Ubiquitin is a 76-residue protein that can be covalently attached to a number of cytoplasmic, nuclear, and integral membrane proteins (for reviews, see Refs. 1–3). Ubiquitinylation of proteins has attracted increasing attention, since genetic analyses have implicated important roles of ubiquitinylation in a number of cellular regulatory processes including DNA repair, induced mutagenesis, sporulation, cell cycle transitions from the G1 to S and from G2 to M, stress resistance and peroxisome induced mutagenesis, and the cooperation between E2 and E3 isozymes and to characterize the ubiquitinylation substrates, we have examined the ubiquitinylation of c-Jun and c-Fos using purified enzymes. In this study, we report the in vitro ubiquitinylation of c-Jun and c-Fos catalyzed by the ubiquitin carrier enzymes E2tyr, E2lys, or E2glu in the presence of the ubiquitin-activating enzyme, E1. Addition of ubiquitin protein ligase E3 substantially enhanced the E2lys-mediated ubiquitinylation of c-Jun and c-Fos. Truncated c-Jun and c-Fos mutant proteins including wbJun and wbFos were also ubiquitinylated under the same conditions, suggesting the sites of ubiquitinylation are located within the dimerization and DNA binding domains of c-Jun and c-Fos. The E3-dependent ubiquitinylation of c-Jun was inhibited upon the heterodimerization of c-Jun with c-Fos. Further addition of E2glu significantly enhanced ubiquitinylation of c-Jun in the heterodimer suggesting a regulatory role of E2glu. Polyubiquitinylated c-Jun, wbFos, and wbJun, but not E2lys ubiquitinylated c-Jun un, were readily degraded by the ATP-dependent 26 S multiscatalytic proteases. These results suggest that the temporal control of c-Jun and c-Fos may be regulated through the ubiquitinylation pathways, and the ubiquitinylation of c-Jun and c-Fos may in turn be regulated in response to the heterodimerization between them and the cooperation between E2lys and E3 mediated polyubiquitinylation.

Recombinant c-Jun and c-Fos were ubiquitinylated by the ubiquitin carrier enzymes E2tyr, E2lys, or E2glu in the presence of the ubiquitin-activating enzyme, E1. Addition of ubiquitin protein ligase E3 substantially enhanced the E2lys-mediated ubiquitinylation of c-Jun and c-Fos. Truncated c-Jun and c-Fos mutant proteins including wbJun and wbFos were also ubiquitinylated under the same conditions, suggesting the sites of ubiquitinylation are located within the dimerization and DNA binding domains of c-Jun and c-Fos. The E3-dependent ubiquitinylation of c-Jun was inhibited upon the heterodimerization of c-Jun with c-Fos. Further addition of E2glu significantly enhanced ubiquitinylation of c-Jun in the heterodimer suggesting a regulatory role of E2glu. Polyubiquitinylated c-Jun, wbFos, and wbJun, but not E2lys ubiquitinylated c-Jun un, were readily degraded by the ATP-dependent 26 S multicatalytic proteases. These results suggest that the temporal control of c-Jun and c-Fos may be regulated through the ubiquitinylation pathways, and the ubiquitinylation of c-Jun and c-Fos may in turn be regulated in response to the heterodimerization between them and the cooperation between E2lys and E3 mediated polyubiquitinylation.

Several enzymes involved in ubiquitinylation have been isolated and characterized (9). The ubiquitin-activating enzyme E13 activates ubiquitin and transfers the activated ubiquitin to one of the ubiquitin-conjugating isozymes, E2, which can then covalently attach the ubiquitin to various protein substrates. One of the E2 isozymes, E2lys, apparently modifies the ubiquitin protein ligase E3, which then catalyzes the transfer of ubiquitin to substrates in a processive manner and forms polyubiquitinylated chains (10). The polyubiquitinylated substrates are selectively degraded by an ATP and ubiquitin-dependent 26 S proteasome (11).

