c-Fos Degradation by the Proteasome

AN EARLY, Bcl-2-REGULATED STEP IN APOPTOSIS*

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c-Fos is a transcription factor that promotes cell growth, differentiation, and transformation. We found that c-Fos was degraded when WEHI7.2 mouse lymphoma cells were induced to undergo apoptosis with the calcium ATPase inhibitor, thapsigargin, or the glucocorticoid hormone, dexamethasone. The degradation of c-Fos preceded caspase-3 activation and apoptotic nuclear chromatin condensation and was inhibited by the proteasome inhibitors MG132, N-acetyl-leucyl-leucyl-norleucinal, and lactacystin. Stable transfection of WEHI7.2 cells with a mutant form of c-Fos that was not degraded by the proteasome inhibited apoptosis. Also, overexpression of Bcl-2 in WEHI7.2 cells blocked c-Fos degradation and inhibited apoptosis. The results indicate that proteasome-mediated degradation of c-Fos is an early, Bcl-2-regulated step in apoptosis induction by thapsigargin and dexamethasone. These findings suggest that c-Fos may have a protective action that is eliminated by proteasome-mediated degradation and preserved by Bcl-2.

Apoptosis, or programmed cell death, is a genetically regulated process necessary for maintenance of normal tissue homeostasis (1). In the nematode, Caenorhabditis elegans, apoptosis is regulated by the death effector gene, ced-3, and the death inhibitor gene, ced-9 (2, 3). The mammalian homologues of ced-3 constitute a family of genes encoding aspartate-specific cysteine proteases, or caspases, that cleave selected proteins, thereby contributing to cell death (reviewed in Ref. 4). The mammalian homologues of ced-9 constitute the bcl-2 family of genes encoding proteins that either enhance (e.g. Bax) or inhibit apoptosis (e.g. Bcl-2, Bcl-XL) by an unknown mechanism (5–7). Bcl-2 and Bcl-XL, the best characterized members of the family, function upstream of caspase activation, inhibiting apoptosis induction by diverse death signals, including radiation, growth factor withdrawal, disruption of calcium homeostasis, and glucocorticosteroids (8–10).

Apoptosis also involves the proteasome, a multicatalytic protease complex located in both cytoplasm and nucleus that degrades proteins targeted for destruction by polyubiquitination (11–13). In the hawkmoth, Manduca sexta, apoptosis of intersegmental muscles is associated with elevated ubiquitin gene expression and proteasome activity (14–16). In mammalian cells, apoptosis can be repressed by antisense oligonucleotides that diminish the level of ubiquitinated nuclear proteins or by specific inhibitors of the proteasome (17–19). Recently, proteasome-mediated degradation of topoisomerase IIa and IκBα have been linked to adenovirus E1A-induced apoptosis and activation-induced T cell death, respectively (20, 21).

The proteasome is responsible for the rapid turnover of key regulatory proteins, including the transcription factor c-Fos (22, 23). c-Fos, the product of the c-fos proto-oncogene, is a major component of the activator protein-1 transcription factor complex, a master switch that regulates expression of genes involved in cell growth, differentiation, and transformation (24–30). We have investigated the involvement of c-Fos in apoptosis in the murine cell line WEHI7.2, a tissue culture model of glucocorticoid-induced apoptosis in immature thymocytes (31). WEHI7.2 cells do not express detectable levels of Bcl-2 and undergo apoptosis in response to thapsigargin (TG), a selective inhibitor of the endoplasmic reticulum-associated calcium-ATPase, and dexamethasone (DX), a synthetic glucocorticoid hormone (32). Here we report that (i) both TG and DX induce rapid degradation of c-Fos by the proteasome, (ii) proteasome-mediated c-Fos degradation precedes caspase activation and apoptotic nuclear chromatin condensation, (iii) Bcl-2 overexpression inhibits c-Fos degradation by the proteasome and increases the level of c-Fos expression, and (iv) a C-terminal truncated version of c-Fos that evades degradation by the proteasome inhibits apoptosis induction by TG and DX.

