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Induction of apoptosis by the transcription factor c-Jun

Ella Bossy-Wetzel[1,2], Latifa Bakiri and Moshe Yaniv[2]

Unité des Virus Oncogenes, URA 1644 du CNRS, Departement des Biotechnologies, Institut Pasteur, 25 rue du Dr Roux, 75724 Paris cedex 15, France

[1]Present address: La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121, USA

[2]Corresponding authors

c-Jun, a signal-transducing transcription factor of the AP-1 family, normally implicated in cell cycle progression, differentiation and cell transformation, recently has also been linked to apoptosis. To explore further the functional roles of c-Jun, a conditional allele was generated by fusion of c-Jun with the hormone-binding domain of the human estrogen receptor (ER). Here we demonstrate that increased c-Jun activity is sufficient to trigger apoptotic cell death in NIH 3T3 fibroblasts. c-Jun-induced apoptosis is evident at high serum levels, but is enhanced further in factor-deprived fibroblasts. Furthermore, apoptosis by c-Jun is not accompanied by an increase in DNA synthesis. Constitutive overexpression of the apoptosis inhibitor protein Bcl-2 delays the c-Jun-mediated cell death. The regions of c-Jun necessary for apoptosis induction include the amino-terminal transactivation and the carboxy-terminal leucine zipper domain, suggesting that c-Jun may activate cell death by acting as a transcriptional regulator. We further show that α-fodrin, a substrate of the interleukin 1β-converting enzyme (ICE) and CED-3 family of cysteine proteases, becomes proteolytically cleaved in cells undergoing cell death by increased c-Jun activity. Moreover, cell-permeable irreversible peptide inhibitors of the ICE/CED-3 family of cysteine proteases prevented the cell death.

Keywords: AP-1/apoptosis/*bcl-2*/c-*jun*/ICE/CED-3-related cysteine proteases

Introduction

Apoptosis is a form of regulated cell death to eliminate unwanted or superfluous cells from the organism (Ellis *et al.*, 1991). It plays an indispensable role in embryogenesis, in adult tissue homeostasis, in the regulation of the immune system and in the development of the nervous system, but can also contribute to the pathogenesis of a number of human diseases when deregulated (reviewed by Wyllie *et al.*, 1980; Ellis *et al.*, 1991; Cohen *et al.*, 1992; Raff, 1992; Raff *et al*., 1993; Schwartz and Osborne, 1993; Steller, 1995; Thompson, 1995). Cells undergoing apoptosis display a series of morphological characteristics including cytoplasmic shrinkage, chromatin condensation, membrane blebbing, DNA fragmentation and generation of apoptotic bodies that are rapidly phagocytosed and digested by neighboring cells (Kerr *et al.*, 1972). Although, the morphological changes of apoptosis are well defined, the genes regulating and executing the process are just beginning to be identified and are currently an area of intense investigations.

Apoptosis depends in some cases on *de novo* RNA and protein synthesis (reviewed by Ellis *et al.*, 1991; Freeman *et al.*, 1993; Martin, 1993). Examples are apoptosis in some neurons (Martin *et al.*, 1988; Oppenheim *et al.*, 1990; Scott and Davies, 1990; D'Mello *et al.*, 1993; Milligan *et al.*, 1994), apoptosis in rodent thymocytes induced by ionizing radiation (Sellins and Cohen, 1987), apoptosis in T cell lines induced by interleukin-2 (IL-2) withdrawal (Duke and Cohen, 1986) and activation-induced apoptosis in T cell hybridomas (Shi *et al.*, 1990; Schwartz and Osborne, 1993). This has led to the concept that apoptosis is an active, gene-directed process that requires, in some instances, the induction of genes in order to proceed. Indeed, a number of proteins with properties of transcription factors have been identified that can trigger apoptotic cell death. For example, c-Myc, a transcription factor implicated in the control of cell proliferation, can also induce apoptosis in growth-arrested fibroblasts (Eilers *et al.*, 1989; Evan *et al.*, 1992). In addition, antisense oligonucleotides corresponding to c-*myc* can block activation-induced apoptosis of T cell hybridomas, indicating that c-*myc* expression is required for the cell death in this situation (Shi *et al.*, 1992). Furthermore, the p53 tumor suppressor gene has been documented to induce either growth arrest or apoptosis when expressed in certain tumor cell lines (Yonish-Rouach *et al.*, 1991; El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Desaintes *et al*., 1996; Ko and Prives, 1996; Lassus *et al.*, 1996). Experiments with p53 knockout mice demonstrated that p53 is essential for apoptosis in response to DNA damage in the skin (Ziegler *et al.*, 1994), in thymocytes (Clarke *et al.*, 1993; Lowe *et al.*, 1993) and in the intestinal epithelium (Clarke *et al.*, 1994; Merritt *et al.*, 1994). Furthermore, evidence was provided indicating that p53 may function as a transcription factor to promote apoptosis (Miyashita and Reed, 1995). In addition, Nurr 77, a zinc finger transcription factor and immediate early gene, is induced and necessary in activation-induced T cell apoptosis (Liu *et al.*, 1994; Woronicz *et al.*, 1994). Moreover, apoptosis can be elicited by the transcription factor E2F (Qin *et al.*, 1994; Shan *et al.*, 1994; Wu and Levine, 1994; Field *et al.*, 1996), by NF-M (Müller *et al.*, 1995) and by c-Rel (Abbadie *et al.*, 1993).

c-Jun is, together with the Fos family of proteins, a major component of the AP-1 transcriptional complex (reviewed by Vogt and Bos, 1990). c-*jun* was identified originally by its homology to v-*jun*, the oncogene captured

in the avian sarcoma virus ASV17 (Maki *et al.*, 1987). Two other c-*jun*-related genes were isolated based on sequence similarity, *junB* and *junD* (Ryder *et al.*, 1989; Hirai *et al.*, 1989). Jun-related protein products can function either as homodimers or as heterodimers bound to partner proteins such as Fos-related proteins, CREB or ATF-2 (Hai and Curran, 1991; van Dam *et al.*, 1993). The dimerization is mediated by a carboxy-terminal coiled-coil structure, termed the leucine zipper, and is necessary for DNA binding to a palindromic sequence known as the TPA-responsive element (TRE) or AP-1 consensus site, contained in many gene enhancers (reviewed in Vogt and Bos, 1990; Angel and Karin, 1991). Thus the different Jun homodimers and Jun heterodimers create a large number of dimer combinations that exhibit distinct transcriptional properties with diverse biological consequences (see, for example, Ryseck and Bravo, 1991; Bossy-Wetzel *et al.*, 1992; Schlingensiepen *et al.*, 1993; Pfarr *et al.*, 1994).

The AP-1 transcriptional complex has been implicated in a number of biological processes (reviewed in Vogt and Bos, 1990; Angel and Karin, 1991). Studies on the c-*jun* expression indicated that it is rapidly and transiently induced, as part of the immediate early growth response when quiescent fibroblasts are stimulated with mitogens, indicating that c-*jun* may have a function in controlling cell proliferation (Angel *et al.*, 1987; Bohmann *et al.*, 1987; Lee *et al.*, 1987; Ryseck *et al.*, 1988; Cater *et al.*, 1991). Support for a role for AP-1 in cell proliferation is provided by experiments where microinjected neutralizing anti-c-Jun and anti-c-Fos antibodies were able to prevent the cell cycle progression of serum-treated fibroblasts (Kovary *et al.*, 1991). In addition, cells lacking a functional c-*jun* allele show retarded growth *in vitro* and *in vivo* (Hilberg and Wagner, 1992; Hilberg *et al.*, 1993; Johnson *et al.*, 1993). However, strong and prolonged induction of c-*jun* has also been reported in response to a variety of stress-inducing stimuli including UV and ionizing irradiation, hydrogen peroxide and tumor necrosis factor (TNF) α, all treatments that can trigger apoptosis (Brenner *et al.*, 1989; Devary *et al.*, 1991; Manome *et al.*, 1993; Davis, 1994). Furthermore, c-*jun* has been implicated in cellular transformation by virtue of its ability to cooperate either with oncogenes such as an activated *ras* gene to transform rat embryo fibroblasts or to transform Rat-1a cells or chick embryonic fibroblasts as a single gene (Schütte *et al.*, 1989a,b; Bos *et al.*, 1990; Castellazzi *et al.*, 1990). In addition, activated Ras can lead to an increase in c-Jun transcriptional activity by phosphorylation of its transactivation domain (Binétruy *et al.*, 1991; Smeal *et al.*, 1991).