Jun and Fos are nuclear proteins encoded by immediate-early genes with transcription activation activities. They are involved in the signaling pathways in regulating cellular growth, differentiation, and neuronal responses (12, 13). These proto-oncoproteins display very short half-lives (14), a feature shared by a number of other proto-oncoproteins, such as Myc, Myb, Erb, and E1a (12), whose degradation might exert a regulatory control of their activities. Inasmuch as cellular transformation results from continuous or deregulated expression of oncoproteins (15, 16), the exact mechanism that controls the turnover of these proteins is important for our understanding of an array of activities regulated by oncoproteins. The mechanism of the temporal control of these proteins is not well understood. The involvement of ubiquitin conjugation in the turnover of nuclear regulatory proteins was suggested by the E1-dependent degradation of in vitro translated proteins in the reticulocyte lysate (17). The ubiquitinylation and degradation of c-Jun (18) and p53 (19) have recently been demonstrated in vivo. In the case of c-Jun, hemagglutinin epitope or oligohistidine labeled proteins were produced in vivo and c-Jun but not v-Jun was found to be selectively ubiquitinylated and degraded in vivo. The enzymes involved in the ubiquitinylation and degradation of c-Jun have not been identified. More recently, ATP-dependent but ubiquitin-independent degradation of c-Jun by the 26 S proteasome was demonstrated in vitro, and opened the possibility of multiple pathways of the degradation of c-Jun (20). In the case of p53, a papilloma viral E6-activated E3 was found to selectively ubiquitinylate p53 (21).

In an attempt to further understand the substrate specificity of the E2 and E3 isozymes and to characterize the ubiquitinylation substrates, we have examined the ubiquitinylation of c-Jun and c-Fos using purified enzymes. In this study, we report the in vitro ubiquitinylation of c-Jun and c-Fos catalyzed by...
Ubiquitylation of c-jun and c-Fos

by reconstituted enzymes purified from reticulocytes. Two enzyme systems that efficiently ubiquitylated c-jun and c-Fos are found. Interestingly, both c-jun and c-Fos can be ubiquitylated efficiently by the same E2 isozymes directly as well as via the protein ubiquitin ligase E3. Furthermore, the E3-ubiquitylated j un and Fos are selectively degraded by the 26 S proteasome. Preliminary results of these studies have been reported earlier (22).

MATERIALS AND METHODS

Rabbit reticulocytes were purchased from Green Hectares (Oregon, WI). Leupeptin, bestatin, and pepstatin A were from Boehringer Mannheim, and TLCK, TPCK, antipain, and chymostatin were from Fluka. Ubiquitin, aprotinin, AEBSF, Arg-Ala, Phe-Ala, and succinyl-Leu-Leu-Arg-Ala were from Sigma. Human c-jun was from Promega. Wild type and mutated c-jun and c-Fos proteins were expressed in Escherichia coli, purified under denaturing conditions by affinity chromatography on nickel-nitriiloacetic acid, and slowly renatured by dialysis as described previously (23). Rabbit antibodies specifically against rat c-jun and rat c-Fos were as described previously (23). Ubiquitin was covalently attached to CH-activated Sepharose 4B (Pharmacia Biotech Inc.) according to the manufacturer’s instruction. SDS-polyacrylamide gel electrophoresis was followed out using the precast Novex 8% or step-gradient 8–16% polyacrylamide gels. Proteins were visualized by the Bio-Rad protein assay.

Purification of Ubiquitylation Enzymes—E1, ubiquitin-conjugating enzymes, E214K, E220K, and E232K, and E3s were purified using the covalent affinity chromatography on ubiquitin-Sepharose (24) and FPLC-Mono Q (25) with modification. Briefly, the ATP-depleted, washed reticulocytes were lysed in the presence of 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM leupeptin, 1 mM bestatin, 0.5 μM pepstatin, 1 μM aprotinin, 30 μM TLCK, 50 μM TPCK, 15 μM AEBSF, and 7 μM calpain I inhibitor. The reticulocyte lysate was centrifuged at 85,000 × g for 1.5 h and the supernatant was first precipitated at 2% polyethylene glycol and subsequently by 9% polyethylene glycol was chromatographed on DEAE-cellulose to obtain fraction I. Fraction II was then affinity-chromatography on ubiquitin-Sepharose (24). Bound enzymes were eluted first using 1 mM AMP and 2 mM sodium pyrophosphate in 50 mM Heps, pH 7.5, 1 mM EDTA, 1 mM EGTA at room temperature (AMP/PPi, eluate), and then 50 mM Heps, pH 9.0, 10 mM DTT, 1 mM EGTA, and 1 mM EDTA DTT/Pi (pH 9 eluate) at 4°C. The AMP/PPi, eluate and DTT/Pi pH 9 eluates were then chromatographed separately on a mono Q column using FPLC as described previously (25) except 50 mM Heps, pH 7.5 was used as the buffer.

