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Ubiquitin-Dependent c-Jun Degradation In Vivo Is Mediated by the δ Domain

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Summary

The role of the ubiquitin-dependent proteolysis system in c-Jun breakdown was investigated. Using in vitro experiments and a novel in vivo assay that utilizes molecularly-tagged ubiquitin and c-Jun proteins, it was shown that c-Jun, but not its transforming counterpart, retroviral v-Jun, can be efficiently multiubiquitinated. Consistently, v-Jun has a longer half-life than c-Jun. Mutagenesis experiments indicate that the reason for the escape of v-Jun from multiubiquitination and its resulting stabilization is the deletion of the δ domain, a stretch of 27 amino acids that is present in c-Jun but not in v-Jun. c-Jun sequences containing the δ domain, when transferred to the bacterial β-galactosidase protein, function as a cis-acting ubiquitination and degradation signal. The correlation between transforming ability and the escape from ubiquitin-dependent degradation described here suggests a novel route to oncogenesis.

Introduction

c-Jun is a signal-transducing transcription factor of the AP-1 family (Bohmann et al., 1987; Angel et al., 1988). It is a basic–leucine zipper protein and must homodimerize or heterodimerize with c-Fos (or related proteins) to recognize a palindromic DNA sequence known as the TPA-responsive element (TRE) or AP-1-binding site. A number of agents, among them many mitogens, induce c-Jun activity, suggesting a role in positive growth control (Angel and Karin, 1991). Results of experiments employing antibody injection to eliminate c-Jun activity, as well as proliferation studies on cells cultured from mouse embryos that lack functional c-*jun* alleles, support a positive regulatory function of this transcription factor for cell growth (Kovary and Bravo, 1991; Johnson et al., 1993). Furthermore, alleles of c-*jun* and c-*fos* (v-*jun* and v-*fos*, respectively) have been identified as oncogenes in acutely transforming retroviruses (Maki et al., 1987; Curran, 1988).

c-*jun* itself has some transforming activity when overexpressed in chicken embryo fibroblasts; however, v-*jun* is inherently more potent in this assay system (Bos et al., 1990; Morgan et al., 1993). The alteration that is most important for the increased transforming potential of v-Jun, at least in the homologous chicken system, is an in-frame deletion of a 27 amino acid stretch, termed the δ domain, from the NH_2-terminal part of the molecule (Bos et al., 1990; Morgan et al., 1993). It has been shown that the δ domain can suppress transcriptional activation by c-Jun in vitro (Bohmann and Tjian, 1989) and that it is required for strong interaction with an as yet unidentified titratable inhibitor that in some cell types decreases c-Jun activity (Baichwal and Tjian, 1990; Baichwal et al., 1991). The functional role of the δ domain and the consequences of its loss in v-Jun are, however, not understood in molecular detail. Here we present evidence that the δ domain is a determinant of c-Jun stability.

A common feature of many regulatory proteins, including transcription factors, is their short half-life (Rogers et al., 1986). c-Jun, for example, has a half-life of approximately 90 min in fibroblast cultures (Lamph et al., 1988). As increased levels of c-Jun, achieved for example by overexpression or by stabilization of the c-*jun* mRNA, can lead to transformation (Schütte et al., 1989; Bos et al., 1990), it might be expected that c-Jun protein instability, and therefore the maintenance of low steady-state levels of the protein, could be important for properly controlled cell proliferation. The relative stability of the AP-1 factors c-Jun and JunD has indeed been suggested to play a role in regulating the growth state of fibroblasts (Pfarr et al., 1994). A further link among transformation, the control of cell growth, and protein stability was discovered with the identification of the *tre-2* oncogene as a dominant negative form of a deubiquitination enzyme that interferes with the ubiquitin-dependent proteolysis system of the cell (Papa and Hochstrasser, 1993). It was suggested that *tre-2* prevents the timely ubiquitin-dependent degradation of (as yet unidentified) positive regulators of cell proliferation, thus promoting the transformed phenotype (Papa and Hochstrasser, 1993).

Ubiquitin-dependent protein degradation is a well-characterized enzymatic process (Finley and Chau, 1991; Hershko and Ciechanover, 1992; Hochstrasser, 1992; Jentsch, 1992). Specific ubiquitin-conjugating enzymes ligate the 76 amino acid ubiquitin polypeptide via an isopeptide bond to lysine residues of target proteins. The ubiquitin moiety of the resulting branched protein is then itself ligated to a second ubiquitin molecule, which can subsequently also be ubiquitinated. Multiubiquitination ensues after several rounds of the reaction (Chau et al., 1989) and marks the substrate protein for rapid degradation by a multicatalytic protease complex. Multiubiquitination has been implicated, for example, in the destabilization of the mating type transcription factor MATα2 in yeast (Hochstrasser et al., 1991; Chen et al., 1993) and in the cell cycle–specific degradation of cyclin B (Glotzer et al., 1991). Here we present evidence that the ubiquitin pathway is involved in regulating the stability of the transcription factor c-Jun, and we propose that the escape of v-Jun from ubiquitination and subsequent degradation promotes the transforming potential of this protein.

Results

Ubiquitination of c-Jun In Vitro

An initial indication that c-Jun might be a substrate for

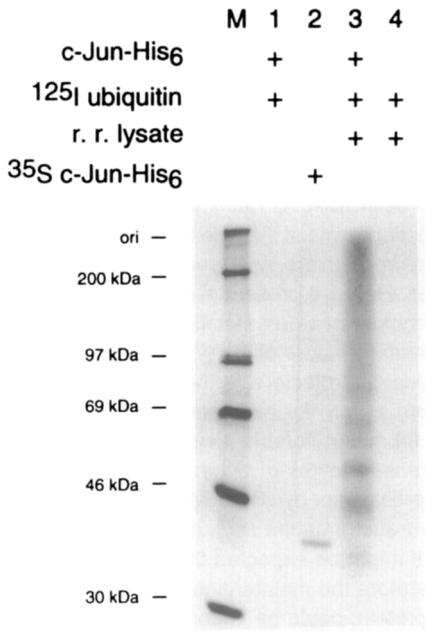

Figure 1. In Vitro Ubiquitination of c-Jun–His_6

^{125}I-labeled ubiquitin was incubated with bacterially expressed c-Jun–His_6 (lane 1), with rabbit reticulocyte lysate (lane 4), or with both (lane 3). His_6-tagged reaction products were purified by NTA chromatography and analyzed by SDS–PAGE and autoradiography. For comparison, metabolically ^{35}S-labeled bacterially expressed c-Jun–His_6 was loaded in lane 2. M, ^{14}C-labeled molecular weight standards. ori, origin of the electrophoresis gel.

ubiquitination originated from attempts to sequence highly purified AP-1 preparations obtained by specific DNA affinity chromatography from HeLa cell nuclear extracts. Surprisingly, these experiments yielded amino acid sequences of ubiquitin, suggesting that at least one component of AP-1 was ubiquitinated (D. B., Arie Admon, and Robert Tjian, unpublished data). To investigate whether c-Jun was such a ubiquitination substrate, we carried out an in vitro experiment employing rabbit reticulocyte lysate as an established source of ubiquitination enzymes (Hershko, 1988). The substrate used was c-Jun–His_6, a bacterially expressed protein that consists of the complete amino acid sequence of c-Jun and, fused to the COOH-terminus, a stretch of six histidine residues. This design endows the recombinant molecule with a high affinity for Ni^{2+}, so that it can be readily purified by nickel–chelate chromatography on nitrilotriacetic acid (NTA)–agarose (Hochuli et al., 1987, 1988). In the experiment presented in Figure 1, c-Jun–His_6 was incubated with reticulocyte lysate in the presence of ^{125}I-labeled ubiquitin. Reaction products containing c-Jun–His_6 were then purified by NTA chromatography and analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) and autoradiography. A ladder of bands with an apparent molecular mass of >50 kDa could be detected only when all three components, namely, c-Jun–His_6, reticulocyte lysate, and radioactive ubiquitin, were present in the reaction (lane 3). Compared with ^{35}S-labeled c-Jun–His_6, which was run as a reference (lane 2), these products correspond in size to c-Jun–His_6 ligated to one or more ubiquitin moieties. We conclude that c-Jun is recognized by the ubiquitination machinery in this in vitro system.

