot) using an anti-proteasome antiserum already shown efficient for such a purpose (30). Depletion was controlled as follows: (i) proteasome proteolytic activity was shown to be reduced >200-fold using the Suc-Leu-Leu-Val-Tyr-NH-7-amido-4-methylcoumarin peptide substrate, which is reported to be a good substrate for the proteasome (31) and (ii) removal of two peptide components of the proteasome was assayed using two specific monoclonal antibodies (Fig. 2A). The latter are the cross-species reactive anti-duck p27K (IB5) antibody and anti-duck p31K (AA4 antibody) (32). c-Jun was no longer degraded in F2 $\Delta$ prot as compared with F2 and F2 $\Delta$ preI (Fig. 2B). Definitely establishing that c-Jun is a target for the proteasome, complementation of F2 $\Delta$ prot with purified rat liver 26 S proteasome (see below) restores quantitative degradation of c-Jun (Fig. 2B).

**ATP-dependent Degradation of c-Jun by the Proteasome Does Not Require any Additional Co-factor**—To determine whether c-Jun is a target for the 20 S and/or the 26 S proteasome and whether a putative co-factor, present in F2, is necessary for c-Jun proteolysis, degradation experiments were conducted with comparable amounts of 20 and 26 S proteasome instead of F2 extracts. Peptide compositions of purified rat liver particles were similar to those published by others (30, 33) (Fig. 3A). Under the conditions tested (50–500 ng/ml of purified particles; for experimental conditions, see legend to Fig. 3 and "Materials and Methods"), c-Jun was not degraded by the 20 S proteasome, whereas it was degraded by the 26 S proteasome, but only in the presence of ATP. As a support to the selectivity of the process, p53 and immunoglobulins revealed a resistance to



both types of particles, even in the presence of ATP (Fig. 3, B and C). Importantly, (i) addition of calpastatin did not stabilize c-Jun, ruling out contamination by calpain (not shown), (ii) about 10% of c-Jun is consistently degraded without any addition of exogenous ATP, perhaps on account of 0.02 mM ATP present in the reaction mixture (since the 26 S proteasome is stored in the presence of 2 mM ATP), (iii) c-Jun is susceptible to higher concentrations (above 5  $\mu$ g/ml) of 20 S proteasome in an ATP-independent but selective manner since p53 and immunoglobulins remained insensitive under the same conditions (not shown), and (iv) c-Jun degradation is still selective and ATP-dependent at a higher concentration of 26 S proteasome (5  $\mu$ g/ml of purified 26 S proteasome; not shown).

#### DISCUSSION

The ubiquitin-proteasome proteolytic pathway is thought to account for the degradation of the bulk of short-lived and ab-

normal cellular proteins (18, 26, 27). It has also been implied in antigen processing for subsequent presentation of antigenic peptides by major histocompatibility complex class I molecules (34, 35) and protein processing as in the case of the p105 NF $\kappa$ B1 precursor protein (36). On the other hand, Rock *et al.* (35) have shown that the proteasome is involved in the degradation of the bulk of long lived intracellular proteins, whereas Gropper *et al.* (37) have reported that inhibition of the E1 ubiquitin-activating enzyme does not affect their breakdown. This suggests that degradation of this class of proteins is likely proteasome-dependent but ubiquitin-independent. Finally, little information is available on the involvement of both the proteasome and of the ubiquitin pathway in the degradation of specific cellular proteins (16–18).

We report here that c-Jun protein can be degraded in a selective and ATP-dependent manner involving the proteasome in rat liver extracts since immunoglobulins and p53 protein (along with bovine serum albumin; data not shown) are not degraded under the same experimental conditions. Thus, this adds one example to the very few characterized cellular substrates of the proteasome. Moreover, we also show that tagging by ubiquitin is not an absolute requirement for recognition and degradation of c-Jun by the 26 S proteasome. Ornithine decarboxylase, which is one of the most rapidly turned over proteins, is the only other example of such a situation. Using the reticulocyte lysate as a cell-free degradation assay, Bercovitch *et al.* (38) have shown that ornithine decarboxylase breakdown is ATP-dependent but ubiquitin-independent. More recently, using both different cell extracts and purified proteasome, Murakami *et al.* (30) have identified the 26 S proteasome as the protease responsible for ornithine decarboxylase degradation. It is worth emphasizing that if breakdown of ornithine decarboxylase does not require tagging by ubiquitination, triggering of proteolysis is dependent upon association of ornithine decarboxylase with a specific peptide co-factor, called antizyme. At variance, with ornithine decarboxylase, no specific co-factor is needed for degradation of c-Jun by purified 26 S proteasome. To our knowledge, c-Jun is thus the first demonstration that a cellular protein can bear intrinsic signal(s) for selective recognition and quantitative ATP-dependent degradation by the 26 S proteasome. This, of course, does not exclude that some co-factors may be involved in the regulation of c-Jun breakdown *in vivo*.

c-Jun appears unique with regard to protein catabolism since several proteolytic systems have been proposed to operate on it. First, as mentioned in the introduction, *in vitro* and *in vivo* lines of evidence suggest that c-Jun degradation can be initiated in the cytoplasm by calpains (13–15). However, since