Assays of E1, E2s, and E3—E1 activity was monitored by the ATP-PP exchange assay and the ubiquitin thioester assay as described below. The activity of E2 was monitored by the ubiquitin thioester assay in the presence of E1. E3 was monitored by the ubiquitin-conjugating assay in the presence of E1 and E214K using oxidized ribonuclease A as the substrate as described subsequently.

ATP-PP Exchange Assay—E1 catalyzed ubiquitin-dependent ATP-PP exchange. The assay mixture contained 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 1 mM AMP, 1 mM DTT, 5 mM MgCl2, 1 mM (32P)pyrophosphate (DuPont NEN), and 1 μM ubiquitin in a volume of 20 μl. The reaction was initiated by the addition of a limiting amount of E1. After incubation for various lengths of time at 37°C, aliquots of 3 μl were spotted onto polyethyleneimine thin layer chromatographic plates (Brinkmann). The TLC plates were developed in 1.5 M potassium phosphate, pH 3.5. Radioactive ATP was scraped off from the plates, and the radioactivity was monitored with a Packard model 2200CA scintillation counter.

Ubiquitin Thioester Assay—E1 and E2s catalyze thioester formation with ubiquitin. The assay was carried out as described elsewhere (24) with the following modifications. The assay mixtures contained, in 20 μl, 50 mM Heps, pH 7.5, 5 mM MgCl2, 25 mM ATP, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% glycerol, and 10–30 μM [32P]-ubiquitin. The reactions were initiated by the addition of 50 to 70 nm each of E1 and E2s, and incubated at 37°C. Reactions were stopped by the addition of SDS to 2% and heated at 100°C for 3–5 min. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis in the absence and presence of 10% mercaptoethanol. Gels were stained with Coomassie Brilliant Blue R-250 and dried, and radioactive activity was quantitated by PhosphorImager (Molecular Dynamics) analysis.

Ubiquitin Conjugation Assays—Conjugation of ubiquitin to c-jun, c-Fos, or oxidized ribonuclease A was carried out under conditions as described above for the ubiquitin thioester assay except with the addition of substrate protein. Reactions were carried out in the presence of 1 μM c-jun or c-Fos, and 50 mM E1, 50 mM specific E2, 50 mM E3, and 10–30 μM [32P]-ubiquitin as specified. Reactions were terminated by the addition of 10 μl of SDS gel electrophoresis sample buffer containing 10% β-mercaptoethanol. Various ubiquitylated species and unmodified proteins were resolved by SDS-polyacrylamide gel electrophoresis. Gels were stained, dried, and radioactivity quantitated by PhosphorImager analysis.

PhosphorImager Analysis—Dried gels from thioester assays and ubiquitin conjugation assays were placed in contact with phosphorescence screens and the phosphorescence was subsequently scanned using a PhosphorImager. The total counts of individual radioactive bands were summed up and calibrated with known amounts of [32P]-ubiquitin.

Purification of the Multicatalytic Proteases—The multicatalytic proteases were purified according to the procedure described by Kanayama et al. (26). Briefly, reticulocytes were washed and lysed in 1 mM DTT, and the lysate was centrifuged at 35,000 rpm in a Beckman Ti50.2 rotor. The supernatant was precipitated with 10% polyethylene glycol followed by centrifugation at 35,000 rpm for 30 min. The pellet was resuspended in 50 mM Heps, pH 7.5, which contained 5 mM MgCl2, 2 mM ATP, 1 mM DTT, and 20% glycerol. The dissolved pellet was chromatographed on a column of Spectral Gel A4 (Spectrum) in the same buffer. Protease activity was assayed using the fluorogenic peptide Suc-Leu-Leu-Val-Tyr-methyl coumarin. High molecular weight and ATP-dependent active fractions were pooled and chromatographed on a column of hydroxyapatite (Bio-Gel). The proteases were eluted with 0.1 M potassium phosphate, pH 6.8, in 1 mM DTT, 2 mM ATP, 5 mM MgCl2, and 20% glycerol. Active fractions were pooled and adjusted to 50% glycerol and 2 mM ATP and stored at -20°C.