Ubiquitination of c-Jun In Vivo

The results obtained in vitro prompted us to develop a cell-culture assay system to examine whether c-Jun ubiquitination also occurs in vivo. Detection of multiubiquitinated proteins in cells or cell lysates has been encumbered by the fact that these species represent intermediates of degradation and are thus both rare and labile. Moreover, even if enough material can be obtained, definitive detection of a specific protein–ubiquitin conjugate is problematic, owing to the difficulty of raising highly specific antibodies against the extremely conserved ubiquitin molecule. The approach we chose in order to overcome these obstacles is outlined in Figure 2a. As in the in vitro experiments, we used hexahistidine-tagged c-Jun as a substrate. c-Jun–His_6 or His_6–c-Jun (carrying the hexahistidine tag fused to the COOH- or the NH_2-termini of c-Jun, respectively) were introduced into the test cells by transient transfection of cytomegalovirus (CMV)-based expression vectors. In addition, the cells received a vector that expresses high levels of a modified version of human ubiquitin from an octameric precursor protein (see Experimental Procedures). This recombinant ubiquitin carries at its NH_2-terminus an 11 amino acid peptide, derived from influenza virus hemagglutinin (HA), that is recognized with high affinity and specificity by a commercially available monoclonal antibody. The cells were lysed in a buffer containing 6 M guanidinium hydrochloride (guanidinium–HCl), 24–48 hr after cotransfection into HeLa thymidine kinase–negative (TK^-) (Figures 2–7), 293, or NIH 3T3 cultures (which gave essentially the same results; data not shown). These denaturing conditions prevent degradation or deubiquitination of protein–ubiquitin conjugates as well as any noncovalent protein–protein interactions. Since the interaction of His_6 sequences with the NTA affinity resin is not impeded by the presence of guanidinium–HCl (Hochuli et al., 1987; Hochuli et al., 1988), tagged c-Jun and its derivatives could be directly purified from the lysates by NTA chromatography. The Jun cross-reactive material present in the obtained eluates was quantitated by Western blotting, and identical amounts were loaded onto Laemmli gels, followed by electrotransfer to nitrocellulose membranes. The left panel of Figure 2b shows such a blot probed with anti-Jun serum using a phosphatase-conjugated secondary antibody and a color reaction for detection. As demonstrated by this blot, His_6–c-Jun and c-Jun–His_6 are efficiently expressed and recovered from the transfected cells by NTA chromatography. Cotransfection of the HA–ubiquitin vector does not interfere with this expression. The same filter, immunostained with the anti-HA monoclonal antibody and the sensitive enhanced chemoluminescence protocol, reveals those proteins that both bind to the nickel column and contain the HA-epitope derived from the exogenous ubiquitin. Hence, this Western blot would visualize the covalent, guanidine-resistant conjugates between His_6–c-Jun

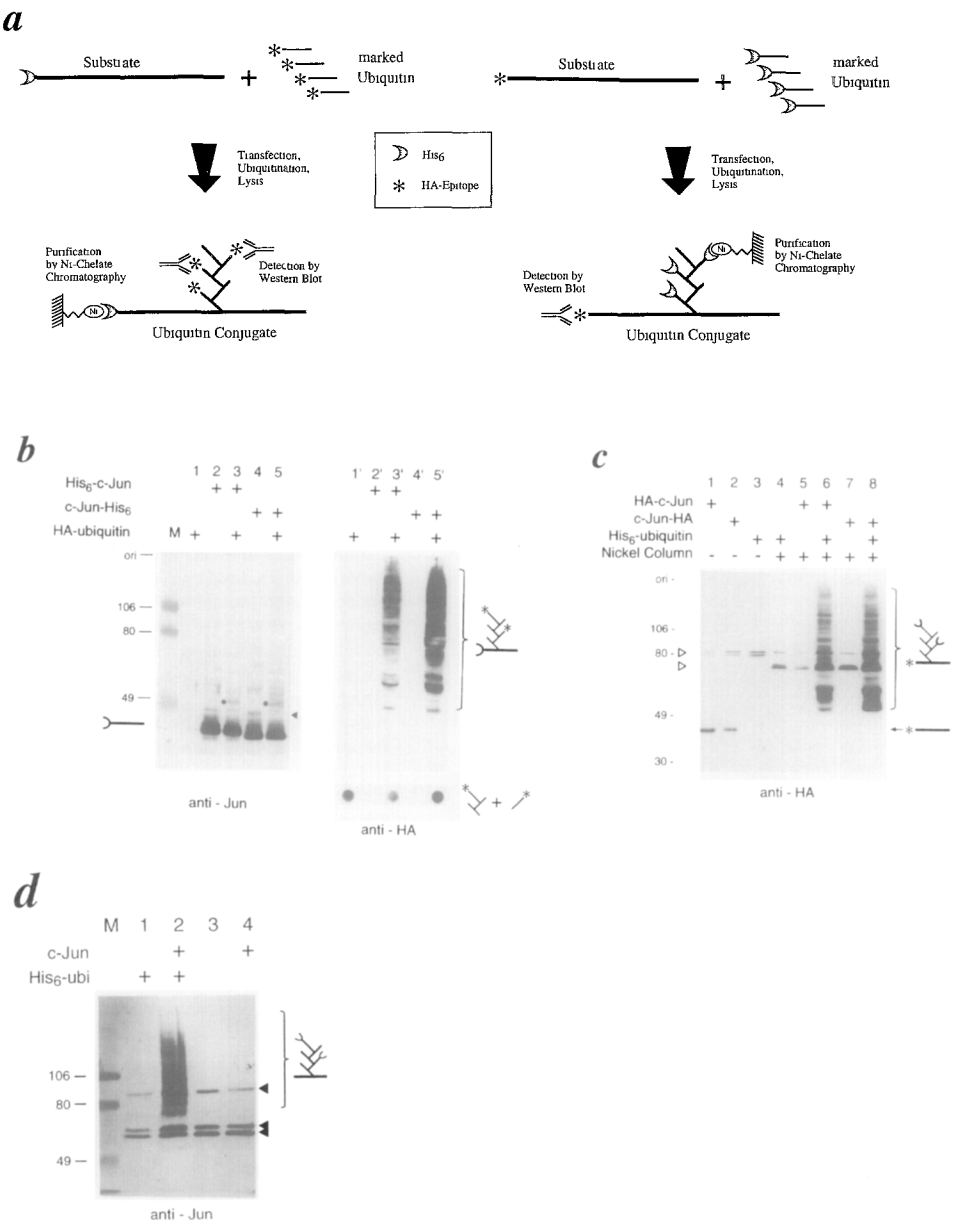

Figure 2. Ubiquitination of c-Jun In Vivo

(A) Schematic depiction of the in vivo ubiquitination assay.

(B) In vivo ubiquitination of His_6-tagged c-Jun. HeLa TK^- cells were transfected with c-Jun–His_6, His_6–c-Jun, and HA–ubiquitin as indicated. His_6-tagged proteins were purified from lysates of the transfected cells, run on SDS–PAGE gels, and transferred to nitrocellulose membrane. The left panel shows the resulting blot immunostained with rabbit anti-Jun antibodies to confirm that the expressed Jun proteins are of the expected size and that the same amounts are loaded in all lanes. The position of His_6-tagged c-Jun is indicated on the left of the panel. The right panel shows the same filter probed with a mouse anti-HA monoclonal antibody. The position of conjugates between His_6-tagged c-Jun and HA–ubiquitin in the gel is indicated on the right. Aliquots of the cell lysates corresponding to samples 1–5 before NTA chromatography were applied to a dot blot and probed with anti-HA antibodies as shown on the lower right hand side of the figure. This control confirms that HA-crossreactive material is present in all cells transfected with HA–ubiquitin.

(C) In vivo ubiquitination of HA-tagged c-Jun. HeLa TK^- cells were transfected with c-Jun–HA, HA–c-Jun, and His_6–ubiquitin as indicated. Aliquots of lysates prepared from the transfected cells were either loaded directly (minuses, Nickel Column) or first subjected to NTA purification and then loaded onto an SDS–PAGE gel (pluses, Nickel Column). Free HA-tagged c-Jun (indicated at the right of the panel) does not bind to the NTA resin and is only detectable in the crude lysate (lanes 2 and 3). Conjugates between HA-tagged c-Jun and His_6-tagged ubiquitin that become visible after purification are marked on the right of the panel. Arrowheads point to unspecifically cross-reacting proteins originating from the cell lysates independent of transfection. Note that some of these products are lost after NTA chromatography, while one is enriched.