Although c-Jun activity is induced by a large variety of external or internal signals, known to exert effects on cell proliferation, differentiation, cellular transformation and apoptosis, what the precise role of c-Jun is in these processes remains unclear. For this reason, we generated a conditional allele for c-Jun, whose activity is dependent upon the availability of exogenous β-estradiol. We find that increased c-Jun activity transmits an apoptotic death signal in immortalized NIH 3T3 fibroblasts. Apoptosis by c-Jun is not associated with cell cycle progression, indicating that the role of c-Jun in apoptosis might be distinct from its function in cell proliferation.

Results

Activation of the c-Jun–ER protein results in a marked decrease in cell numbers

To investigate the functional properties of c-Jun, a conditional allele was generated, in which the full-length mouse c-*jun* cDNA was fused at the 3′ end with the hormone-binding domain of the human estrogen receptor (ER) and cloned into a retroviral vector, containing the *neo* resistance gene as a selectable marker. The principle of this inducible system is that, although the chimeric protein is constitutively expressed, it is in an inactive state in the absence of hormone, but can be activated by addition of exogenous β-estradiol (for review, see Picard, 1994). NIH 3T3 immortalized fibroblasts were transfected with the expression vector containing the c-*jun–ER* fusion gene, and several independent clones with stable expression of the c-Jun–ER fusion protein were isolated and used for further characterization.

A number of reports have suggested that c-*jun* may induce cell proliferation (Ryseck *et al.*, 1988; Castellazzi *et al.*, 1990; Cater *et al.*, 1991; Kovary and Bravo, 1991; Hilberg and Wagner, 1992; Johnson *et al.*, 1993; Pfarr *et al.*, 1994). For this reason, we examined the effect of activation of the c-Jun–ER protein by β-estradiol on the growth of NIH 3T3 fibroblasts. Control cells (neo-resistant NIH 3T3 cells, transfected with the empty expression vector) and two independent clones, expressing different steady-state levels of the c-Jun–ER chimeric protein (C3 and C19), were seeded at low densities in medium containing 10% calf serum and in the presence or absence of exogenous β-estradiol, and the cell numbers were determined daily. The growth curves of β-estradiol-treated or untreated control fibroblasts were virtually identical, demonstrating that β-estradiol had no effect on the growth rate of these cells (Figure 1A). This was in marked contrast to NIH 3T3 cells expressing the c-Jun–ER protein. Activation of the c-Jun–ER protein by β-estradiol resulted in a substantial decrease in cell numbers (Figure 1A).

The extent of decrease in cell numbers was dependent upon the expression levels of the c-Jun–ER protein present in these cells (Figure 1B). Clone C19, expressing moderate levels of the c-Jun–ER protein, revealed a relatively mild effect on reducing cell numbers after β-estradiol treatment (Figure 1A and B). In contrast, clone C3, with higher expression levels of the c-Jun–ER protein, exhibited a strong decrease in cell numbers in the presence of β-estradiol (Figure 1A and B). Furthermore, this also correlated with the ability of the two c-Jun–ER-expressing clones to activate transcription from a reporter plasmid containing an AP-1-binding site in a hormone-dependent manner. The collagenase gene promoter was stimulated only 2.4-fold in clone C19, whereas it was activated up to 7-fold in clone C3, upon activation of the c-Jun–ER protein by β-estradiol (Figure 1C). Because the ER domain of the chimeric construct contains a TAF-2 transactivation region, we were concerned that this activity could have contributed to the observed effects on growth and transactivation. For this reason, we tested the effects of the estradiol antagonist 4-hydroxytamoxifen (4-HT) that permits DNA binding of the ER but fails to activate TAF-2-dependent transactivation (Berry *et al.*, 1990). 4-HT mimicked the effects of β-estradiol on growth and trans-

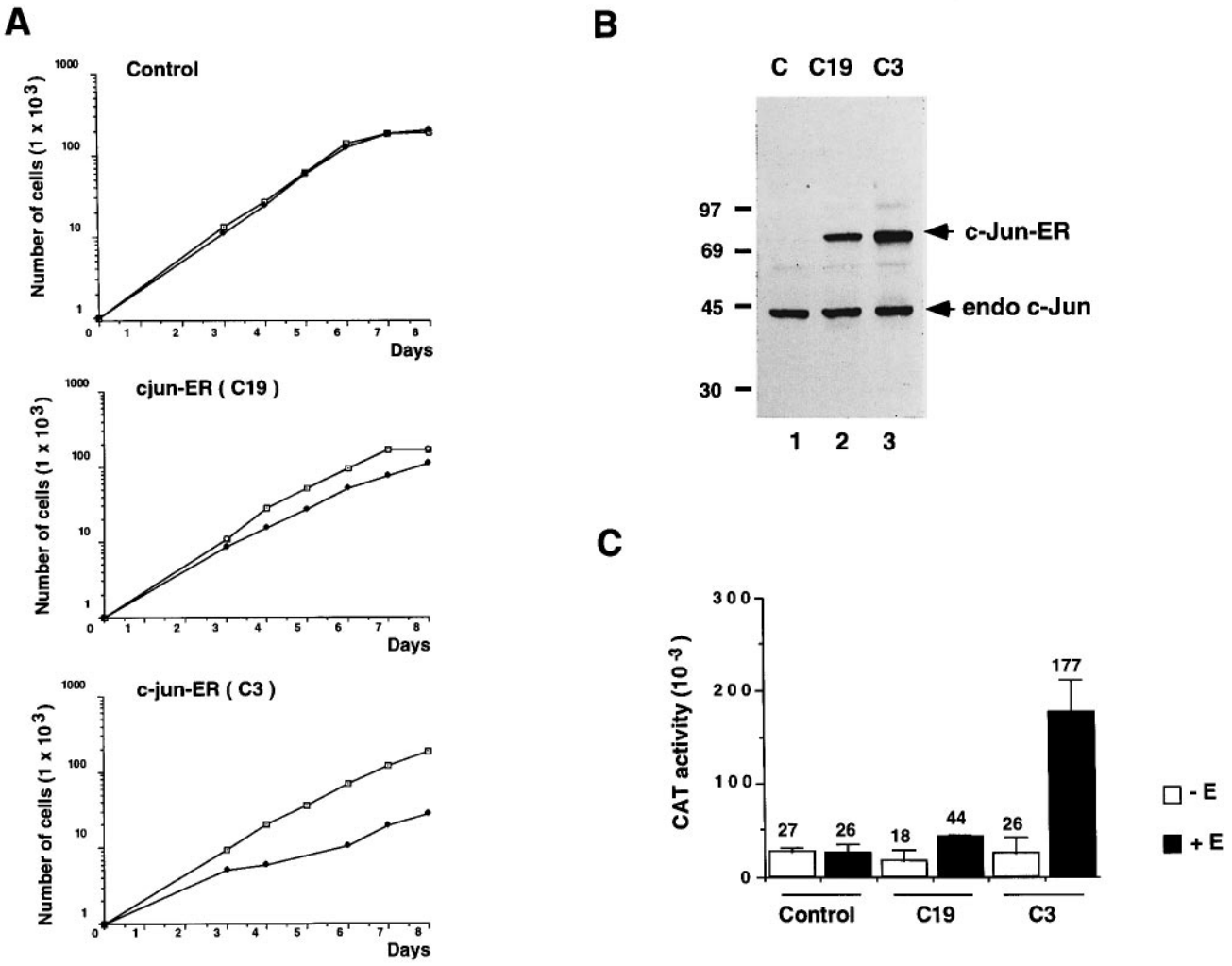

Fig. 1. (**A**) Effect of the activation of the c-Jun–ER protein by β-estradiol on the growth of NIH 3T3 cells. Triplicate cultures of NIH 3T3 control or NIH 3T3/c-jun–ER cells (clone C19 and clone C3) were cultured in medium containing 10% calf serum in the presence or absence of β-estradiol (2 mM), and live cells excluding trypan blue were counted at daily intervals. The mean values of triplicate cultures are shown plotted against time. The open squares represent the values for untreated cultures, and closed diamonds are the values for β-estradiol-treated cultures. (**B**) Immunoblotting analysis of the c-Jun proteins. Total cell extracts from control NIH 3T3 cells (lane 1), NIH 3T3/c-jun–ER cells, clone C19 (lane 2) and clone C3 (lane 3) were separated by SDS–PAGE, transferred to nitrocellulose filters and probed with affinity-purified anti-c-Jun polyclonal antibodies (Pfarr *et al.*, 1994). c-Jun-specific protein bands were visualized by the ECL chemiluminescence reagent (Amersham). Arrows indicate the endogenous c-Jun protein and the ectopically expressed c-Jun–ER fusion protein of ~80 kDa size. (**C**) The c-Jun–ER protein activates transcription of a transiently transfected CAT reporter plasmid, containing the AP-1-responsive collagenase I gene promoter, in a hormone-dependent fashion. NIH 3T3 fibroblasts and c-jun–ER cells (clones C19 and C3) were transiently transfected with the plasmid Coll(-514)-CAT. Following transfection, plates were left either untreated or were treated with 2 μM β-estradiol. After 24 h, cell extracts were prepared and assayed for CAT activity. The values and the standard deviation are based on three independent experiments.