RESULTS

Purification of Ubiquitylation Enzymes—The general procedure of Haas and Bright (25) for purifying ubiquitylation enzymes was used and modified by the inclusion of a battery of protease inhibitors in the lysis buffer, and by the additional step of polyethylene glycol fractionation of the 100,000 × g supernatant. These modifications minimized proteolysis by endogenous proteases during purification, and shortened the total time for processing large volumes of lysates. The entire procedure can be completed in 3 days and afforded purified E1, E22AK, E2217K, E2224K, E2220K, E2232K, and partially purified E3s. Typical elution profiles of the FPLC Mono-Q chromatography of the AMP/PP, eluates and DTT/Pi pH 9 eluates from the ubiquitin-Sepharose are shown in Fig. 1, a and b, respectively. The results are almost identical to those reported by Haas and Bright (25), except for the following differences. E1 was eluted at 250 mM KCl from Mono Q together with E22AK and a 26-kDa protein. The 26-kDa protein did not have any detectable ubiquitin-conjugating activity after further purification by chromatography on Superose 12. E1 and E22AK were separated by chromatography on Superose 12 in the presence of 0.5 M NaSCN, which was needed to resolve E1 from E22AK. The ubiquitin protein ligases, E3s, were present only in the DTT/Pi pH 9 eluates and were eluted at 300 mM KCl from Mono Q together with a molecular mass 94-kDa protein. The 94-kDa protein was resolved from E3 by FPLC gel filtration on Superose 12 and the 94-kDa protein thus purified did not have any E3 activity. Addition of the 94-kDa protein did not affect the E3-dependent ubiquitylation.

Specificity of the Ubiquitin Carrier Isozymes—The purified E1, E2s, and E3s were first used to examine their capability to catalyze the ubiquitylation of c-jun and c-Fos and the substrate specificity of various E2 isozymes. In the presence of E1, E22AK showed the highest ubiquitylation activity toward both c-jun and c-Fos among all E2 isozymes purified (14K, 17K, 20K, 24K, and 32K). Fig. 2, A and B, shows the E22AK-mediated ubiquitylation of c-jun (lane 2) and c-Fos (lane 2), respectively. E217K and E22AK did not exhibit any activity toward c-jun. At 50 μM E1, 50 μM E22AK, and 1 μM c-jun, 22.5% of c-jun was ubiquitylated as three distinct ubiquitylated products.
species determined under nonreducing conditions. Similarly, 5.1% of c-Jun was found to be monoubiquitinylated by 50 nM E214K. However, when the products were analyzed by SDS-gel electrophoresis in the presence of 10% mercaptoethanol, the extents of ubiquitinylation were lower under reducing conditions. Thus, E220K ubiquitinylated 20% c-Jun while E214K ubiquitinylated 2.5% c-Jun under reducing conditions (Table I). The ubiquitin moieties covalently attached to c-Jun not affected by mercaptoethanol were apparently attached through Lys residues in c-Jun. The slight excess of ubiquitin obtained under nonreducing conditions is likely due to ubiquitin attached to cysteine residues in c-Jun. Since the amount of thio-linked ubiquitin to c-Jun was low, the following studies were focused on the isopeptide-linked ubiquitinylation. When the ubiquitinylation of c-Fos was examined under the same conditions as those used for c-Jun, similar results were obtained, except that the extents of ubiquitinylation were lower than those of c-Jun. As shown in Table I, E220K and E214K ubiquitinylated c-Fos to 3.4 and 1.9% of the total protein used after 30 min of incubation, respectively, and only monoubiquitinylated c-Fos was detected in both cases.