(D) Ubiquitination of nontagged and endogenous c-Jun. HeLa TK^- cells were transfected with CMV vectors directing the synthesis of nontagged c-Jun and His_6–ubiquitin as indicated. NTA-purified material from the transfected cell lysates was analyzed by Western blotting with anti-Jun antisera. The bracket on the right side of the panel marks a smear of cross-reactive material that corresponds in size to ubiquitinated c-Jun. The arrowheads indicate cellular proteins that unspecifically cross-react with the secondary antibody.

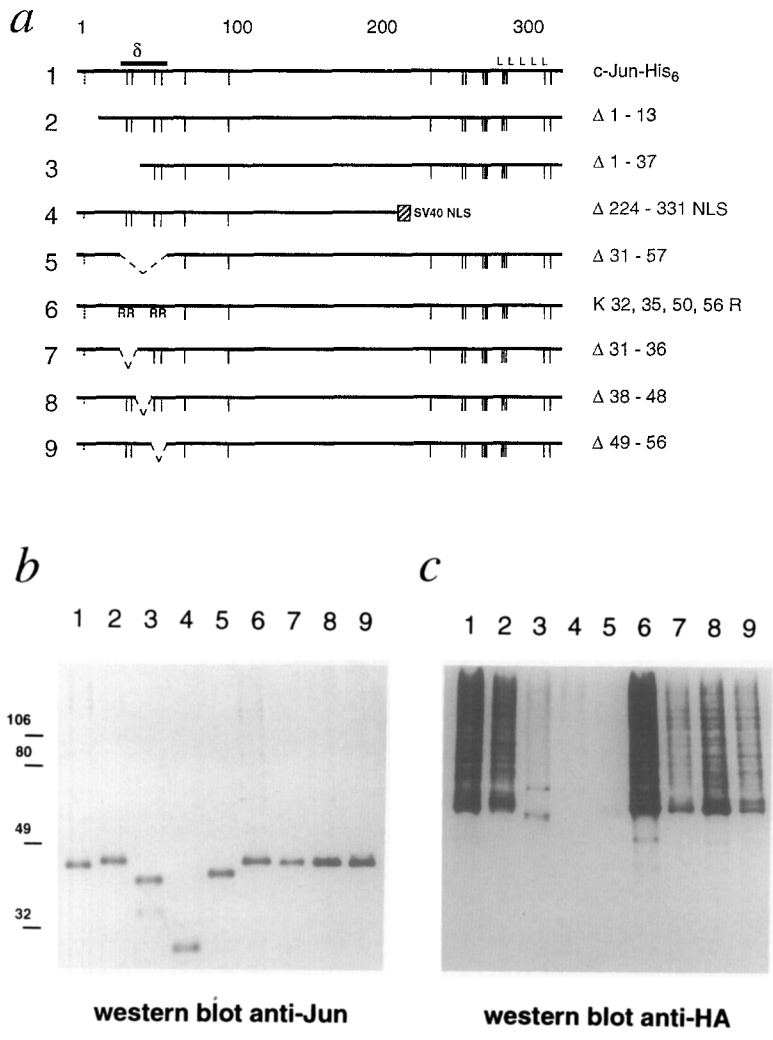

Figure 3. NH_2- and COOH-Terminal Sequences of c-Jun Are Required for Efficient Ubiquitination

(A) c-Jun mutants subjected to the in vivo ubiquitination assay. Wild-type c-Jun is represented on the top. Coordinates are given in amino acids. The locations of the leucine repeat (LLLLL) and of the δ domain are indicated. The positions of the 17 lysine residues of c-Jun are marked by vertical ticks. The deviation from the wild-type structure in the mutants, either deletions or substitutions of lysine with arginine (R) residues, is schematically depicted. The name of the mutation is printed on the right. The Δ224–331 NLS construct that lacks the c-Jun nuclear localization sequence (Alani et al., 1991) was targeted to the nucleus by addition of a constitutive SV40 NLS.

(B) Expression of mutant c-Jun proteins. Lysates of cells transfected with the nine constructs shown in (A) were loaded onto NTA columns, and the different Jun proteins were purified. Equal amounts of Jun cross-reactive material were loaded onto an SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. This blot was immunostained with both anti-Jun antibodies and anti-HA antibodies (see [C]). Lane numbers correspond to construct numbers in (A). c-Jun(Δ1–13), lane 2, has a slightly slower electrophoretic mobility because of a longer spacer between the His_6 tag and the c-Jun sequences. Apparent molecular weights (in kilodaltons) of marker proteins are indicated.

(C) Ubiquitination of c-Jun mutants. The same Western blot used in (B) was probed with mouse anti-HA antibody to reveal c-Jun–ubiquitin conjugates.

or c-Jun–His_6 and HA–ubiquitin, should they have been generated in the transfected cell. A group of bands with the apparent molecular mass predicted for c-Jun–ubiquitin conjugates is indeed seen when His_6-tagged c-Jun and HA-tagged ubiquitin have been coexpressed. No such products can be observed upon cotransfection of HA–ubiquitin with nontagged c-Jun (data not shown). The smallest band stained by the anti-HA antibody runs at approximately 8 kDa (the molecular mass of a single HA–ubiquitin molecule) above the c-Jun band (Figure 2b; compare lanes 3 and 3′, 5 and 5′). The slower migrating bands form a more complex pattern that transforms into a smear toward the top of the gel. These bands most likely represent multiubiquitin conjugates of c-Jun that consist of mixtures of HA-tagged ubiquitin and the endogenous, untagged ubiquitin. A further cause of complexity might arise from multiple ubiquitin attachment sites in the Jun substrate and from the formation of branched so-called ubiquitin trees (Chau et al., 1989). Closer inspection of the anti-Jun-stained filter (Figure 2b, left panel) reveals a faint band visible above the main c-Jun-band (arrowheads in Figure 2b, lanes 2 and 4). This band corresponds in size to a conjugate between histidine-tagged c-Jun and endogenous ubiquitin. In experiments where HA–ubiquitin has been cotransfected, part of this band is shifted into a slower-migrating, higher molecular mass form that is also stained with the anti HA-antibody and thus represents a conjugate containing the slightly larger tagged form of ubiquitin (marked with a dot in Figure 2b, lanes 3 and 5). The fact that both His_6–c-Jun and c-Jun–His_6 exhibit a comparable ubiquitination pattern indicates that the native NH_2- or COOH-terminus is not essential for the ubiquitination process. Expression of the HA-ubiquitin construct alone does not yield detectable HA immunoreactivity in the NTA eluate fraction, even though the protein is present in the corresponding cell lysates (dot blots in Figure 2b). This indicates that HA–ubiquitin, either monomeric or in conjugates with proteins other than His_6-tagged c-Jun, is not retained on the NTA resin to a detectable extent.

If the interpretation of the data presented above is correct, the in vivo ubiquitination assay should also work if the tags are reversed, i.e., if the substrate is HA-tagged c-Jun and the affinity for the NTA resin is mediated by His_6-tagged ubiquitin (Figure 2a, right). Figure 2c shows

such an experiment. A group of bands of the molecular mass expected for c-Jun–ubiquitin conjugates can be visualized in the population of NTA-purified proteins from cells that have been cotransfected with either HA-c-Jun or c-Jun-HA and His_6–ubiquitin. Again, the smallest species of the HA-Jun–His_6–ubiquitin complexes migrates like a protein 8 kDa larger than free HA-tagged c-Jun, which can be visualized when a small aliquot of the nonpurified lysate of the transfected cells is analyzed by anti-HA Western blotting (lanes 1 and 2). As expected, the intensity distribution of the HA signal in this experiment is different from that presented in Figure 2b: the higher forms give rise to a weaker signal than before, because every HA–c-Jun ubiquitin conjugate contains only one HA epitope, regardless of how many ubiquitin moieties are attached. The experiment performed with HA-tagged Jun as substrate allows a rough quantitation of the ratio of ubiquitinated versus nonubiquitinated c-Jun at equilibrium. Considering that, in lanes 1 and 2, lysates from 4×10^4 cells each and, in lanes 6 and 8, 4×10^7 cell equivalents of the NTA eluate were loaded, and assuming an ideal 100% recovery on the NTA column, the ubiquitinated fraction of HA-Jun is minimally between 0.1 and 1.0%. These figures are comparable to those obtained for bona fide ubiquitination targets and compatible with the ubiquitin system being a major determinant for c-Jun instability in vivo (see Discussion). The ubiquitination efficiency did not detectably change when the c-Jun expression vector was titrated from 1 μg (as in the presented figures) down to 10 ng per transfected plate (data not shown).