activation (data not shown), thus arguing that the observed effects are due to the c-Jun portion and not to the ER domain of the fusion protein.

c-Jun signals apoptotic cell death

At least two possible explanations could account for the decrease in cell numbers upon activation of the c-Jun–ER protein. One possibility may be that increased c-Jun activity results in growth inhibition. Alternatively, c-Jun may trigger apoptotic cell death in these cells. To explore these possibilities further, the cell morphology of untreated and β-estradiol-treated fibroblasts was examined in high and low serum. Control NIH 3T3 fibroblasts grew as flat and normal looking monolayers, irrespective of whether they were cultured in the presence or absence of hormone (Figure 2a, c, e and g). Similarly, untreated c-jun–ER cells displayed a normal morphology in medium containing 10% calf serum (Figure 2b). By contrast, c-Jun–ER activation resulted in morphological changes reminiscent of the phenotype that has been described for apoptotic cells (Figure 2d). Hormone treatment of c-jun–ER cells revealed not only fewer cell numbers as shown in Figure 1A, but also many cells with phase bright cell bodies, shrunken cytoplasm and condensed chromatin (Figure 2d). In addition, fragmented cells were visible, suggestive of apoptotic bodies (Figure 2d). In low serum, activation of the c-Jun–ER protein by β-estradiol resulted in an even more substantial change in cell morphology (Figure 2h). Many cells lost their adhesion to the matrix, and cells with a refractile look formed aggregates

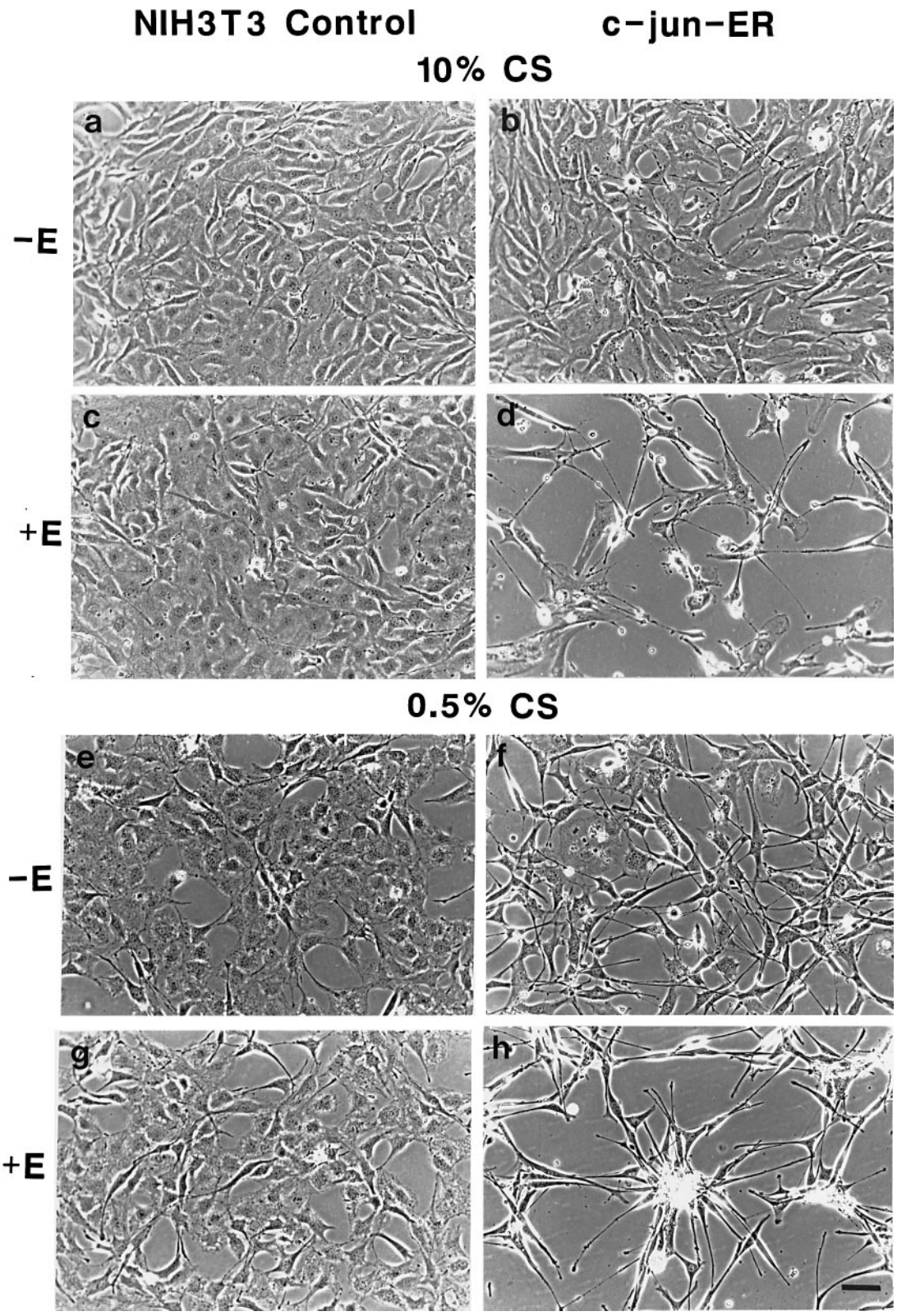

Fig. 2. Changes in cell morphology induced by activation of the c-Jun–ER protein. Control cells and c-jun–ER cells (clone C3) were seeded in DMEM plus 10 or 0.5% calf serum and in the absence (–E) or presence (+E) of β-estradiol (2 mM). Photographs were taken under phase contrast from cells cultured in 10% serum after 6 days and from cells cultured in 0.5% serum after 1 day. Bar, 50 μm.

(Figure 2h). It is of note that in low serum even untreated c-jun–ER cells appeared less healthy looking (Figure 2f). A likely explanation for this observation may be leakiness of the c-Jun–ER fusion protein.

To investigate further whether increased c-Jun activity may induce apoptosis, the occurrence of DNA fragmentation was evaluated by the TUNEL technique (Gavrieli *et al.*, 1992). Only a few TUNEL-labeled cells could be