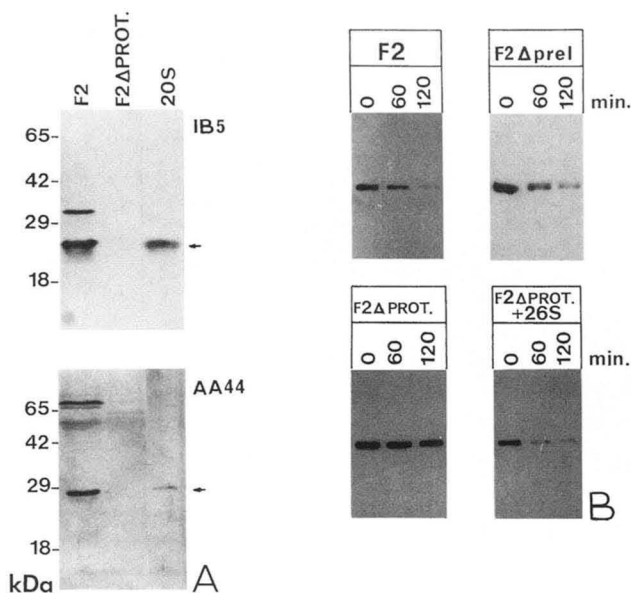
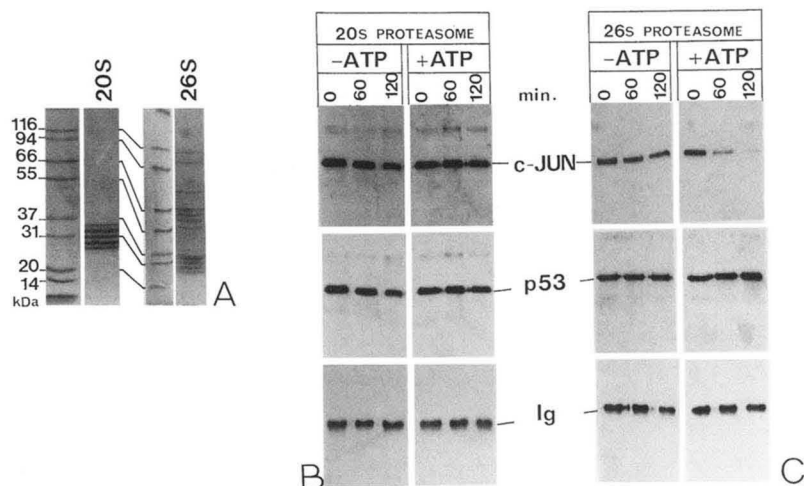


FIG. 2. Involvement of the proteasome in c-Jun degradation in rat liver F2 fraction. A, immunodepletion of proteasome was performed with the anti-proteasome rabbit antiserum described by Murakami *et al.* (30). For control of depletion, immunoblots were prepared with 100  $\mu$ g of protein/sample, and analysis was performed with the cross-species reactive anti-duck p27K (IB5 antibody) and anti-duck p31K (AA4 antibody) mouse monoclonal antibodies (19). B, degradation of c-Jun was conducted as in Fig. 1C. 10 ng of 26 S proteasome (see Fig. 3) was added for complementation. Comparable results were obtained with 2 ng.

FIG. 3. 26 S, but not 20 S, proteasome degrades c-Jun in an ATP-dependent manner. A, purification of rat liver 20 and 26 S proteasomes was conducted essentially as described in Ref. 20 (see "Materials and Methods"). 1  $\mu$ g of protein samples were analyzed by SDS-polyacrylamide gel electrophoresis and stained with Coomassie Blue. B and C, degradation experiments by 20 and 26 S proteasomes were conducted as described in Fig. 1 with 50 ng of purified particles (20 and 26 S proteasome displayed comparable proteolytic activities as assayed with the Suc-Leu-Leu-Val-Tyr-NH-7-amido-4-methylcoumarin peptide substrate) instead of F2 extract. 10-fold more 20 S and 5-fold less 26 S proteasome gave the same results (not shown).



cleavage of c-Jun by calpains is limited, it is likely that other proteolytic activities are involved in the completion of degradation. Preliminary experiments by our group point to the involvement of the proteasome. Second, Trier *et al.* (12) have recently shown that (i) a small fraction of c-Jun is ubiquitinated *in vivo*, (ii) a 27-amino acid domain ( $\delta$  domain), located in the N-terminal part of c-Jun and which is deleted from the stabilized v-Jun<sup>ASV17</sup> protein, is determining for the conjugation of ubiquitin at multiple sites on the protein, and (iii) the capability to be ubiquitinated correlates with rapid turnover since v-Jun<sup>ASV17</sup> is not, or little, ubiquitinated. However, the formal demonstration that degradation of c-Jun is actually triggered by conjugation of ubiquitin has not been brought, and the involvement of the proteasome has not been investigated. It cannot thus be ruled out that the  $\delta$  domain is instrumental for rapid degradation of c-Jun through another mechanism. Third, we report here that c-Jun can be degraded by the 26 S proteasome in the absence of ubiquitinylation, at least *in vitro*. An important question to solve is obviously to determine whether ubiquitin-dependent and ubiquitin-independent pathways actually act *in vivo* for c-Jun breakdown and whether they act synergistically or independently under different physiological conditions or in different cell types. To our knowledge, the possibility that an unstable protein can be degraded according to different pathways has not yet been reported. It is, however, worth noting that the situation has already been described at the RNA level since two destabilizers of the *c-fos* mRNA have been shown to exert their effects under different cell culture conditions (39). Addressing the issue of the possible differential contributions of the different proteolytic machineries involved in c-Jun degradation *in vivo* will, however, be difficult. As a matter of fact, little is known about the activation of calpains *in vivo* (40), on one hand, and the 26 S proteasome might have variable subunit structures according to its cellular origin and, perhaps, intracellular localization on the other hand. One can thus speculate that certain isoenzymes degrade c-Jun in an ubiquitin-dependent manner, whereas others act independently of ubiquitinylation. Purification and characterization of the different 26 S proteasomes will thus be necessary for solving this point.

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