To address the important question of whether the multiubiquitination of overexpressed, tagged versions of c-Jun reflects a property of the endogenous protein, we performed the following experiment (Figure 2d). His_6–ubiquitin was expressed in HeLa TK^- cells either alone or along with full-length, nontagged c-Jun. A large quantity of His_6-tagged protein was isolated from these cells 48 hr after transfection and was analyzed by Western blotting with anti-c-Jun serum. In the material from cells cotransfected with c-Jun and His_6–ubiquitin, a smear of bands migrating similarly to the ubiquitinated forms of HA-Jun could be seen. No such smear was detected when c-Jun was expressed, without His_6–ubiquitin. This result provides evidence that ubiquitination can occur on native c-Jun and that it is not an artifactual consequence of His_6 or HA tagging. In lane 1, where the sample from cells transfected with His_6–ubiquitin alone was analyzed, a faint smear of Jun-cross-reactive material indicates the presence of His_6–ubiquitin conjugates with endogenous c-Jun, thus confirming that ubiquitination of c-Jun is not simply a result of overexpression of the protein.

Both the δ Region and the DNA-Binding/Dimerization Domain Are Required for Jun Ubiquitination

To investigate the cis requirements for c-Jun ubiquitination, we tested a number of His_6-tagged deletion mutants of c-Jun in the assay system described above (Figure 3). A truncation of the molecule that removes the NH_2-terminal 13 amino acids (c-Jun(Δ1–13)–His_6, lane 2) has no effect on the pattern or extent of ubiquitination. Extending the deletion up to amino acid 37 in the derivative c-Jun(Δ1–37)–His_6 (lane 3), however, causes a dramatic reduction in ubiquitination. The missing amino acids in this mutant include the first 7 of a stretch of 27 residues that has been termed the δ region, because it is missing in v-Jun. To investigate whether the integrity of the δ region is required for ubiquitination, it was precisely excised by site-directed mutagenesis. The resulting internal deletion mutant, c-Jun(Δ31–57)–His_6, exhibits a strikingly reduced activity as a multiubiquitination substrate (lane 5). The requirement of the δ region for efficient c-Jun multiubiquitination could mean that it contains either the major ubiquitination site(s), or a recognition signal for the ubiquitination machinery, or both. A mutant of c-Jun in which all four lysines contained within the δ region have been converted to nonubiquitinatable arginines displays a wild-type pattern of ubiquitination (lane 6). We conclude that the δ deletion does not remove the principal ubiquitin attachment sites, but destroys a cis-acting signal on c-Jun that is required for efficient multiubiquitination. To examine this potential element further, we constructed three deletion mutants that remove subfragments of the δ region. These three mutations all reduce ubiquitination to a level intermediate between wild type and c-Jun(Δ31–57)–His_6 (lanes 7–9).

c-Jun(Δ224–331 NLS)–His_6 (lane 4), a construct that lacks the 107 COOH-terminal amino acids, including 11 of the 17 lysine residues present in c-Jun, is no longer efficiently ubiquitinated. This is apparently not caused by the inability of c-Jun(Δ224–331 NLS)–His_6 to dimerize or to bind to DNA, because point mutants of c-Jun that abolish these functions do not show impaired ubiquitination (data not shown). One explanation for this effect is that the COOH-terminal region of c-Jun contains the principal substrate site(s) and thus may be required for efficient ubiquitination.

No Single Lysine Is Essential for Ubiquitination

In an attempt to determine which 1 of the 17 lysines present in c-Jun could represent a ubiquitination site, we converted all of them individually or in clusters to arginines (Figure 4a). These mutations were introduced into the CMV c-Jun–His_6 vector and analyzed by cotransfection with CMV HA–ubiquitin. Surprisingly, none of the mutants showed a striking quantitative or qualitative change in the observed ubiquitination pattern (Figures 4b and 4c). Only c-Jun(K101R)–His_6 seems to lose a minor ubiquitination site, evident from the disappearance of one band visible in the ubiquitination ladder of wild-type c-Jun–His_6 (dot in lane 5 of Figure 4c). We conclude that either ubiquitination of c-Jun occurs at multiple points, so that mutation of single lysine residues or lysine clusters has no dramatic effect, or that there is no strict sequence requirement for the ubiquitin-attachment site, and removal of a major substrate lysine shifts the machinery to alternative sites. Similar conclusions have been drawn from ubiquitination studies using artificial substrates in yeast (Bachmair and Varshavsky, 1989).

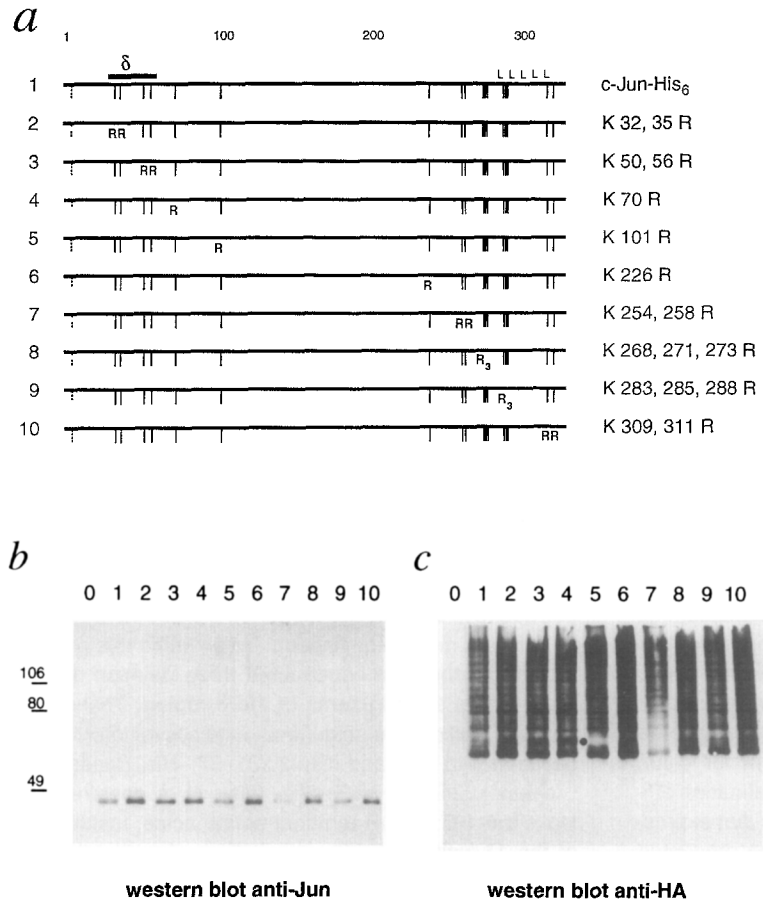

Figure 4. Mutational Analysis of Lysine Residues in c-Jun

(A) Lysine to arginine substitutions in c-Jun–His_6 mutants. For details, see legend to Figure 3.

(B) Expression of lysine substitution mutants. Western blot of purified Jun–His_6 mutants as described in the legend to Figure 3. Lane 0 is a control in which HA–ubiquitin was transfected without a c-Jun–His_6 derivative.

(C) Ubiquitination of lysine substitution mutants. For details, see legend to Figure 3. The dot next to lane 5 marks the position of a ubiquitinated species that is missing when mutant K101R is subjected to the ubiquitination assay.