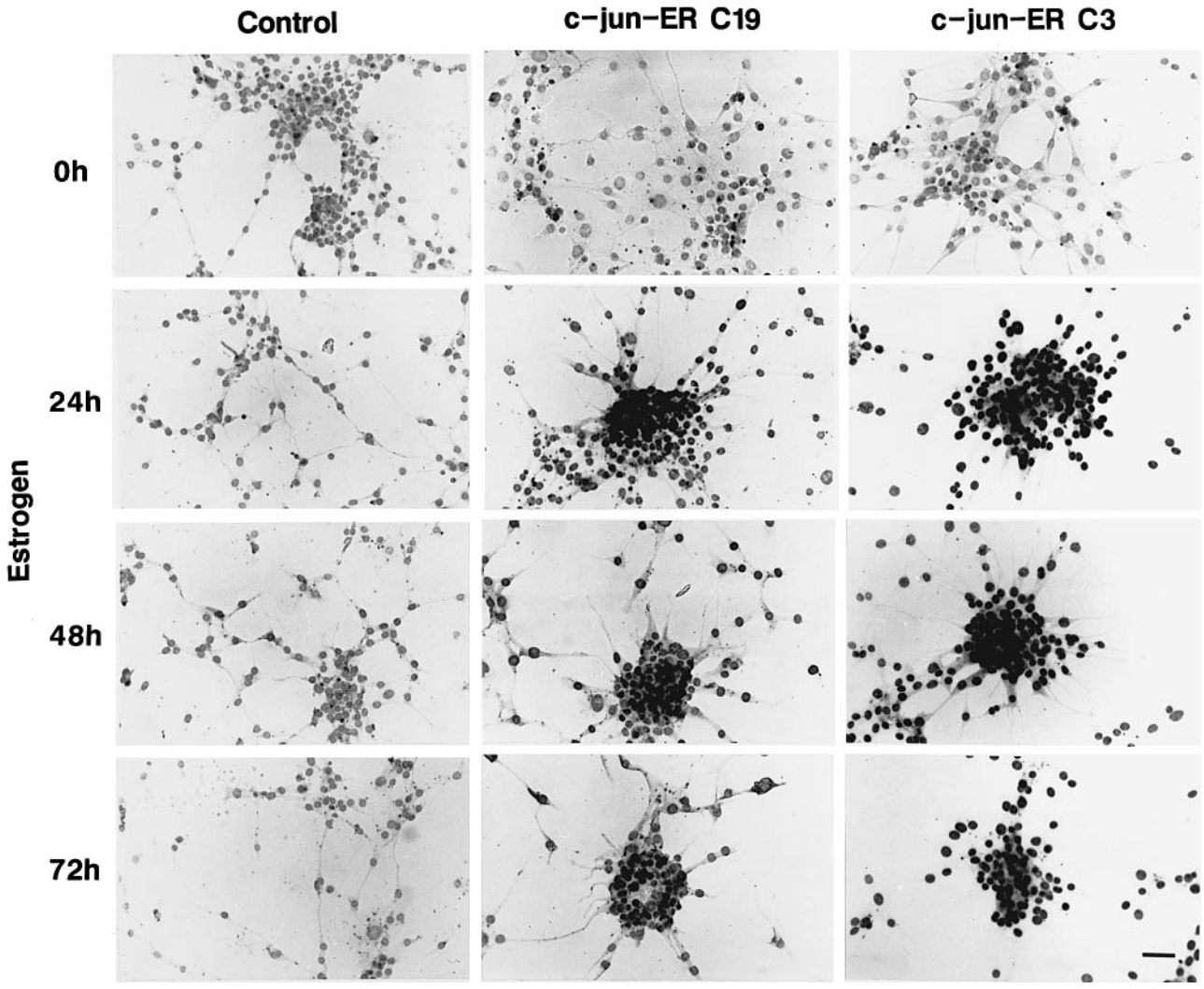

Fig. 3. Assessment of DNA breaks using the TUNEL assay. For control cells, only a very few TUNEL-positive cells are visible, even after 72 h of treatment with β-estradiol. By contrast, extensive TUNEL labeling is seen for c-jun–ER cells, already after 24 h of treatment with β-estradiol and in 0.75% calf serum, as visualized by the black nuclear staining. Bar, 50 μm.

seen for NIH 3T3 control cells, even after 72 h of treatment with β-estradiol (Figure 3). In contrast, extensive DNA fragmentation was observed for hormone-treated c-jun–ER cells after 24 h, visualized by the black nuclear staining (Figure 3). In particular, cells participating in aggregates exhibited strong DNA fragmentation. Clone C19, which expresses lower levels of the c-Jun–ER protein than clone C3, revealed fewer TUNEL-positive cells in the presence of hormone, indicating that the extent of DNA fragmentation is dependent upon the amount of c-Jun–ER protein expressed in these cells (Figure 3).

Because apoptosis frequently is accompanied by DNA cleavage at internucleosomal sites, DNA was isolated from hormone-treated and untreated c-jun–ER cells and analyzed by gel electrophoresis. Figure 4 demonstrates that DNA extracted from untreated c-jun–ER cells did not show any DNA laddering, whereas typical oligonucleosomal DNA laddering, characteristic of apoptotic cells, was evident after 48 and 72 h of c-Jun–ER activation by β-estradiol.

Collectively, these data indicate that c-Jun induces apoptosis in NIH 3T3 fibroblasts. Apoptosis was clearly evident in serum-rich medium, but was accelerated further in growth factor-deprived fibroblasts, when inspected microscopically. It is of note that some cell clones that initially underwent apoptosis upon activation of the c-Jun–ER protein became resistant to cell death on continued culture. However, when we again tested the expression of the c-Jun–ER protein, it was found to be no longer expressed (data not shown). Hence, these results indicate that apoptosis is dependent upon the expression of the c-Jun–ER protein.

c-Jun-induced apoptosis is delayed by Bcl-2 expression

The *bcl-2* proto-oncogene increases cell survival and blocks apoptosis in many systems (reviewed by Korsmeyer, 1992; Reed, 1995). To investigate the effect of *bcl-2* expression on the c-Jun-mediated apoptosis, c-jun–ER fibroblasts (clone C3) were transfected with an expression vector containing the human *bcl-2* gene together with a hygromycin-resistant marker. Several hygromycin-resistant colonies were identified by immunoblotting with abundant expression levels of the human Bcl-2 protein and were used for further analysis (Figure 5A). To test the effect of Bcl-2 expression on the

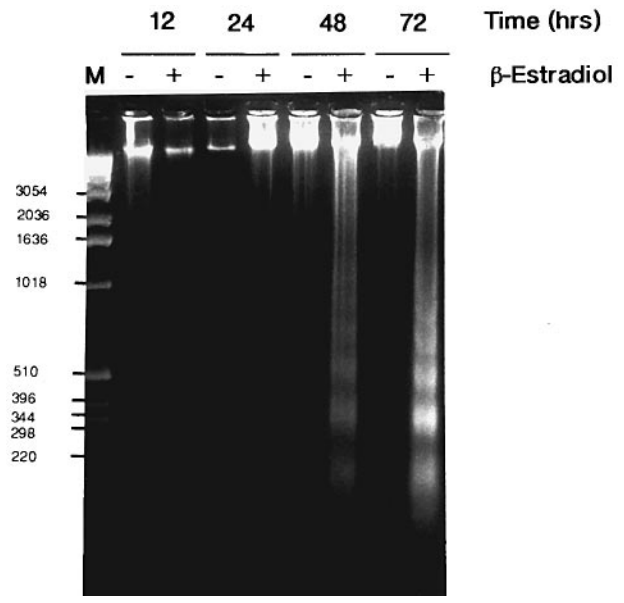

Fig. 4. Internucleosomal DNA cleavage after activation of the c-Jun–ER protein. NIH 3T3/c-jun–ER cells (clone C3) were cultured in DMEM, 0.5% calf serum with or without β-estradiol for the indicated periods of time. DNA was separated by agarose gel electrophoresis. DNA fragmentation into multimers of 200 bp is seen only in DNA samples from hormone-treated cells, but not in samples of untreated cells, after 48 and 72 h.

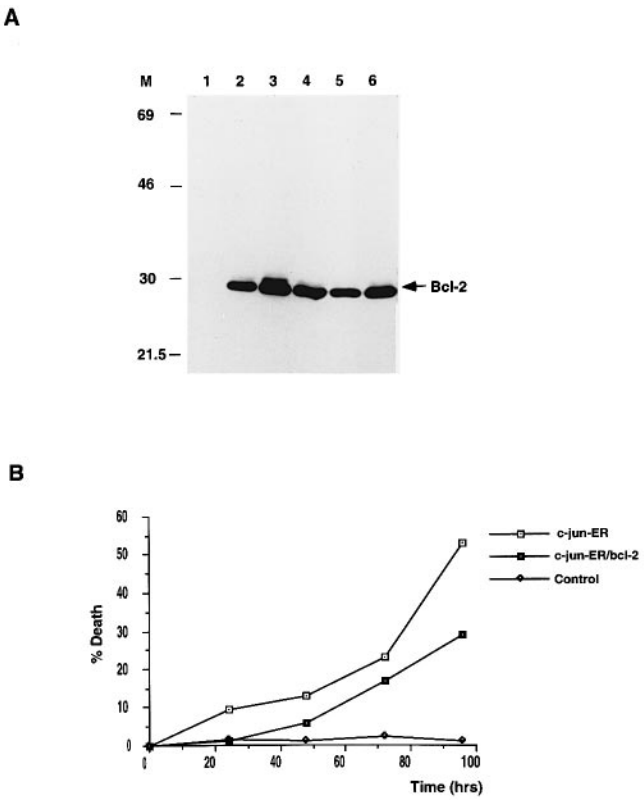

Fig. 5. Effect of Bcl-2 overexpression on the cell death induced by c-Jun. (**A**) Detection of Bcl-2 protein by immunoblotting analysis. Cytoplasmic extracts from NIH 3T3/c-jun–ER fibroblasts (lane 1) and NIH 3T3/c-jun–ER/bcl-2 clones, bulk cell population (lane 2); clone 4 (lane 3); clone 15 (lane 4); clone 18 (lane 5); clone 19 (lane 6); were separated by SDS–PAGE, transferred to nitrocellulose filters and probed with polyclonal antibodies against human Bcl-2 protein (DAKO; 1:60). Bcl-2-specific protein bands were visualized by the ECL chemiluminescence reagent (Amersham). (**B**) Constitutive Bcl-2 expression attenuates the c-Jun-induced apoptosis in serum-deprived NIH 3T3/c-jun–ER fibroblasts. Cells were seeded in triplicate into medium containing 0.75% calf serum and with or without 2 mM β-estradiol. Live cell numbers were determined after the indicated periods of time by the MTT assay. The c-jun–ER/bcl-2 data represent the average of five independent NIH 3T3/c-jun–ER/bcl-2 cell clones with relatively high expression levels of the transfected human *bcl-2* gene as presented in (A).

