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 δ domain, confers increased transforming potential (8). cjun 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 ubiquitinylated in vivo (12). However, although the correlation between the capability to be ubiquitinylated 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), 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 × g for 30 min and 70,000 × 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 hereby marked "advertisement" in accordance with 15 U.S.C. Section 1734 solely to indicate this fact.

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carried out as described in Hershko et al. (19). F2 extracts (20 mg/ml) were aliquoted and kept at -20 °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 Superdex 200 columns (20). Purified proteasomes were incubated for 2-4 h at 0-4 °C in 20 mM Tris-HCl, pH 7.5, 2 mM ATP, 5 mM MgCl₂, 10 mM phosphocreatine (Sigma), 1 mg/ml creatine kinase (Sigma), 2 mM DTT, 5 μg of 125I-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 °C in a volume of 100 μl in the presence of 20 mM Tris-HCl, pH 7.5, 350 μg of recombinant rat c-Jun (or other proteins), and usually 40 μg of proteins from different extracts (similar results were obtained with 10-fold less proteins). When needed, ATP and MgCl₂ (5 mM each), ubiquitin (500 μg/ml), and the calpastatin peptide (180 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 multiubiquitinylated 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 conjugation 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 en-bodies, which by the same test are shown to be competent to ensure the 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 (F2ΔUb extract). In the other case, the ubiquitinlyation pathway was inactivated by E1 immunodepletion (F2ΔE1 extract). Western blotting analysis (Fig. 1A) shows that free ubiquitin and intermediate molecular weight ubiquitin-protein conjugates were quantitatively eliminated from F2 and F2ΔUb. 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 F2ΔUb 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). F2ΔE1 extract does not allow ubiquitinylation, either, as tested by lack of conjugation of exogenous 125I-labeled ubiquitin to proteins of the extract in contrast to both F2 and F2 processed with a preimmune serum (F2ΔPre), 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-
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 ubiquitylation of p53 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 ubiquitylation 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 proteasome 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 contrast 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 (F2prot) using an anti-proteasome antibody serum 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-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 (1B5) antibody and anti-duck p31K (AA4 antibody) (32). c-Jun was no longer degraded in F2prot as compared with F2 and F2prel (Fig. 2B). Definitely establishing that c-Jun is a target for the proteasome, complementation of F2prot 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 μg/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 μg/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 NFKB1 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 ubiquitin, 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
cleavage of c-Jun by calpains is limited, it is likely that other proteolytic activities are involved in the completion of degrada-
tion. Preliminary experiments by our group point to the in-
volvement of the proteasome. Second, Trier et al. (12) have
recently shown that (i) a small fraction of c-Jun is ubiquitinyl-
ated in vivo, (ii) a 27-amino acid domain (Δ domain), located in
the N-terminal part of c-Jun and which is deleted from the
protein, is determining for the conjugation of ubiquitin at multiple sites on the protein, and (iii) the capability to be ubiquitinylated correlates with rapid turnover since v-JunΔSV17 is not, or little, ubiquitinylated. However, the formal demonstration that degradation of c-Jun is actually
incorporated into the proteasome has not been investigated. It
cannot thus be ruled out that the Δ 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 protea-
some 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 ac-
tually 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-JunΔSV17 protein, is determining for the

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