Loss of the δ Domain Stabilizes c-Jun

The most prominent role of protein ubiquitination is to mark the affected proteins for rapid degradation (Chau et al., 1989). It has, however, been suggested that ubiquitination might also serve other functions in the cell (Levinger and Varshavsky, 1982; Paolini and Kinet, 1993). To investigate whether ubiquitination-dependent proteolysis is involved in the control of c-Jun stability, we measured the half-life of the ubiquitination-deficient δ deletion mutant in comparison with the wild-type c-Jun protein. From the pulse–chase experiment shown in Figure 5, the half-life of c-Jun–His_6 was estimated at 90 min (lanes 2–7), in good agreement with the value of 90–120 min previously reported for endogenous c-Jun in fibroblasts (Lamph et al., 1988). This result shows that overexpression of c-Jun does not interfere with efficient degradation by the cellular proteolysis machinery. In striking contrast, the nonubiquitinatable variant of c-Jun–His_6 that lacks the δ domain displays a strongly increased half-life of 4 hr or longer (Figure 5, lanes 9–14). The correlation between the ability of c-Jun to serve as a substrate for multiubiquitination and its instability in the cell indicates that the multiubiquitination pathway is involved in the regulation of c-Jun half-life in vivo.

The δ Domain as a Degradation Signal

The results of the above experiments imply a requirement of the δ domain both for multiubiquitination and destabilization of c-Jun. Next, we wanted to determine which sequences are sufficient for these functions. Consequently, we employed β-galactosidase (β-gal), which is normally very stable in eukaryotic cells. We fused the NH_2-terminal 67 amino acids of c-Jun that contain the δ domain plus 40 flanking amino acid residues to β-gal (Figure 6, number 3). A constitutive nuclear localization signal (NLS) was added to the construction to ensure that the test protein would be exposed to the same subcellular environment as c-Jun itself. The resulting fusion protein, $c\text{-}Jun_{1\text{–}67}$–NLS–β-gal, was expressed in HeLa TK^- cells by transient transfection of a suitable CMV vector, and its stability was measured in a pulse–chase labeling experiment. Two other constructions served as controls in this experiment: $c\text{-}Jun_{1\text{–}67\Delta\delta}$–NLS–β-gal, which is equivalent to $c\text{-}Jun_{1\text{–}67}$–NLS–β-gal but lacks the 27 amino acids of the δ domain, and NLS–β-gal, which does not carry any Jun sequences. Whereas NLS–β-gal was essentially stable during the time course of the experiment (8 hr; Figure 6, number 1), the c-Jun fusion product containing the δ domain decayed quickly (Figure 6, number 3). $c\text{-}Jun_{1\text{–}67\Delta\delta}$–NLS–β-gal was not destabilized (Figure 6, number 2). Importantly, this control rules out a δ domain–independent effect mediated for example by the c-Jun NH_2-terminus. Consistent with the above result, the δ domain–containing β-gal derivative ($c\text{-}Jun_{1\text{–}67}$–NLS–β-gal, number 3 in Figure 6) exhibited multiubiquitination in vivo. We suggest that the β-gal part of the hybrid protein contains lysine residues that can be targeted for ubiquitination after attachment of the δ do-

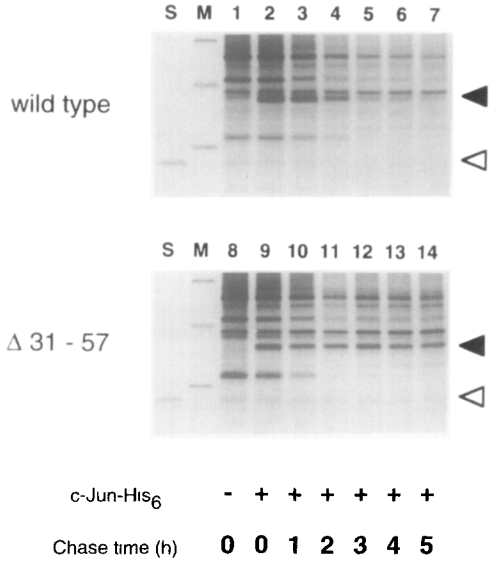

Figure 5. c-Jun Ubiquitination Correlates with Instability In Vivo

A day (24 hr) after transfection of c-Jun–His_6 and c-Jun(Δ31–57)–His_6 into six identical plates of HeLa TK^- cells, these were metabolically labeled with ^{35}S-Express-Label for 30 min and then chased with cold methionine and cysteine for the time periods indicated at the bottom of the figure. After the chase, c-Jun–His_6 and c-Jun(Δ31–57)–His_6 were purified by NTA chromatography and analyzed by SDS–PAGE and autoradiography. Lanes 1 and 8 show a lysate of mock-transfected HeLa TK^- cells to visualize the background of cellular proteins bound to the NTA resin. The closed arrowheads indicate the positions of the respective c-Jun derivatives. The open arrowhead points at a ^{35}S-labeled His_6-tagged so-called spike protein, produced by in vitro translation, that was added in equal amounts to each lysate before NTA chromatography as a recovery control. M, ^{14}C-labeled molecular weight markers (30, 47, and 69 kDa); S, spike protein.

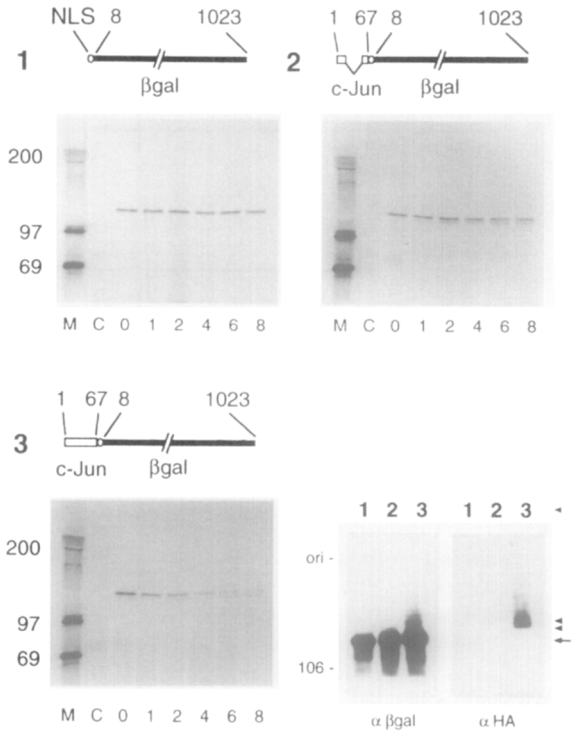

Figure 6. Degradation and Ubiquitination of β-Gal Induced by the δ Domain

The stabilities of NLS–β-gal (1) and of two derivatives carrying at their NH_2-termini either the first 67 amino acids of c-Jun without (2) or with the δ domain (3), respectively, were compared. The structure of the test proteins is schematically indicated on the top of each panel. c-Jun sequences are depicted as white boxes, the SV40 NLS as an open circle, and the β-gal sequences are shown as a black line. The amino acid coordinates of c-Jun and β-gal amino acid sequences are indicated. A pulse–chase experiment was carried out 36 hr after transfection of CMV vectors encoding these three protein fusions into HeLa TK^- cells. Plates of transfected cells were labeled with ^{35}S-labeled amino acids and then chased with cold methionine and cysteine for 0–8 hr. Labeled β-gal proteins were purified from transfected cells (lanes 0–8) and from a mock-transfected control (C) by immunoprecipitation and analyzed by SDS–PAGE and autoradiography. The minor, slightly smaller band migrating underneath the c-Jun_{1-67}–NLS–β-gal and the c-$Jun_{1-67\Delta\delta}$–NLS–β-gal bands consists of discrete, apparently in vivo–produced cleavage products. The amount of material loaded was adjusted so that approximately the same signal was obtained in the 0 hr lanes. This was necessary, because the stable proteins (NLS–β-gal and c-$Jun_{1-67\Delta\delta}$–NLS–β-gal) accumulate to higher steady state levels than c-Jun_{1-67}–NLS–β-gal (data not shown). (A simple correlation between protein stability and steady state levels had not been observed for the Jun derivatives shown in Figures 3 and 4, because the expessed proteins fed back on the CMV enhancer and thus on the rate of their own synthesis; data not shown.) The molecular masses (in kilodaltons) of the protein markers (M) are shown on the left of the panel. The chase times (in hours) are indicated under the panel. The bottom right hand panel shows an in vivo ubiquitination assay on the three β-gal derivatives (1, NLS–β-gal; 2, c-$Jun_{1-67\Delta\delta}$–NLS–β-gal; 3, c-Jun_{1-67}–NLS–β-gal) performed by coexpression with HA–ubiquitin. Loading of identical amounts of substrate proteins was monitored by Western blotting with anti-β-gal antibody. The ubiquitination was determined by Western blotting with the anti-HA monoclonal antibody. The arrow marks the native β-gal fusions, the arrowheads the ubiquitinated forms.