cell viability, control NIH 3T3, c-jun–ER and c-jun–ER/bcl-2 cells were plated in low serum in the presence or absence of β-estradiol and the numbers of live cells were determined by the MTT assay at daily intervals. As presented in Figure 5B, 50% of the cells expressing the c-Jun–ER protein alone died under these conditions after 96 h in the presence of hormone. In contrast, only 27% of the cells with co-expression of Bcl-2 plus c-Jun–ER underwent apoptosis when treated similarly. No significant loss in cell viability was noticed for control cells under the same conditions (Figure 5B). Hence, these data indicate that constitutive expression of Bcl-2 results in a considerable attenuation of the c-Jun-mediated apoptosis in serum-deprived NIH 3T3 fibroblasts.

Apoptosis by c-Jun is not linked to cell proliferation

A number of reports have suggested that c-*jun* may play a role in cell cycle progression (Ryseck *et al.*, 1988; Cater *et al.*, 1991; Kovary and Bravo, 1991; Johnson *et al.*, 1993; Pfarr *et al.*, 1994). We therefore considered the possibility that the c-Jun-activated apoptosis may be coupled to an increase in DNA synthesis, as has been documented for the c-Myc- or E2F-mediated apoptosis in fibroblasts (Eilers *et al.*, 1991; Evan *et al.*, 1992; Qin *et al.*, 1994; Shan and Lee, 1994; Wu and Levine, 1994). To explore this issue further, the distribution of cell cycle phases was determined by flow cytometry. Unexpectedly, the activation of the c-Jun–ER protein by β-estradiol had no effect on the cell cycle profile of NIH 3T3 fibroblasts. The number of cells in the G_0/G_1, the S or the G_2/M phase of the cell cycle was very similar for β-estradiol-treated or untreated c-jun–ER fibroblasts (Figure 6A and Table I). Instead of changes in the cell cycle phases, c-Jun activation resulted in an increase in cells with less than diploid DNA content, representative of apoptotic cells (Figure 6A and Table I). The fraction of apoptotic cells was ~25% at high serum levels, whereas it was increased further up to 40% in low serum, confirming our initial observation that the c-Jun-mediated cell death is more profound for factor-deprived fibroblasts (Table I). It is of note that the percentage of apoptotic cells was higher for untreated c-jun–ER cells as compared with control cells (Table I). As mentioned above, a likely explanation for this finding may be the leakiness of the c-Jun–ER protein. Finally, these data suggest that the c-Jun-mediated apoptosis is not confined to a specific phase of the cell cycle.

To confirm that the c-Jun-induced apoptosis was not coupled to DNA synthesis, the [^{3}H]thymidine incorporation was analyzed. c-jun–ER cells (clone C3) were growth arrested by serum deprivation for 3 days and treated either with β-estradiol to activate the c-Jun–ER protein, or with serum, as a control. No appreciable increase in thymidine incorporation was measured after the activation of the

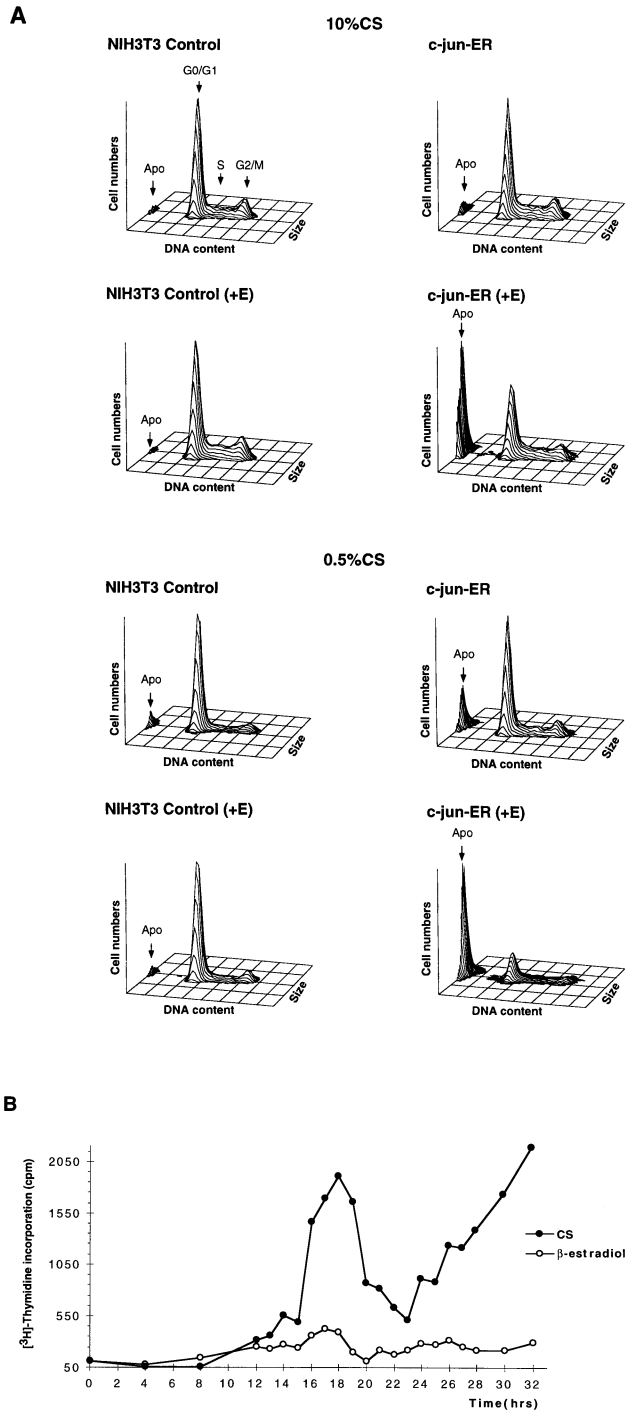

Fig. 6. (**A**) Flow cytometric analysis of control NIH 3T3 cells and NIH 3T3/c-jun–ER cells (clone C3). Cells were incubated for 48 h with or without β-estradiol and in medium containing 10 or 0.5% calf serum, stained with propidium iodide and analyzed by flow cytometry. The DNA content is presented as relative fluorescence. The apoptotic cell population is shown by the first peak (Apo), cells in G_0/G_1 are in the second peak and cells in G2/M phase are in the third peak. Cells in S phase are in the area between the G_0/G_1 and G2/M phase peak. A quantitative representation of the cell cycle distribution is demonstrated in Table I. (**B**) Activation of the c-Jun–ER protein does not induce DNA synthesis in quiescent NIH 3T3 fibroblasts. NIH 3T3/c-jun–ER fibroblasts (clone C3) were growth arrested by serum deprivation for 3 days and thereafter treated every hour either with β-estradiol (2 mM) or with calf serum (15%). One hour before cell harvest, cells were treated with a pulse of [^{3}H]thymidine and incorporated counts were determined. Each value represents the mean of triplicate measurements.

c-Jun–ER protein when tested over a period of 32 h (Figure 6B), yet apoptosis was clearly evident already after 24 h of β-estradiol treatment (Figures 2 and 3). That the growth-arrested c-jun–ER cells were not defective in their ability to synthesize DNA is shown by control treatment with serum (Figure 6B). Serum stimulation resulted in a 40-fold increase in [^{3}H]thymidine incorporation after 18 h, representing the peak in DNA synthesis. These results clearly demonstrate that c-Jun is insufficient to permit growth-arrested fibroblasts to transit through the cell cycle. Thus, the apoptotic function of c-Jun seems to be unrelated to its other functions such as the induction of cell proliferation.