EXPERIMENTAL PROCEDURES

Materials—The anti-c-Fos antibody was purchased from Upstate Biotechnology Inc. The anti-CPP32 p17 subunit antibody (SK398) was raised to amino acids 85–94 of caspase-3 (VRNKNDLTRE) and provided by Kristy Kikly (SmithKline Beecham Pharmaceuticals). MG132 (Z-Leu-Leu-Leu-H aldehyde) was purchased from Peptides International, Inc. Lactacystin was purchased from E. J. Corey (Harvard University). N-Acetyl-leucyl-norleucinal (ALLN) and N-acetyl-leucyl-methylional (ALLM) were purchased from Enzyme Systems Products. E64d ((2S,3S)-trans-epoxy succinyl-I-leucylamido-3-methyl-L-butaneethyl-ester) was purchased from Sigma. Stock solutions of these reagents were prepared in dimethyl sulfoxide and stored in aliquots at −80 °C, except for lactacystin, which was dissolved in water. DX was purchased from Sigma, and TG was purchased from LC Laboratories.

Cell Lines and Culture Conditions—Cells were cultured in Dulbecco’s modified Eagle’s medium (BioWhittaker) supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin, 0.4 mM nonessential amino acids, and 10% (v/v) heat-inactivated horse serum (HyClone) at 37 °C in a 7% CO2 atmosphere. IκB-Tat, a nuclear factor of activated T cells (NF-κB), have been linked to adenovirus E1A-induced apoptosis and activation-induced T cell death, respectively (20, 21).

The abbreviations used are: TG, thapsigargin; DX, dexamethasone; ALLN, N-acetyl-leucyl-norleucinal; ALLM, N-acetyl-leucyl-methylional; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin.

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c-Fos Degradation by the Proteasome in Apoptosis

Cell Counting and Apoptosis Assay—Viable cells, defined as cells that exclude trypan blue dye, were counted using a hemocytometer. The percentage of cells with an apoptotic nuclear morphology was assessed by fluorescence microscopy after staining with ethidium bromide and acridine orange (32).

Protein Preparation—The c-fos cDNA was removed from the pMSVS-fos plasmid (provided by Michael Simonson, Case Western Reserve University) by digestion with EcoRI and ligated in sense orientation into the pcDNA-3 expression vector (Invitrogen) downstream of the cytomegalovirus promoter, producing a pcDNA-3-sense-fos vector. The orientation of inserts was confirmed by analysis with four groups of restriction enzymes. The c-fos cDNA was cloned by polymerase chain reaction using the 5' primer sequence AAAGGGGATTCTAGTACCCATAGGCCGCTACGCTATGGTTCTCGGGTTTACCCGGGAC and the 3' primer sequence ACAGTGGATCTTCTTTTTGCCGTTTGCGTGGCAGCTA. The c-fos cDNA was cloned into the pcDNA-3 expression vector in the sense orientation, confirmed by DNA sequencing. The empty pcDNA-3 vector and the pcDNA-3-fosAC vectors were transfected into WEHI7.2 cells by electroporation and selected with G418 (1 mg/ml). Findings were confirmed in experiments in which the c-fos cDNA was cloned in sense orientation in the pSFFV-neo expression vector.

Western Blotting—To recover c-Fos, cells were washed twice with cold phosphate-buffered saline, resuspended in a 5-fold volume of modified Tris-HCl buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mm EDTA, 5 mm EGTA, 1 mm NaN3, 5 mm NaF, 20 mm N-ethylmaleimide, 1 mm phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin), and boiled immediately for 10 min. To recover caspase-3, cells were lysed in a buffer containing 10 mm HEPES/KOH, pH 7.4, 2 mm EDTA, 0.1% CHAPS, 5 mm dithiothreitol, 1 mm phenylmethylsulfonyl fluoride, 10 mg/ml pepstatin A, 1 mg/ml leupeptin, and 1 mg/ml aprotinin. The cell lysates were centrifuged at 14,000 × g for 10 min. The protein concentration of supernatant samples was determined by the Bio-Rad protein assay (Bio-Rad Laboratories). 70 µg of each protein lysate was separated by SDS-polyacrylamide gel electrophoresis under reducing and denaturing conditions and transferred to polyvinylidene difluoride membrane (Millipore Co.). Equal loading of protein lysates was confirmed by staining membranes with Ponceau S or staining gels with Coomassie Blue after protein transfer. Membranes were incubated in 5% nonfat dry milk in T-TBS (18 mm Tris-HCl, pH 7.6, 122 mm NaCl, 0.1% Tween 20) at room temperature for 2 h and then incubated with the primary antibodies (anti-c-Fos at 1:5000 dilution or anti-caspase-3 at 1:4000 dilution) at 4 °C overnight. Membranes were then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (Life Technologies, Inc.) at a 1:2000 dilution. Immune complexes were detected with the ECL Western blotting detection reagent (Amersham Pharmacia Biotech) according to the manufacturer's protocol, followed by exposure to x-ray film (Sigma).