In the presence of E3, the E214K-mediated ubiquitinylation of c-Jun or c-Fos was appreciably enhanced, while E220K-mediated ubiquitinylation of c-Jun as expected was not affected. Fig. 2, A and B, show the ubiquitinylination of c-Jun (lane 1) and c-Fos (lane 1), respectively, by E214K and E3. Quantitation of the ubiquitinylated species showed that 8% of c-Jun and 4% of c-Fos were ubiquitinylated in 30 min by 50 nM E1/E214K and E3, as compared to 2.5% and 1.9%, respectively, in the absence of E3 (Table I). Furthermore, four distinct ubiquitinylated c-Jun and c-Fos species together with some high molecular weight polyubiquitinylated species were observed (Fig. 2). The total amount of ubiquitin covalently attached to c-Jun in the presence of E3 was at least 10-fold higher than that in the absence of E3, when high molecular weight species were included in the quantitation.

Ubiquitinylation of Truncated c-Jun and c-Fos Proteins—

FIG. 1. FPLC Mono Q chromatography of the AMP/PPi and DTT/pH 9 elutes. The AMP/PPi, (a) and DTT/pH 9 (b) eluates from covalent affinity chromatography on ubiquitin-Sepharose were loaded separately onto a Mono Q column at 4°C. The column was washed with 5 ml of buffer A (50 mM Hepes, pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mM EGTA), eluted with a 40 ml linear gradient of 0–0.5 M KCl in buffer A, a 4-ml linear gradient of 0.5–1 M KCl and 5 ml of 1 M KCl in buffer A. Fractions of 2 ml were collected at a flow rate of 1 ml/min. Absorbance at 280 nm was monitored.

FIG. 2. Ubiquitinylation of c-Jun and c-Fos. Ubiquitinylation of c-Jun (A) and c-Fos (B) was carried out under the standard ubiquitin conjugation assay conditions using 125I-ubiquitin, followed by SDS-polyacrylamide gel electrophoresis and PhosphorImager analysis. A, lane 1, 1 μM c-Jun and 50 nM each of E1, E214K, and E3; lane 2, 1 μM c-Jun with 50 nM E1 and 50 nM E220K. The radioactively labeled 55-kDa protein band between J un(ub1) and J un(ub2) was immunoprecipitated by monospecific anti-c-Jun antibodies and was evidently due to impurity in the c-Jun preparation. B, lane 1, 1 μM c-Fos and 50 nM each of E1, E214K, and E3; lane 2, 1 μM c-Fos with 50 nM E1 and 50 nM E220K.

TABLE I

| Protein | Percent of c-Jun or c-Fos (no. of conjugates) |
|---------|----------------------------------------------|
| E3      |                                              |
| c-Jun   | 2.5 (1)                                      |
| c-Fos   | 1.9 (1)                                      |
| +E3     |                                              |
| c-Jun   | 8 (4)*                                       |
| c-Fos   | 4 (4)*                                        |

*The values include ubiquitinylated species up to four ubiquitin moieties and do not include the high molecular weight species.

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Ubiquitinylation of truncated c-Jun and c-Fos mutants.

A, c-Jun mutant proteins were ubiquitinylated with 50 nM E1, 50 nM E214K, and 50 nM E3 (lanes 1–3) or at 50 nM E1 and 50 nM E220K (lanes 4–6) under the standard ubiquitin-conjugating conditions and analyzed as described under "Materials and Methods." The lanes labeled as 187, 199, and 241 correspond to reaction products from c-Jun(187–334), c-Jun(199–334), and c-Jun(241–334), respectively. B, c-Fos mutant proteins were ubiquitinylated by E1, E214K, and E3 (lanes 1–3) or by E1 and E220K (lane 4). Lane 1, c-Fos; lane 2, wbFos(116–211); lane 3, wbFos(127–224); and lane 4, wbFos(224–334).

The above results demonstrated that c-Jun and c-Fos are good substrates of the ubiquitinylating enzymes, and that E1/E220K and E1/E214K/E3 are the most efficient enzyme systems for c-Jun and c-Fos.