Functional regions of c-Jun required for apoptosis

The biochemical and biological functions of c-Jun have been mapped to certain regions within the protein. These regions include the amino-terminal transactivation region, the carboxy-terminal basic DNA-binding region and the leucine zipper region, that is necessary for dimerization. To analyze which portions of the c-Jun protein are necessary for the induction of apoptosis, several c-*jun* mutant constructs were tested in a transient transfection assay for their ability to display morphological characteristics of apoptotic cell death. Transfected cells were identified by co-transfection with a plasmid containing the β-galactosidase gene (RSV-β-gal). After histochemical detection of the β-galactosidase activity, many of the blue cells transfected with the wild-type c-*jun* gene (RSV-c-jun) or a mutation in which the unconserved region of c-*jun* is deleted (RSV-c-junD131–220) exhibited shrunken cell size, condensed chromatin and cytoplasmic blebs (Figure 7b and c). By contrast, transfection with RSV-β-gal either alone (Figure 7a) or in combination with two dominant-negative c-*jun* mutants, lacking the transactivation domain (RSV-c-junΔ169; RSV-c-junΔ196) or with a leucine zipper deletant (RSV-c-junΔ238–311) revealed mainly normal and healthy looking β-gal-positive cells (Figure 7d, e and f).

A quantification of three independent transient transfection experiments is presented in Figure 8. Approximately 40% of the blue cells arising from co-transfection of c-*jun*, c-*jun–ER* or *c-junΔ131–220* (PMV-7-c-jun; PMV-7-c-jun–ER; RSVc-junΔ131–220) plus β-*gal* displayed an apoptotic cell morphology, whereas only 6–10% of the blue cells that had been transfected with β-*gal* alone or in combination with various c-*jun* mutants (RSV-c-junΔ169; RSV-c-junΔ196, RSV-c-junΔ238–311) exhibited morphological characteristics of apoptotic cell death. Thus, cell death induced by overexpression of c-*jun* seems to be dependent upon an amino-terminal region, containing the transactivation region and a carboxy-terminal region that includes the leucine zipper domain. Furthermore, these data demonstrate that the native c-*jun* gene and the c-*jun–ER* chimeric gene share similar functional properties.

Activation of ICE/CED-3-related cysteine proteases during c-Jun-induced apoptosis

There is increasing evidence indicating that the proteolytic cleavage of critical cellular substrates, by a family of cysteine proteases related to the interleukin 1β-converting enzyme (ICE) and the *Caenorhabditis elegans* CED-3 gene, plays a central role in the executionary phase of the

Table I. Cell cycle distribution of NIH 3T3/c-jun–ER cells

Cell line	Serum %	β-estradiol	Apo (%)	G_0/G_1 (%)	S (%)	G2/M (%)
Control	10	–	2.2 ± 1.1	54.3 ± 2.1	26.6 ± 3.5	18.9 ± 2.1
Control	10	+	1.2 ± 1.2	50.7 ± 1.8	31.8 ± 3.3	17.2 ± 2.1
c-jun–ER	10	–	8.2 ± 3.0	50.8 ± 1.8	28.7 ± 1.7	20.5 ± 1.2
c-jun–ER	10	+	29.7 ± 9.9	51.6 ± 2.4	29.6 ± 0.3	17.8 ± 2.7
Control	0.5	–	1.2 ± 1.3	82.1 ± 5.9	12.0 ± 3.8	5.1 ± 2.1
Control	0.5	+	0.6 ± 1.4	82.6 ± 6.7	12.6 ± 4.8	4.8 ± 2.4
c-jun–ER	0.5	–	10.6 ± 4.4	75.6 ± 6.3	15.8 ± 6.5	8.5 ± 1.5
c-jun–ER	0.5	+	40.2 ± 9.8	72.8 ± 10.6	17.9 ± 8.9	9.2 ± 2.8

Cell cycle distribution of cells after 48 h of treatment with β-estradiol in 10 and 0.5% calf serum. Values and standard deviations represent the results of three independent flow cytometry expertiments. Representative histograms are shown in Figure 6A.

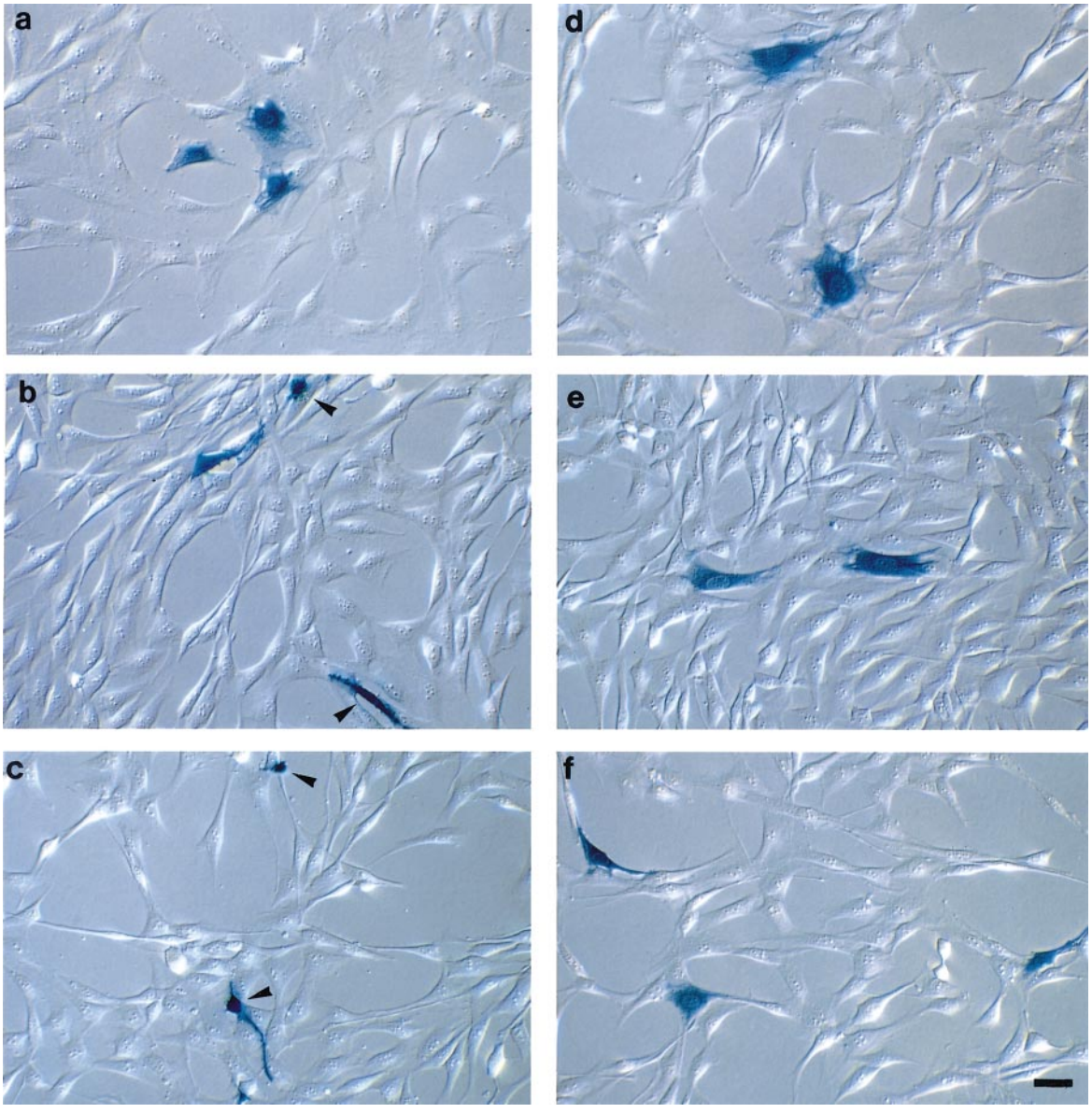

Fig. 7. Apoptotic changes in NIH 3T3 cells transiently transfected with c-*jun* or various c-*jun* mutants together with a plasmid expressing the bacterial β-galactosidase gene. After 24 h of transient transfection, cells were fixed and incubated with buffer containing X-gal to visualize β-galactosidase-positive cells. Representative images of transfections with (**a**) RSV-β-gal alone; co-transfections of RSV-β-gal with (**b**) RSV-c-jun; (**c**) RSV-c-junΔ131–220; (**d**) RSV-c-junΔ169; (**e**) RSV-c-junΔ196; (**f**) RSV-c-junΔ284–311; photographs were taken with a Zeiss Axiovert microscope and Hoffmann optics. Cells with apoptotic phenotype are indicated by the arrows. Bar, 50 μm.