Caspase Activity—Cells were lysed in 0.25 ml of lysis buffer (100 mm HEPES, 10% sucrose, 0.1% CHAPS, 1 mm phenylmethylsulfonyl fluoride, 100 µM PMSF, 100 µM leupeptin, 1 mm EDTA at pH 7.5), followed by a 30-min incubation on ice. Lysis was completed by two 10-s sonication pulses (Sonicator XL, Heat Systems, Farmingdale, NY). Cellular debris was removed by centrifugation, and the cytosolic extract was stored at −20 °C. Protein content of the lysates was determined by the Bio-Rad Protein assay according to the manufacturer's instructions. 20 µg of cytosolic protein and 50 µl of DEVD-AMC (Biomol Research Laboratory, Plymouth Meeting, PA) were incubated in a total volume of 500 µl in 100 mm HEPES, 10% sucrose, 0.1% CHAPS, pH 6.8, for 30 min on an orbital shaker at 37 °C. Samples were diluted with lysis buffer to a final volume of 1 ml immediately before measurement of fluorescence by fluorospectrophotometry (Perkin-Elmer LS-5; excitation, 380 nm; emission, 480 nm; slit width, 5.0 nm). Standards containing 0–5000 pmol of AMC were used to determine the amount of fluorochrome released. Measurements were recorded over the linear range of the assay. Bovine serum albumin (50 µg) served as negative control.

Protease Inhibitors—Cells were pretreated with inhibitors for time periods described in the text at the following concentrations: MG132, 25 µM; lactacystin, 10 µM; ALLN, 50 µM; ALLM, 50 µM; benzoyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone, 50 µM; E64d, 10 µM.

RESULTS

The level of c-Fos was measured by Western blotting in wild type WEHI7.2 cells, which lack Bcl-2, and in stable transfectants, W.Hb12, which were derived from WEHI7.2 cells and express a high level of Bcl-2 (32). Findings were confirmed in replicate experiments using vector only control transfectants, WEHI7.2-neo, and another clone that stably expresses a high level of Bcl-2, W.Hb15 (not shown). The basal level of c-Fos, before treatment with either TG or DX, was 4.6 ± 2.1-fold (mean ± S.E.) higher in W.Hb12 cells than in WEHI7.2 cells (Fig. 1A). After adding 100 nM TG to WEHI7.2 cells, the level of 66-kDa c-Fos declined markedly, giving rise to a 46-kDa fragment (Fig. 1A), which in many experiments was further degraded to smaller size fragments. In contrast, the level of c-Fos was stable following TG treatment in W.Hb12 cells (Fig. 1A). A decline in c-Fos level was also induced when WEHI7.2 cells were treated with 1 µM DX, although the appearance of the 46-kDa fragment was more variable than in TG-treated cells, perhaps due to the longer time course of DX-induced cell death (Fig. 2A). In the case of both TG- and DX-treated WEHI7.2 cells, the decline in c-Fos preceded caspase-3 (CPP32, apopain, and Yama) activation, detected both by generation of a p17 caspase-3 activation fragment (Figs. 1B and 2B) and by cleavage of a synthetic tetrapeptide substrate, DEVD-AMC (Figs. 1C and 2C). Furthermore, the decline in c-Fos preceded apoptotic nuclear chromatin condensation detected by fluorescence microscopy (Figs. 1D and 2D). Consistent with its known antiapoptotic action, Bcl-2 inhibited both caspase-3 activation (Figs. 1, B and C) and nuclear chromatin condensation (Figs. 1D and 2D). Thus, both TG and DX induced a marked decline in the level of c-Fos, detected during the early phase of apoptosis and inhibited by Bcl-2.

To confirm that the decline in c-Fos induced by TG was due to accelerated degradation and to identify the responsible protease, WEHI7.2 cells were treated with protease inhibitors
were prepared from WEHI7.2 cells (−Bcl-2) and W.Hb12 cells (+Bcl-2) at time intervals after adding 1 μM DX and Western blot analysis was done with anti-c-Fos antibody. B, Western blot of caspase-3 activation fragment in protein extracts from cells treated with DX. Whole cell extracts were prepared from WEHI7.2 cells (−Bcl-2) and W.Hb12 cells (+Bcl-2) at time intervals after adding 1 μM DX and Western blot analysis was done with an antibody that recognizes the 17-kDa activation fragments of caspase-3 but not the intact proenzyme. C, Fluorogenic substrate assay measuring caspase-3-mediated cleavage of the synthetic peptide substrate DEVD-AMC. Protein extracts were prepared from WEHI7.2 cells (−Bcl-2) and W.Hb12 cells (+Bcl-2) at time intervals after adding 1 μM DX. The data represent the means ± S.E. of three separate experiments. D, Summary of morphological assays for apoptosis in DX-treated cells. WEHI7.2 cells (−Bcl-2) and W.Hb12 cells (+Bcl-2) were stained with acridine orange and ethidium bromide at time intervals after adding 1 μM DX. The percentage of cells displaying apoptotic nuclear chromatin condensation is shown as the mean ± S.E. of three experiments.