A number of truncated proteins of c-Jun were previously constructed to examine their DNA binding and the dimerization characteristics (23). These truncated c-Jun proteins were found to be good substrates of ubiquitinylating enzymes. As shown in Fig. 3A, truncated c-Jun proteins were ubiquitinylated by E220K to similar extents as the full-length c-Jun. However, primarily monoubiquitinylated species were obtained from the truncated proteins. When the amounts of truncated c-Jun proteins that were ubiquitinylated by E220K were quantitated, amounts very similar to those for c-Jun were found (Table I, right column). Since the extent of ubiquitinylation of c-Jun by E220K changed little after truncation, the primary sites of ubiquitinylation in intact c-Jun and those in mutant c-Jun proteins are likely to be the same.

Ubiquitinylation of c-Jun truncation mutants by E3 was appreciably more efficient than those of the full-length protein. As shown in Table I, 50% of wbJun was ubiquitinylated by E220K and E3. The majority of the ubiquitinylated proteins appeared as high molecular weight conjugates, suggesting the polyubiquitinylated nature of the truncated c-Jun proteins. The high efficiency of ubiquitinylation exhibited by wbJun suggested that the sites of ubiquitinylation in c-Jun are likely located within the 110 residues in wbJun that contain the DNA binding domain and the leucine repeats, although sites within the deleted regions cannot be excluded. The large increase of ubiquitinylation mediated by E220K and E3 in the truncated c-Jun indicated that either additional sites in c-Jun became available in the truncated proteins or the presence of additional E3 isozymes in the E3 preparation which catalyzed the ubiquitinylations of the truncated c-Jun and c-Fos proteins. As shown in Table II, deletion of N-terminal 90 residues in c-Jun was sufficient to bring about the enhanced ubiquitinylation of c-Jun. The partial deletion of the basic region in wbJun(1260–266) (Table II) did not appreciably affect the extents of ubiquitinylations of wbJun catalyzed by E3. Thus the deleted residues in the basic region were not required for the ubiquitinylations of wbJun.

When the ubiquitinylations of truncated c-Fos proteins by E220K and by E214K and E3 were examined (Fig. 3B), results similar to those obtained with the truncated c-Jun proteins were observed (Table III). The truncated c-Fos proteins, similar to full-length c-Fos, were good substrates of E220K. Similar to that observed in c-Jun, the truncated c-Fos proteins including wbFos(116–224) and wbFos(127–224) were more efficiently ubiquitinylated by E3 than the full-length c-Fos. These results suggest that residues deleted from c-Fos were not required for efficient ubiquitinylation by E220K or by E214K and E3.

Specificity of E3 isozymes—The observation that E3 ubiquitinylated the truncated c-Jun or c-Fos proteins appreciably more efficiently than their respective full-length proteins raised the question as to the identity of the E3 isozymes that ubiquitinylated these proteins. The dipeptides, Phe-Ala and Arg-Ala, were shown to inhibit the E3α isozyme but to have relatively little effects on the E3β isozyme (27). As a control, ubiquitinylation of oxidized ribonuclease was shown to be inhibited by Arg-Ala and little affected by Phe-Ala. This observation is consistent with E3α as the catalyst (Fig. 4A, lanes 4–6). The E3-dependent ubiquitinylations of wbFos and wbJun were similarly examined in the presence of Arg-Ala or Phe-Ala. As shown in Fig. 4B, the levels of ubiquitinylation of wbFos (lanes 7–9) and wbJun (lanes 10–12) were appreciably enhanced by Arg-Ala and were inhibited by Phe-Ala. The observed stimulation by Arg-Ala on the wbFos and wbJun ubiquitinylations was not expected for E3α or E3β, suggesting the existence of a different E3 isozyme or unusual effects of known E3s on these novel substrates. When the effects of the dipeptides on the E3-dependent ubiquitinylations of full-length c-Jun were similarly examined, relatively little effects were found (Table IV), and thus the ubiquitinylations of c-Jun was likely carried out by E3β. Similarly, ubiquitinylations of c-Fos was not significantly inhibited by Arg-Ala or Phe-Ala, indicative of E3β as the enzyme involved (data not shown).