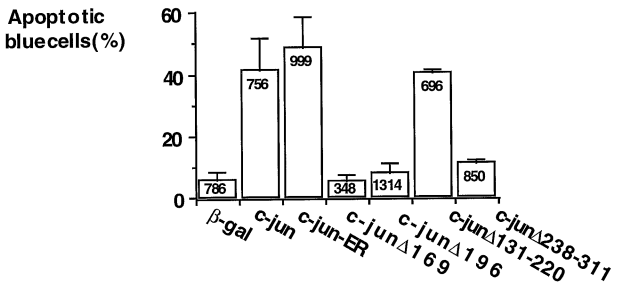

Fig. 8. Quantitative representation of c-*jun*-induced cell death in NIH 3T3 cells after transient transfection. Shown are the results of three independent transient transfection experiments. The data represent the percentage of blue cells with apoptotic cell phenotype relative to the total number of blue cells counted, which is given within each column.

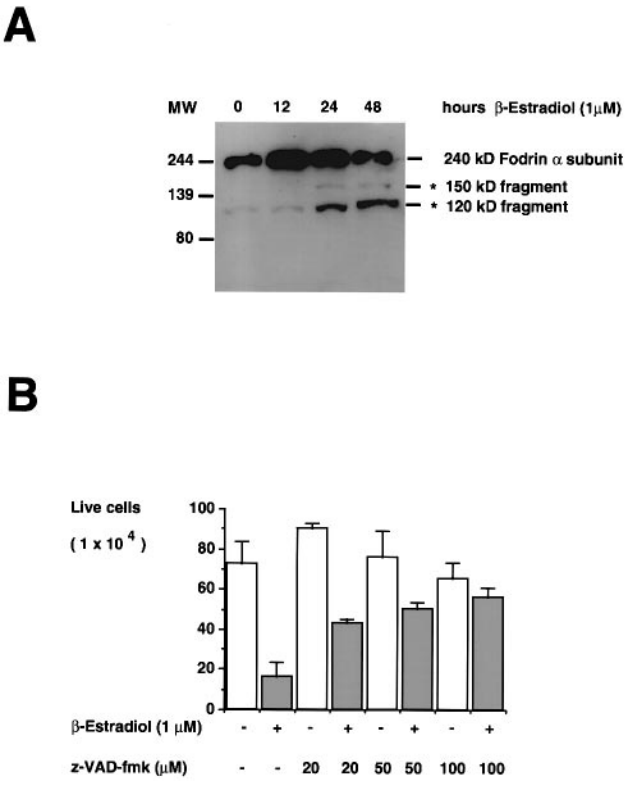

Fig. 9. (**A**) Fodrin cleavage during c-Jun-induced apoptosis. Cell lysates from untreated c-jun–ER and c-jun–ER cells cultured in the presence of β-estradiol were prepared as described in Materials and methods and subjected to electrophoresis in an 8% polyacrylamide gel followed by immunoblotting. The blot was probed with a monoclonal anti-fodrin antibody (Chemicon International, 1:2000). Proteolytic cleavage of the 240 kDa fodrin subunit into two fragments of 150 and 120 kDa (indicated by the asterisks) is evident after 24 and 48 h of c-Jun activation by β-estradiol. (**B**) Inhibition of c-Jun-mediated apoptosis by the peptide inhibitor of the ICE/CED-3 family of cysteine proteases, z-VAD-fmk. c-jun–ER cells were seeded into DMEM containing 0.5% fetal bovine serum and treated with the peptide inhibitor at the indicated concentrations. After 4 h of pre-treatment with the peptide inhibitor, β-estradiol was added to the cultures to activate the c-Jun–ER protein. Surviving cells were harvested by trypsinization after 68 h, and live cell numbers were determined by trypan blue exclusion. z-VAD-fmk increases considerably the survival of fibroblasts expressing the activated c-Jun–ER protein. The values of the standard deviation are based on triplicate cultures.

apoptotic process (reviewed by Martin and Green, 1995; Thornberry *et al.*, 1995; Whyte, 1996). This family of proteases cleave their target proteins after aspartic acid (Thornberry *et al.*, 1992; Nicholson *et al.*, 1995; Tewari *et al.*, 1995; Xue and Horwitz, 1995). During apoptosis, a discrete subset of cellular proteins become cleaved by the ICE/CED-3-related proteases, including poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), protein kinase Cδ (PKCδ), sterol regulatory element-binding protein SREBP-1 and SREBP-2, U1 small nuclear ribonucleoprotein (U1snRNP), α-fodrin, Gas2 and lamin A, B and B1 (reviewed by Whyte, 1996). To investigate whether c-Jun may induce a *bona fide* apoptotic program by activating members of the ICE-related proteases, we tested whether α-fodrin, a known substrate of the ICE-like proteases (Martin *et al.*, 1995), would be proteolytically cleaved in cells undergoing c-Jun-mediated cell death. Cell lysates from control untreated c-jun–ER cells and from c-jun–ER cells treated with β-estradiol were probed for α-fodrin cleavage by immunoblotting. As indicated in Figure 9A, α-fodrin cleavage was detected in lysates from β-estradiol-treated cells visualized by the appearance of two fragments of 150 and 120 kDa. By contrast, cell lysates from untreated cells revealed only a major band of 240 kDa corresponding to the intact α-fodrin protein. These data suggest that members of the ICE-like proteases are activated during the c-Jun-induced cell death.

We then tested whether the apoptosis by c-Jun could be inhibited by z-Val-Ala-Asp-fluoromethylketon (z-VAD-fmk), a cell-permeable, irreversible tripeptide inhibitor of the ICE/CED-3-related cysteine proteases. As shown in Figure 9B, z-VAD-fmk provides considerable protection against c-Jun-induced cell death starting at concentrations of 20 μM. Collectively, these data provide evidence that c-Jun induces a *bona fide* apoptotic program in fibroblasts that involves the activation of ICE/CED-3 cysteine proteases.

Discussion

The functional roles of the transcription factor c-Jun were evaluated using a conditionally active allele. Here we provide evidence that c-Jun can be a potent inducer of apoptotic cell death in immortalized NIH 3T3 fibroblasts. Five criteria were employed to demonstrate apoptosis, including cell morphology, TUNEL labeling, oligonucleosomal DNA laddering, flow cytometry and the activation of ICE/CED-3-related cysteine proteases. c-Jun-mediated apoptosis was clearly detectable in high serum, but was even more pronounced in low serum, indicating that serum may contain factors that can either abolish or delay the c-Jun-mediated apoptosis. Indeed, cytokines such as epidermal growth facor (EGF), insulin-like growth factor (IGF) I and IGFII were able to increase the cell viability of serum-deprived fibroblasts expressing the activated c-Jun protein (E.Bossy-Wetzel, unpublished data). It is notable that in low serum the dying cells adhered to each other and formed cell aggregates that are bound through living cells to the culture matrix (Figure 2). A possible explanation for this phenotype may be that live cells are recognizing the dying cells and trying to engulf them, but the cell death is so profound in low serum that the phagocytosis is incomplete. Consistent with this idea is the observation that these aggregates were not detectable

in high serum, where the cell death is reduced. Moreover, such an interpretation is in agreement with the finding that the size of the cell aggregates decreases over time.

There is precedent for a role of c-*jun* and c-*fos* in apoptosis, and previous studies have suggested that AP-1 may be an essential component of programed cell death in diverse cell types and provoked by different cell death-inducing stimuli. For example, when rat sympathetic neurons undergo apoptosis upon nerve growth factor (NGF) withdrawal, the levels of c-*jun* mRNA and c-Jun protein increase (Estus *et al.*, 1994; Ham *et al.*, 1995). In addition, the c-Jun protein becomes phosphorylated, resulting presumably in an enhancement of its transactivation activity (Ham *et al.*, 1995). Microinjection of neutralizing antibodies specific for either c-Jun or Fos proteins, as well as the expression of a dominant-negative c-*jun* mutant, were able to protect NGF-deprived sympathetic neurons from apoptosis (Estus *et al.*, 1994; Ham *et al.*, 1995). Furthermore, neurons undergoing apoptosis induced by hypoxia-ischemia or status epilepticus show a strong increase of c-Jun protein *in vivo* (Dragunow *et al.*, 1993).