Fig. 3. Inhibition of c-Fos degradation by proteasome inhibitors. A, WEHI7.2 cells were pretreated with protease inhibitors for 60 min and then incubated for another 4 h with or without 100 nM TG. Western blot analysis was done with anti-c-Fos antibody. B, WEHI7.2 cells were treated with or without 1 μM DX for 6 h and then incubated with protease inhibitors for another 6 h. Western blot analysis was done with anti-c-Fos antibody.

before adding TG (Fig. 3A). The decline in c-Fos and the appearance of the 46-kDa c-Fos fragment were inhibited by protease inhibitors with the specific proteasome inhibitors, lactacystin and MG132 (N-carboxybenzoyl-L-leucinyl-L-leucinyl) (34, 35) and also by the less specific proteasome inhibitor, ALLN (36).

ALLN also exhibits significant inhibitory activity for the cysteine protease calpain (36). Although c-Fos is subject to degradation by calpain (37), it is unlikely that calpain played a significant role, because c-Fos degradation was not prevented by the calpain inhibitors, ALLN and E64d (36) (Fig. 3A). Pretreatment with benzylxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethylketone, a cell permeant inhibitor of the caspase family of cysteine proteases (38), did not inhibit c-Fos degradation (Fig. 3A), indicating that proteasome-mediated degradation of c-Fos is not secondary to caspase activation.

The decline in c-Fos observed in DX-treated WEHI7.2 cells was also due to proteasome-mediated degradation, based on evidence that c-Fos was stabilized by pretreatment with the proteasome inhibitors lactacystin, MG132, and ALLN (Fig. 3B) and not by E64d (not shown). In DX-treated WEHI7.2 cells, pretreatment with proteasome inhibitors stabilized 46–48 kDa degradation intermediates as well as intact c-Fos (Fig. 3B). Also, several higher molecular mass forms were detected in DX-treated cells in the presence of proteasome inhibitors (Fig. 3B). The appearance of both degradation intermediates and presumably polyubiquitinated high molecular weight forms in the presence of proteasome inhibitors has been reported previously in the case of other proteasome target proteins (39, 40).

In view of the preceding findings suggesting a correlation between proteasome-mediated c-Fos degradation and apoptosis in WEHI7.2 cells, we wished to test whether c-Fos degradation by the proteasome contributes to apoptosis induction. It was not possible to answer this question with the use of proteasome inhibitors, because treatment of WEHI7.2 cells with lactacystin and MG132 induces apoptosis. Therefore, as an alternative approach, WEHI7.2 cells were stably transfected with either a cDNA encoding wild type c-Fos or a cDNA encoding a truncated version of c-Fos, c-FosΔC, missing 21 C-terminal amino acids. This strategy was based on evidence that deletion of the C-terminal sequence stabilizes c-Fos by reducing proteasome-mediated degradation (23). Wild type c-Fos was still degraded following TG treatment, even when expressed at levels 2–4-fold higher than the endogenous level (Fig. 4A), and therefore did not provide protection against apoptosis (Fig. 4C). In contrast, c-FosΔC was stable for 16–20 h after TG treatment (Fig. 4A) or DX treatment (Fig. 5A). In both situations, c-FosΔC expression inhibited the induction of both caspase-3 activation (Figs. 4B and 5B) and apoptotic nuclear chromatin condensation (Fig. 4C and 5C). The protective effect of c-FosΔC is not associated with alterations in the level of expression of endogenous Bcl-2 (data not shown). In summary, a mutant form of c-Fos that evades degradation by the proteasome inhibits apoptosis induction by both TG and DX. Thus, it appears that inhibiting the degradation of c-Fos prevents apoptosis, suggesting that accelerated degradation of c-Fos contributes to the generation of apoptotic cell death in WEHI7.2 cells.