main. In agreement with this hypothesis, the recognition of a lysine by the ubiquitination machinery has been suggested not to require a special sequence context (Bachmair and Varshavsky, 1989; see also Figure 4). The demonstration that a short stretch of c-Jun encompassing the δ domain can confer multiubiquitination and a short half-life to a previously stable heterologous protein identifies it as a necessary and sufficient degradation signal.

v-Jun Escapes Multiubiquitination and Is More Stable Than c-Jun

The finding that the δ domain is a necessary part of the c-Jun degradation signal gains special significance, because it suggests a mechanistic explanation for the transforming activity of v-Jun. The deletion of the δ domain is the main oncogenic mutation of v-Jun (Bos et al., 1990; Morgan et al., 1993). From the previous results, one might speculate that loss of the δ domain might abrogate ubiquitin-dependent degradation that would otherwise curb the transforming function of the oncoprotein. To test this hypothesis directly, we subjected v-Jun and c-Jun to the in vivo ubiquitination assay (Figure 7). Because v-*jun* is an avian oncogene, we used its direct cognate, the chicken c-*jun* gene, for this comparison. Both products

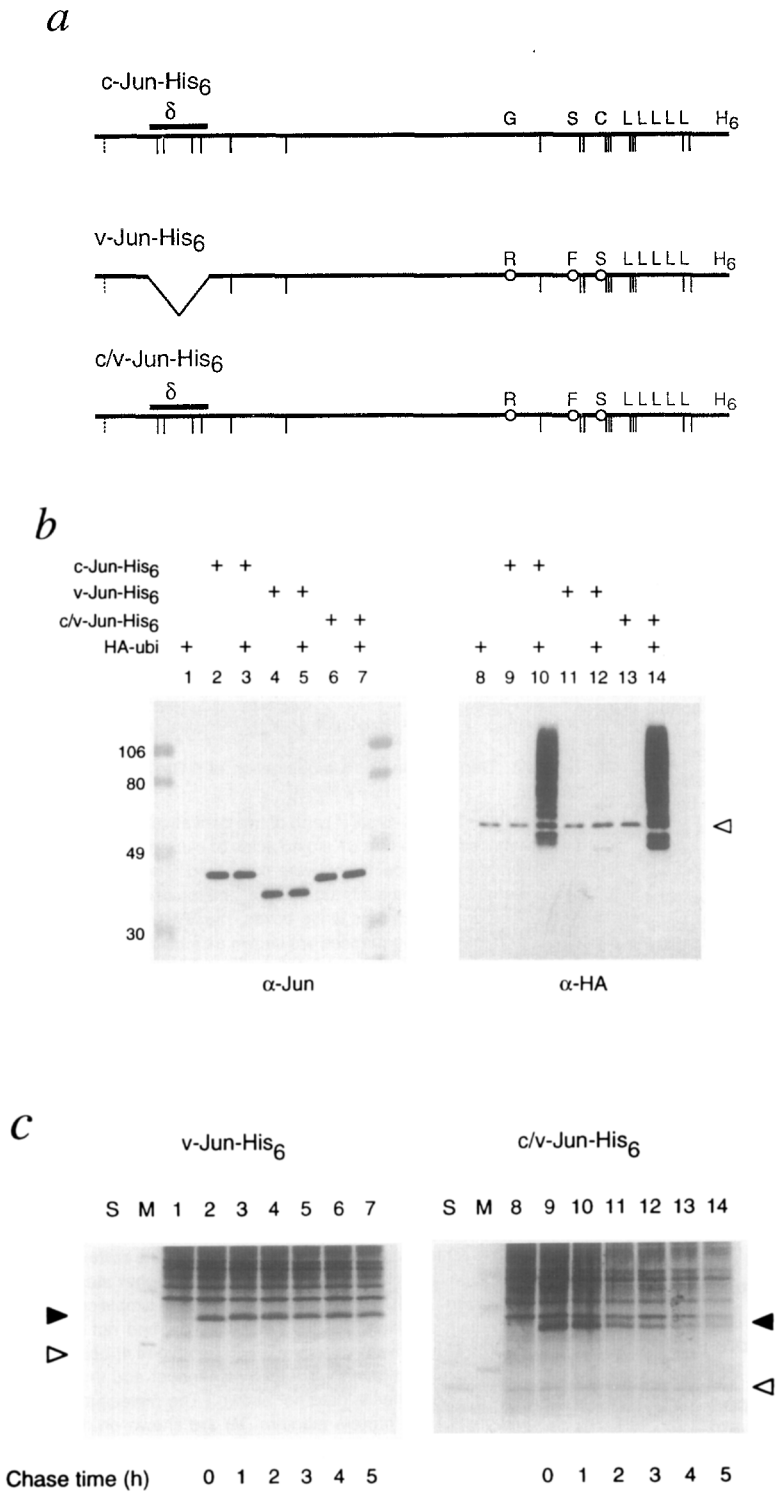

Figure 7. v-Jun Is Inefficiently Ubiquitinated and More Stable Than c-Jun

(A) Schematic description of the examined substrates. Proteins are represented as horizontal black lines; vertical ticks indicate the positions of lysine residues. The positions of the δ domain (δ), the leucine zipper (LLLLL), and the hexahistidine tag (H_6) are indicated. v-Jun differs from chicken c-Jun, which was used in these experiments, by the deletion of the δ domain and three point mutations, marked by open circles. The original amino acid residues at these positions and the mutated substitutions in v-Jun are indicated in single letter code. c/v-Jun is a c-Jun–v-Jun hybrid that resembles v-Jun with the δ domain reinserted.

(B) Ubiquitination assay. Chicken c-Jun–His_6, v-Jun–His_6, and c/v-Jun–His_6 were transiently expressed in HeLa TK^- cells with or without HA-tagged ubiquitin as indicated above the panel. His-tagged material was purified from lysates of the transfected cells, and equal amounts were loaded onto SDS gels as analyzed by Western blot with anti-Jun peptide (α-Jun). Subsequent probing of the same Western blot with anti-HA antibodies (α-HA) visualizes ubiquitinated forms. Strong ubiquitination is only detected when c-Jun–His_6 or c/v-Jun–His_6 has been coexpressed with HA–ubiquitin (lanes 10 and 14). The arrowhead indicates an unspecified cross-reactive band. The fact that the three v-Jun-specific point mutations cause c/v-Jun to run slightly faster in the SDS gel than c-Jun has previously been described by Bos et al. (1990).

(C) Pulse–chase experiment. The stability of v-Jun and of c/v-Jun was determined in a pulse–chase experiment as described in the legend to Figure 5. S marks a lane with spike protein recovery control alone. The masses of the marker proteins (M) are 30, 47, and 69 kDa. Lanes 1 and 8 are mock-transfected controls. The chase time is indicated at the bottom of the figure. The closed arrowheads indicate the running positions of v-Jun–His_6 and c/v-Jun–His_6, respectively. The open arrowheads point to the spike protein.

were expressed as His_6-tagged proteins together with HA–ubiquitin. It is clearly apparent from Figure 7b that ubiquitination of v-Jun is drastically reduced in comparison with c-Jun, indicating that indeed v-Jun has lost the ability of c-Jun to function as an effective ubiquitination substrate. To confirm that this functional change is a consequence of the loss of the δ domain and not of any other mutation suffered in the course of retroviral transduction, we also analyzed a c-Jun–v-Jun hybrid protein that is identical to v-Jun but contains the δ domain; i.e., it carries all mutations but the δ domain deletion. The resulting protein (c/v-Jun) shows the ubiquitination pattern of c-Jun (Figure 7b). This result reiterates the role of the δ domain in the control of ubiquitination and predicts that

v-Jun should be stabilized relative to its cellular progenitor. This supposition was confirmed when v-Jun–His_6 and c-Jun–His_6 were transiently expressed in HeLa TK^- cells and analyzed by a pulse–chase experiment (Figure 7c). Indeed, v-Jun displays a significantly longer half-life than c-Jun (compare Figure 5). The prolonged half-life is a consequence of the lack of the δ domain and not of any of the v-Jun-specific point mutations, because reintroduction of the δ domain restores rapid degradation (c/v-Jun).