Ubiquitinylations of Fos and Jun Heterodimers—Ubiquitinylations of truncated Jun and Fos mutant proteins suggested that the sites of ubiquitinylations could be located within the dimerization domain in the respective proteins. J un homodimer and the J un/Fos heterodimer showed different physiological activ-
were carried out separately in the presence of 50 nM each of c-Jun and c-Fos. The E220K-mediated ubiquitinylation of c-Jun and c-Fos was reduced by 50% upon the heterodimerization of c-Jun-Fos heterodimer, the E3-dependent ubiquitinylation of c-Jun and c-Fos was not affected by heterodimerization or by E3. The results represent the total ubiquitination of c-Jun and c-Fos species observed in the presence of E220K and E1/E214K/E3. **Table V**

When c-Jun and c-Fos were partially purified from 26 S and 20 S proteasomes from rabbit reticulocytes. As a control, the 26 S proteasome as expected catalyzed the degradation of the oxidized ribonuclease A that was ubiquitinated by E3, while the 20 S proteasome did not (Fig. 5a), indicating that the 26 S proteasome preparation was indeed specific for the ubiquitinated proteins. The present investigation provides direct evidence for the ubiquitinylation of c-Jun and c-Fos. The E2 and E3 isozymes that inherent structural features in c-Jun and c-Fos were recognized. The recombinant c-Jun and c-Fos are evidently good substrates for E220K and E1/E214K/E3. The high levels of ubiquitinylation of c-Jun or c-Fos suggest that ubiquitinylation of this family of transcription factors are catalyzed by E2 and E3 under the standard ubiquitin conjugation assay conditions in the absence of 1 mM Arg-Ala or 1 mM Phe-Ala for 30 min at 37 °C. The products were analyzed by SDS-polyacrylamide gel electrophoresis followed by PhosphorImager analysis. The results represent the total ubiquitination of c-Jun and c-Fos species observed in the presence of E220K and E1/E214K/E3. **Table V**

The ubiquitinylation of c-Jun and c-Fos by E220K was not affected by heterodimerization or by E3. The results represent the total ubiquitination of c-Jun and c-Fos species observed in the presence of E220K and E1/E214K/E3. **Table V**

| Protein       | c-Jun | c-Fos | c-Jun/c-Fos |
|---------------|-------|-------|-------------|
|               | −E220K | +E220K |             |
| c-Fos(1–380)  | 0.55  | 3.79  |             |
| wbFos(116–211)| 0.74  | 2.27  |             |
| wbFos(119–145)| 0.92  | ND    |             |

**DISCUSSION**

The present investigation provides direct evidence for the ubiquitinylation of c-Jun and c-Fos. The E2 and E3 isozymes that ubiquitinated this family of transcription factors are identified. The recombinant c-Jun and c-Fos are evidently good substrates for E220K and E1/E214K/E3. The high levels of ubiquitinylation of c-Jun or c-Fos suggest that inherent structural features in c-Jun and c-Fos were recognized by both E220K and E3. The same two enzyme systems, E1/E220K and E1/E214K/E3 were found to be the most efficient among various reconstituted enzyme systems for both c-Jun and c-Fos. Ubiquitinylation of deletion mutant proteins of c-Jun and c-Fos suggested that most of the sites of multi- and polyanlyubiquitination are most likely located in the heterodimerization and the DNA binding domains. Heterodimerization of c-Jun and c-Fos affected the ubiquitinylation of c-Jun by E3. The ubiquitinylation of c-Jun and c-Fos is susceptible to the 26 S but not to the 20 S proteasome for degradation.

With the inclusion of a battery of protease inhibitors in the reticulocyte lysate, E1 and E220K were copurified throughout the purification suggesting that at least part of the E1 and
E214K may be physically associated as a multienzyme complex. Chemical cross-linking using a cleavable heterobifunctional cross-linking reagent, 3,3'-dithiobis(sulfosuccinimidylpropionate), suggested that indeed E1 and E214K were physically associated. However, the association appears to be weak since gel filtration of the complex partially separated E1 and E214K. Gel filtration of the complex in the presence of 0.5 M NaSCN completely separated E1 and E214K (data not shown) (22). The association of E1 and E214K is biochemically important since it may provide a mechanism for the direct transfer of ubiquitin from E1 to E214K without first dissociating from E1 followed by reassociation with E214K in solution. We consistently obtained somewhat higher levels of ubiquitinylation using the copurified E1/E214K preparation than those using recombined purified E1 and E214K.