DISCUSSION

Our findings indicate for the first time that c-Fos degradation by the proteasome contributes to the execution of the apoptotic death program. It appears that c-Fos may confer protection against TG- and DX-induced apoptosis that is abrogated by proteasome-mediated degradation and preserved either by expressing a C-terminal truncated version of c-Fos that is not degraded by the proteasome or by overexpressing Bcl-2, which inhibits c-Fos degradation by the proteasome. The concept that c-Fos has an antiapoptotic action is consistent with other recent evidence that c-Fos mediates expression of genes that protect against apoptosis (41–43) and with evidence that

2. H. He and C. W. Distelhorst, unpublished data.
cells from c-fos−/− knockout mice are hypersensitive to apoptosis induction (42).

Earlier reports suggested that c-fos may be a mediator of apoptosis, a concept based on evidence that c-fos transcription increases in cells undergoing apoptosis (44–46) and overexpression of a c-Fos-estrogen receptor fusion protein induces apoptosis in colorectal cells (47). However, more recent findings in a c-fos−/− knockout mouse indicate that c-fos induction is not required for apoptosis (48). On the other hand, recent findings indicate that light-induced photoreceptor apoptosis is deficient in the c-fos−/− knockout mouse (49). However, as noted by Hafezi et al., it is unclear whether this is due to a requirement for c-Fos in mediating the cell death process or whether a deficiency of c-Fos throughout development alters the expression of genes required for apoptosis.

The findings of the current study suggest that the induction of c-fos transcription observed in cells undergoing apoptosis may be a stress response that is counterbalanced by proteasome-mediated degradation of c-Fos protein. Indeed, this appears to be the case in TG-treated cells where TG treatment induces c-fos transcription (50), but newly synthesized c-Fos is rapidly degraded by the proteasome, thus preventing an elevation of c-Fos protein. The observation that both TG and DX induce c-Fos degradation indicates that apoptotic signals with diverse mechanisms of action target c-Fos for degradation by the proteasome. It is possible that proteasome-mediated degradation of c-Fos may account for the previously described decline in activator protein-1 activity associated with glucocorticoid-induced apoptosis in thymocytes (51–53). Moreover, accelerated degradation of c-Fos has been described in nitric oxide-mediated neuronal cell death, although the role of the proteasome in mediating c-Fos degradation in this situation has not been tested (54).

The mechanism by which apoptotic signals target c-Fos for degradation by the proteasome is unknown. Polyubiquitination targets proteins for degradation by the proteasome (11–13). Ubiquitination of c-Fos has been detected both in vitro and in vivo (22, 23, 55). In experiments not shown, c-Fos immunoprecipitates from WEHI7.2 cells were analyzed for evidence of ubiquitination by Western blotting using an anti-ubiquitin antibody. Polyubiquitinated c-Fos was detected at similar levels in cell lysates from TG-treated and untreated WEHI7.2 cells and whether or not Bcl-2 was overexpressed. There are at least two alternative explanations for these preliminary observations. First, TG treatment may have increased the ubiquitination of c-Fos, but rapid degradation of the newly ubiquitinated c-Fos may have prevented its detection in immunoprecipitates. Second, there is evidence that c-Fos may be degraded by the proteasome independent of ubiquitination (56); hence, TG may have induced ubiquitin-independent degradation of c-Fos by the proteasome.

Apoptotic signals may generate calcium fluxes, either by increasing the uptake of extracellular calcium or by releasing calcium from intracellular stores. This concept is based in part on the suspected role of calcium in mediating TG- and DX-induced apoptosis, as well as nitric oxide-induced apoptosis (32, 54, 57–59). Calcium fluxes might activate kinases or phosphatases that in turn alter the phosphorylation status of c-Fos, thereby targeting c-Fos for destruction by the proteasome. Alternatively, calcium fluxes may activate the proteasome, a concept that is based on evidence that calcium induces assembly of the 26 S proteasome from the 20 S proteasome (60, 61) and regulates proteasome activity in vivo (62).

c-Fos normally has a very short half-life, an attribute that
c-Fos Degradation by the Proteasome in Apoptosis

25019

 enables c-Fos mediated signals to be switched off rapidly. However, abnormally prolonged c-Fos expression leads to cellular transformation (24, 25, 28, 29, 63, 64) and is associated with increased expression of genes involved in tumor invasion and metastasis (26, 29, 30). In the present study, we find that Bcl-2 stabilizes c-Fos against proteasome-mediated degradation and that cells overexpressing Bcl-2 have a higher constitutive level of c-Fos than wild type cells. Thus, the possibility needs to be considered in future studies that by inhibiting proteasome-mediated c-Fos degradation, Bcl-2 may contribute to cellular transformation and tumor progression.

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