Discussion

The lability of many regulatory proteins is crucially important for their function in the cell. In the case of signal-dependent transcription factors, it is apparent that changes in their half-life could have a significant impact on the activity of the corresponding target genes. Indeed, mutations that alter the stability of transcription factors can have marked phenotypes in the organism (for example Cohn et al., 1990; Kellerman et al., 1990; Tebb et al., 1993). In this regard, it is important to understand the molecular mechanisms that control transcription factor turnover. Here we show that the half-life of c-Jun is regulated by ubiquitin-dependent protein degradation. Previous studies using the reticulocyte lysate in vitro system had suggested that some nuclear protooncogene products, including c-Myc and c-Fos, were degraded in a ubiquitin-dependent fashion (Ciechanover et al., 1991). However, no direct evidence for ubiquitination of these proteins or for a similar mechanism in vivo was presented.

According to a rough estimate, 0.1–1.0% of c-Jun in HeLa TK^- cells is multiubiquitinated. In the few cases of proteins that were shown to be degraded predominantly or exclusively by ubiquitin-mediated proteolysis, comparable figures were reported (Glotzer et al., 1991; Hochstrasser et al., 1991). The reason for this seemingly low abundance of multiubiquitinated degradation intermediates is evidently their extremely rapid turnover, with a half-life that has been estimated in the range of 1 min (Glotzer et al., 1991). We conclude that the observed fraction of multiubiquitinated c-Jun is compatible with the ubiquitin system being the main determinant of the short half-life of c-Jun. Other protein breakdown systems may, however, also operate on c-Jun. It has, for example, been suggested on the basis of in vitro and indirect in vivo experiments that c-Jun and c-Fos are targets for degradation by the Ca^{2+}-dependent protease calpain (Hirai et al., 1991).

With the described in vitro assay system, it should now be possible to dissect the trans-acting components mediating c-Jun multiubiquitination biochemically and to characterize biochemical requirements for this process and its control.

The in vivo ubiquitination assay that is introduced here is similar in approach to that used by Hochstrasser and colleagues to study ubiquitination of the MATα2 repressor in the yeast system (Ellison and Hochstrasser, 1991; Hochstrasser et al., 1991). The employment of NTA chromatography and hexahistidine tags facilitates the method and obviates the requirement for antibodies to the tested proteins. It is important to point out that the addition of His_6 or HA tags does not appear to interfere with such biochemical properties of c-Jun as DNA binding, transcriptional activation, phosphorylation pattern, or half-life (A. Papavassiliou and S. E. Smith, unpublished data; this study). Thus, the possibility that the c-Jun-derived substrates employed here are recognized as aberrant proteins and targeted by the ubiquitination system for this reason can be excluded. In addition, it was found that neither addition of the molecular tags nor overexpression is necessary for multiubiquitination, since endogenous c-Jun is also found in a ubiquitinated form. The described assay thus measures a physiological property of c-Jun and should be generally applicable for the study of other known or suspected ubiquitination substrates in higher eukaryotic cells.

The previously characterized δ domain appears to be a necessary and (together with 40 flanking amino acids) sufficient signal for c-Jun degradation. A deletion of the δ domain stabilizes c-Jun, and fusion of the NH_2-terminus of c-Jun bearing this domain to a heterologous protein targets the latter for degradation. It is plausible that the δ domain is recognized by a component of the cellular ubiquitination machinery, perhaps one or more specific E2 enzymes. This speculation raises the question of whether such a hypothetical E2 might be related to the cell type–specific titratable inhibitor of c-Jun described by Baichwal and Tjian (Baichwal and Tjian, 1990; Baichwal et al., 1992), which requires the δ domain for efficient interaction. This possibility is ruled out by the observation that a mutant of c-Jun lacking the ε domain (amino acids 100–127), which is incapable of interacting with the inhibitor (Baichwal et al., 1992), is ubiquitinated with wild-type efficiency (data not shown). Evidently, regulatory processes targeting the NH_2-terminus of c-Jun are quite complex.

Deletion of the δ domain from c-Jun has been shown to increase its transforming potential in chicken embryo fibroblasts to that of v-Jun. Our finding that the same mutation abrogates ubiquitin-dependent degradation raises the intriguing possibility that the transformation-enhancing effect of the δ deletion in v-Jun is mediated or promoted by the stabilization of the protein. Indeed, stabilization of oncogene products frequently contributes to transformation. Increased stability of the transforming mRNA versus the cellular counterpart is often observed, for example in the case of v-*fos* and c-*fos* (Treisman, 1985). Several protein products of oncogenes have been shown to be more stable than their nontransforming progenitors (Jenuwein and Müller, 1987; Cohn et al., 1990). A role for Jun turnover in the physiological control of cell proliferation was proposed by Pfarr and colleagues, who found a serum-induced breakdown of the otherwise stable JunD protein and a concomitant increase of c-Jun levels during G0–G1 transition (Pfarr et al., 1994). Even though we do not know whether JunD is also subject to ubiquitin-dependent degradation, we observed that this protein is ubiquitinated in fibroblasts (M. T., unpublished data). Further evidence for a participation of the ubiquitin system in the control of stability and thus steady-state concentrations of key regulators of cell growth was recently suggested by Papa and

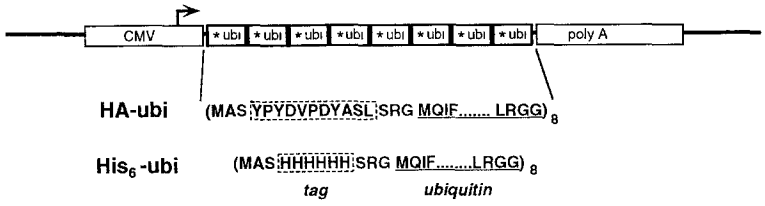

Figure 8. Structure of the Vectors Expressing Tagged Ubiquitin

The vectors consist of the CMV promoter/enhancer region (CMV) driving the transcription of an mRNA encoding a multimeric precursor molecule composed of eight ubiquitin units with NH_2-terminally added tags (asterisks). Polyadenylation is mediated by the SV40 polyadenylation signal (poly A). The protein sequence of His_6-tagged and HA-tagged ubiquitin is shown below.

Hochstrasser, on the basis of the finding that the product of the *tre-2* oncogene acts as a dominant negative mutant of a deubiquitination enzyme and thus can interfere with ubiquitin-dependent protein degradation (Papa and Hochstrasser, 1993). An additional indication for a connection between ubiquitin-dependent regulation of transcription factor stabilty and cell transformation comes from the elegant studies of Scheffner et al., who demonstrated oncogene-induced, ubiquitin-dependent degradation of the tumor suppressor p53 in vitro (Scheffner et al., 1990, 1993). In conclusion, disturbance of ubiquitin-mediated protein degradation either on the level of the enzymatic machinery involved (as in the case of *tre-2*) or on the level of the substrates (as demonstrated here for v-Jun) might be an important route leading to cell transformation and oncogenesis.

Experimental Procedures

Plasmid Construction

Eukaryotic expression vectors were constructed by inserting the corresponding cDNA between the CMV enhancer and promoter and a SV40 polyadenylation signal.

Human c-Jun Constructs

Unless otherwise stated, all c-Jun constructs are derived from the human cDNA sequence. CMV c-Jun–His_6 was constructed by replacing the last two COOH-terminal codons of human c-Jun by an oligonucleotide encoding EFHHHHHH. CMV His_6–c-Jun was constructed by replacing the first 13 NH_2-terminal codons by a sequence coding for MDPHHHHHHDPP. CMV c-Jun–HA bears the sequence encoding EFGASYPYDVPDYASLSR instead of the last two amino acids at its COOH-terminus. CMV HA–c-Jun contains the sequence MASYPYDVPDYASLS added to the NH_2-terminus of full-length c-Jun.

All the following point mutations and deletions were generated by oligonucleotide-directed mutagenesis using the polymerase chain reaction according to the method of Landt et al. (1990). All constructions were verified by dideoxy sequencing.