The occurrence of E3 isozymes raised the possibility that the E3 isozyme that acted on c-Jun and c-Fos may be different from that of wbFos and wbJun. The inhibition patterns with respect to Arg-Ala and Phe-Ala suggested that this may be the case. The observation that the E3-mediated ubiquitinylation of wbFos and wbJun was apparently stimulated by Arg-Ala indicated that there could be a different E3 isozyme that preferentially acts on the fragments of c-Fos and c-Jun. The action of such additional E3 isozyme on wbFos and wbJun could play a concerted role in the degradation of c-Fos and c-Jun along with E3β.

The susceptibility of the E3-ubiquitinated c-Jun and c-Fos to the 26 S multicatalytic proteasome is in accord with the hypothetical in vivo function of ubiquitinylation in the turnover of c-Jun and c-Fos. The regulation of the turnover of c-Jun via ubiquitinylation in HeLa cells has recently been demonstrated (18). Although only 0.1–1% c-Jun was ubiquitinated in vivo, highly suggestive evidence was obtained supporting the selective degradation of c-Jun via the ubiquitinylation pathway. Furthermore, the retroviral counterpart v-Jun cannot be polyubiquitinated nor degraded efficiently in vivo. The ubiquitinylation enzymes involved in the modification of c-Jun in HeLa cells have not been identified. Since c-Jun but not v-Jun was efficiently ubiquitinated, the in vivo ubiquitinylation is likely mediated through the δ-domain in c-Jun which is absent in v-Jun. The present studies are consistent with the observed ubiquitination of c-Jun and the degradation of ubiquitinated c-Jun. However, since wbJun, in which the δ-domain was deleted, was also efficiently ubiquitinated by E3, our results suggest that c-Jun may have undergone different ubiquitinylation pathways under different conditions. The ubiquitinating enzymes in reticulocytes that acted on c-Jun may be different from those in HeLa cells. Alternatively, different E3 isozymes or other cofactors may be involved in the in vivo ubiquitinylation of c-Jun. Furthermore, the subcellular location of the overexpressed recombinant c-Jun is not known at present. The recent demonstration of ubiquitin-independent degradation of c-Jun by the 26 S proteasome (20) provoked the question as to the role of ubiquitination in the in vivo degradation of c-Jun in HeLa cells. However, our results show that c-Jun and c-Fos can be degraded by the 26 S proteasomes via the ubiquitin-dependent pathway.

The stability of c-Fos was shown to be regulated by phosphorylation (29). Whether phosphorylation regulates the ubiquitinylation of c-Fos and c-Jun is not known. It is known in the case of the oncoprotein Mos that the stability is regulated by ubiquitination (29). Whether phosphorylation regulates the ubiquitinylation which is in turn regulated by phosphorylation (30, 31). Preliminary studies of the effects of phosphorylation of c-Fos and c-Jun by a number of known protein kinases on the ubiquitinylation were inconclusive. One of the known mechanisms that regulate the DNA binding and transcription activation activities of c-Jun and c-Fos is the heterodimerization via the leucine repeats (28). The present results show that heterodimerization affected ubiquitination of c-Jun. The stimulatory effect of E220K on the ubiquitinylation of the heterodimer of c-Jun/c-Fos suggests that E220K could add an additional level of regulation in the E3-dependent ubiquitinylation of the heterodimeric c-Jun/c-Fos. It is intriguing that E220K was found to be the most efficient isozyme among various E2 isozymes in the ubiquitinylation of c-Jun and c-Fos. The present results open the possibility of an interplay of the transcription activation by these transcription factors and their ubiquitinylation, which may act in a concerted fashion in the temporal regulation of transcription.

The reconstitution of the ubiquitinylation enzymes for c-Jun...
and c-Fos provided a route for a systematic studies of the mechanism and the regulation of ubiquitinylation. Further studies on the kinetics and mechanisms of E2- and E3-mediated ubiquitinylation of c-Jun will be reported.

Acknowledgment—We thank Dr. L. Patel (Roche) for help in the preparation of bacteria-expressed proteins.

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J. Biol. Chem. 1996, 271:4930-4936.
doi: 10.1074/jbc.271.9.4930

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