Lysine to Arginine Mutations

CMV c-Jun(K 32, 35 R)–His_6
CMV c-Jun(K 50, 56 R)–His_6
CMV c-Jun(K 70 R)–His_6
CMV c-Jun(K 101 R)–His_6
CMV c-Jun(K 226 R)–His_6
CMV c-Jun(K 254, 258 R)–His_6
CMV c-Jun(K 268, 271, 273 R)–His_6
CMV c-Jun(K 283, 285, 288 R)–His_6
CMV c-Jun(K 309, 311 R)–His_6
CMV c-Jun(K 32, 35, 50, 56 R)–His_6

Deletions

CMV c-Jun(Δ1–37)–His_6
CMV c-Jun(Δ31–57)–His_6
CMV c-Jun(Δ31–36)–His_6
CMV c-Jun(Δ38–47)–His_6
CMV c-Jun(Δ49–56)–His_6

CMV c-Jun(Δ224–331 NLS)–His_6 encoding amino acids 1–223 of c-Jun plus EFPKKKRKVKFHHHHHH, including SV40 large T nuclear localization signal (underlined) (Kalderon et al., 1984).

Chicken c-Jun and v-Jun Constructs

CMV chicken c-Jun–His_6 was constructed by replacing the last two COOH-terminal codons of chicken c-Jun by an oligonucleotide encoding EFHHHHHH. CMV v-Jun–His_6 was constructed by replacing the last two COOH-terminal codons of v-Jun by an oligonucleotide encoding EFHHHHHH. CMV c-Jun–v-Jun–His_6 was constructed by fusing the NH_2-terminus of chicken c-Jun, including the δ domain, to the COOH-terminus of v-Jun–His_6, which carries three point mutations.

Nuclear β-Gal Constructs

All β-gal constructs were inserted into the CMV vector. The SV40 large T nuclear localization signal was fused to the NH_2-terminus of the β-gal portion to target the protein to the nucleus. The exact sequence is MGDPTPPKKKRKVKGPIP plus β-gal from amino acids 8 to 1023. c-$Jun_{1–67}$–NLS–β-gal was constructed by fusing amino acids 1–67 of human c-Jun to the NH_2-terminus of nuclear β-gal. c-$Jun_{1–67Δδ}$–NLS–β-gal is identical to the above construct but missing amino acids 31–57 corresponding to the δ domain in the Jun part of the molecule. The nuclear localization of the NLS–β-gal derivatives was confirmed by indirect immunofluorescence analysis of transfected cells with anti-β-gal sera.

Ubiquitin Constructs

The design of the expression vectors for tagged ubiquitin is described in Figure 8. An octameric ubiquitin precursor protein is expressed from the CMV enhancer–promoter. Each ubiquitin unit contains at its NH_2-terminus an HA or His_6 tag as indicated in the figure. The precursor is expressed and efficiently processed by cellular ubiquitin–COOH-terminal hydrolases as confirmed by anti-ubiquitin or anti-HA Western blotting (data not shown).

Cell Culture and Transient Transfections

HeLa TK^- cells were cultured in Dulbecco's modified Eagle's medium (DMEM plus 4.5 g/l glucose) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin in a humidified atmosphere at 37°C with 5% CO_2. HeLa TK^- cells were transfected with DOTAP (Boehringer Mannheim) according to the instructions of the manufacturer, with up to 5 μg of CMV-based expression vector, up to 1 μg of substrate expression vector, and up to 4 μg of ubiquitin expression vector per 100 mm dish.

Protein Purification

Purification of ubiquitin–Jun conjugates: 24 hr after transfection, cells were lysed in 2 ml of 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0) plus 5 mM imidazole per 100 mm dish. The lysate was sonicated with a Branson microtipped sonifier at setting 4 for 30 s to reduce viscosity. Lysate (6 ml) was mixed on a rotator with 0.2 ml (settled volume) of Ni^{2+}–NTA–agarose (Qiagen) for 4 hr at room temperature. The slurry was applied to a Bio-Rad Econo-Column. The column was successively washed with the following: 1 ml of 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0); 2 ml of 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 5.8); 1 ml of 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0); 2 ml of (6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 [pH 8.0]:protein buffer) 1:1; 2 ml of (6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 [pH 8.0]:protein buffer) 1:3; 2 ml of protein buffer; 1 ml of protein buffer plus 10 mM imidazole. Elution was 1 ml of protein buffer plus 200 mM imidazole. Protein buffer is 50 mM Na_2HPO_4/NaH_2PO_4 (pH 8.0), 100 mM KCl, 20% glycerol, and 0.2% NP-40. The eluate was TCA precipitated for further analysis.

Iodination of Ubiquitin and In Vitro Ubiquitination of c-Jun

Bovine ubiquitin from Sigma was iodinated with IODO-GEN (Pierce)

according to the instructions of the manufacturer. Bacterially expressed c-Jun–His_6 was incubated with ^{125}I-labeled ubiquitin in 50 μl of degradation mix, final concentrations 70% (v/v) untreated reticulocyte lysate (Promega), 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, 5% (v/v) glycerol, for 1 min at 37°C. The reaction was stopped by addition of 1 ml of 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0). Ubiquitin–c-Jun conjugates were purified by Ni–NTA columns and analyzed by electrophoresis and autoradiography.

Western Blot Analysis

Following SDS–PAGE, proteins were electroblotted to Hybond–ECL Nitrocellulose (Amersham) and immunostained with one or more of the following antibodies. Polyclonal anti-Jun serum was raised in rabbits against full-length bacterially expressed human c-Jun. The Jun antipeptide antibody (Ab-2) used in Figure 7 was purchased from Oncogene Science. The monoclonal antibody 12CA5, specific for a 9 amino acid HA peptide sequence (YPYDVPDYA) from influenza HA was purchased from BabCo. The β-gal antibody was a monoclonal purchased from Promega. The antigen–antibody complexes were visualized using appropriate secondary antibodies and either alkaline phosphatase or the ECL detection system as recommended by the manufacturer (Amersham).

Pulse-Chase Analysis

HeLa TK^- cells were plated the day before transfection to a density of 8×10^5 per 60 mm dish in 4 ml of DMEM. One μg of expression vector per plate of the appropriate CMV Jun–His_6 constructs was transfected with DOTAP. On the following day, the dishes were washed twice with DMEM plus 4.5 g of glucose plus 2% dialyzed serum without methionine and cysteine (pH 7.6). Cells were incubated for 1 hr in the above medium and then labeled for 30 min at 37°C with 0.3 mCi of ^{35}S-Express-Label (New England Nuclear). The plates were washed twice with chase medium (DMEM plus 4.5% glucose, 2.0% FCS, 2 mM methionine, 2 mM cysteine) and incubated for indicated times. The cells were lysed in 3 ml of 6 M guanidinium–HCl, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0) plus 5 mM imidazole. Equal amounts of acid-insoluble cpm were applied to Ni–NTA columns together with an in vitro–translated ^{35}S-labeled His-tagged protein as an internal standard. The columns were run as described above. The eluates were TCA precipitated and separated on a 10% SDS–polyacrylamide gel. The dried gels were exposed for autoradiography and quantitated on the phosporimager (Molecular Dynamics).

Analysis of β-Gal Fusion Proteins

Pulse-Chase

Transfections of cells and labeling after 2 days was done as for the His_6 constructs. Cells were washed twice with ice-cold PBS and lysed on ice in 1.5 ml of RIPA–buffer (10 mM Tris [pH 7.5], 0.5% deoxycholate, 1% NP-40, 0.1% SDS, 150 mM NaCl, 2 mM EDTA) plus 5 mM PMSF and 10 μg/ml each of the following protease inhibitors: aprotinin, leupeptin, pepstatin, and E64. Lysates were cleared by centrifugation (10 min at 12,000 rpm, minifuge). Equal amounts of acid-insoluble cpm were subjected to immunoprecipitation with a monoclonal anti-β-gal antibody. Immunoprecipitated proteins were separated on a 6% SDS–polyacrylamide gel and analyzed by autoradiography.

Detection of β-Gal–Ubiquitin Conjugates

HeLa TK^- cells were cotransfected with 2 μg of the different β-gal constructs plus 4 μg of the HA-tagged ubiquitin expression vector per 100 mm dish. After 36 hr, cells were lysed on ice in RIPA buffer plus 10 mM N-ethylmaleimide and proteinase inhibitors as above. After harvesting, cysteine was added to a final concentration of 0.1%. Immunoprecipitation was carried out as above; proteins were separated on 6% SDS–polyacrylamide gel and blotted onto nitrocellulose. The blots were immunostained successively with HA antibody and with β-gal antibody, respectively. Reactive products were visualized with ECL (Amersham).

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