Together, these data suggest that c-Jun may mediate apoptosis in some neurons under certain conditions. In addition, several reports have implicated c-*jun* and c-*fos* in lymphocyte apoptosis elicited by diverse stimuli. For instance, c-*jun* and c-*fos* mRNAs are induced rapidly when certain lymphoid cells lines are deprived of IL-6 and IL-2. Antisense oligonucleotides corresponding to the c-*fos* and c-*jun* mRNA were capable of partially preventing the apoptosis in this system (Colotta *et al.*, 1992). In addition, c-*jun* and c-*fos* mRNAs are increased in the lymphocyte apoptosis induced by dexamethasone (Grasselli *et al.*, 1992; Sikora *et al.*, 1993; Goldstone and Lavin, 1994). A similar correlation between increased expression of different members of the AP-1 family and apoptosis has been documented for the cell death in the involuting mouse mammary gland and the rat prostate *in vivo* upon hormone depletion (Buttyan *et al.*, 1988; Marti *et al.*, 1994). Furthermore, experiments with *fos–lacZ* transgenic mice showed that c-*fos* expression precedes developmental cell death in many tissues *in vivo* (Smeyne *et al.*, 1993). In addition, using different inducible systems, it could be demonstrated that c-Fos is able to induce apoptosis in certain cell lines *in vitro* (Smeyne *et al.*, 1993; Preston *et al.*, 1996). However, despite these observations, a recent report on embryos lacking a functional c-*fos* gene suggested that c-*fos* may not be essential for developmental cell death (Roffler-Tarlov *et al.*, 1996). A similar analysis on c-*jun* knockout mice was limited in its interpretation due to the early embryonic death of these mice (Hilberg *et al.*, 1993; Johnson *et al.*, 1993; Roffler-Tarlov *et al.*, 1996). Finally, an increase in AP-1 activity correlates with apoptosis induced by genotoxic insults such as radiation and chemotherapeutic agents (for example, Devary *et al.*, 1991; Hallahan *et al.*, 1991; Bhalla *et al.*, 1992; Manome *et al.*, 1993; Gillardon *et al.*, 1994; Goldstone and Lavin, 1994).

That increased c-Jun activity exhibits apparently opposing consequences by triggering either cell proliferation or apoptosis, seems to be paradoxical. One possible explanation could be that c-Jun is activating genes that are involved in cell cycle progression, and apoptosis is the consequence of entering a defective cell cycle, when coupled with a block in cell proliferation. Alternatively, the role of c-Jun in cell proliferation and apoptosis may be unlinked, and the decision for either response may be dictated by the availability of other Jun dimerization partners such as Fos-related proteins, ATF-2 and CREB proteins, or by the relative abundance of external or internal survival signals. Our data support the latter model. First, apoptosis by c-Jun was elicited even in the presence of serum, thus the implementation of cell death was not strictly confined to conditions where external growth signals were missing. Second, apoptosis mediated by c-Jun was not linked to cell cycle progression (Figure 6A and B). Hence, cell proliferation and apoptosis may represent two independent pathways both regulated by c-Jun. AP-1 activity can be induced by two distinct signal transduction pathways mediated by different mitogen-activated protein kinases (MAPK) (reviewed by Davis, 1994; Karin, 1994). The Ras/Raf/ERK kinase cascade induces the expression of c-*fos* and thereby increases AP-1 activity. The MEKK/SEK/JNK (SAPK) kinase pathway results in the phosphorylation and an increase in transactivation activity of c-Jun and ATF-2. Both the ERK and the JNK kinase pathways have been implicated in apoptosis (Wyllie *et al.*, 1987; Ridley *et al.*, 1988; Arends *et al.*, 1994; Xia *et al.*, 1995; Verheij *et al.*, 1996).

It is tempting to speculate that the dynamic balance between Fos- and Jun-related proteins may play a decisive role in whether the cell survives or undergoes apoptosis. It is noteworthy that we also generated Balb/c 3T3 cells expressing the c-Jun–ER protein. Although some of the Balb/c 3T3 cell lines expressed even higher levels of the c-Jun–ER chimeric protein than we could isolate from NIH 3T3 fibroblasts, these cells were resistant to apoptosis induced by c-Jun activation (data not shown). These findings suggest that c-Jun is not cytotoxic *per se*, but that the susceptibility to respond in apoptosis by c-Jun signaling is dependent on the cellular context. This is also true for other cell death-inducing stimuli, where the sensitivity to apoptosis frequently is dependent on the cell type and the availability of external or internal survival factors. One can speculate that during the immortalization process of the Balb/c 3T3 cell line, genetic changes have occurred that provide a protective effect against a cell death-inducing stimulus delivered by increased c-Jun activity.

What might be the molecular mechanism by which c-Jun is triggering apoptosis? Given that c-Jun is a sequence-specific transcriptional activator or repressor and directly regulates the expression of several genes (reviewed by Karin, 1992), it seems conceivable that c-Jun may trigger apoptosis either by inducing the expression of *bona fide* death genes or by repressing the expression of genes that code for proteins that exhibit survival-promoting activities. Several observations made here are consistent with this notion. First, dominant-interfering alleles of c-*jun*, lacking the transactivation domain, were defective in inducing apoptosis. Second, by screening suspect genes that are known to be implicated either in the regulation or execution of apoptosis, two genes were found to be induced upon c-Jun activation by β-estradiol (E.Bossy-Wetzel, unpublished data). The first one is *bad*, a member of the *bcl-2* gene family with cell death-enhancing properties (Yang *et al.*, 1995), thus raising the intriguing possibil-

ity that c-*jun* may activate apoptosis by differentially regulating the levels of death-preventing versus death-enhancing members of the *bcl-2* gene family. The second gene is tissue transglutaminase (*tTG*) (E.Bossy-Wetzel and L.Bakiri, unpublished data). The *tTG* gene codes for a cross-linking enzyme that has been suggested to be involved in the stabilization of apoptotic bodies before they can be cleared by phagocytosis, thus limiting the leakage of cellular components by the dying cell (Fesus *et al.*, 1987; Piacentini *et al.*, 1991). Finally, cycloheximide prevented the c-Jun-induced apoptosis in NIH 3T3 fibroblasts, indicating that macromolecular synthesis is required for the cell death in this situation (E.Bossy-Wetzel and L.Bakiri, unpublished data). Taken together, these results are in agreement with the notion that c-Jun may induce apoptosis by regulating gene expression.

Constitutive overexpression of *bcl-2* considerably attenuated the c-Jun-induced apoptosis (Figure 5). However, *bcl-2* expression was not sufficient to overcome the apoptosis-inducing effects of c-Jun signaling completely and the cells entered delayed apoptosis. Similar weak protection by Bcl-2 was reported for apoptosis in a variety of other systems (Nunez *et al.*, 1990; Sentman *et al.*, 1991; Vaux *et al.*, 1992; Cuende *et al.*, 1993; Chiu *et al.*, 1995). Several possible explanations could account for the limited protective effect of *bcl-2* in some situations of apoptosis. It has been suggested that *bcl-2*-independent pathways may exist (Cuende *et al.*, 1993; Strasser *et al.*, 1995). Alternatively, it is possible that the cell death induced by certain stimuli and in certain cell types can be blocked more efficiently by other members of the *bcl-2* family, such as *bcl-xl*. Furthermore, some cell death signals may inactivate the anti-apoptotic functions of *bcl-2*. Support for such a view has been provided recently by a report suggesting that increased phosphorylation may interfere with the ability of *bcl-2* to prevent apoptosis (Haldar *et al.*, 1995). Moreover, some cell death signals may result in changes in the relative expression levels of death-preventing versus death-enhancing *bcl-2* family members, and competing interactions between these gene products may dictate the outcome. As mentioned above, the expression of *bad*, a cell death-enhancing member of the *bcl-2* gene family, becomes induced upon c-Jun activation. Thus it is tempting to speculate that c-Jun signaling may increase the susceptibility to apoptosis by changing the ratios of death-inhibiting versus death-enhancing Bcl-2-related proteins, providing a possible explanation for why *bcl-2* overexpression may block apoptosis only partially in this system.

Results in this study suggest that the ICE/CED-3-related cysteine proteases are activated during c-Jun-induced cell death, demonstrated by the proteolytic cleavage of α-fodrin. In addition, cell-permeable, irreversible inhibitors considerably increased the cell survival of β-estradiol-treated c-jun–ER cells. Thus c-Jun acts upstream of the ICE/CED-3 family of cysteine proteases in the apoptosis pathway.

In conclusion, our data provide evidence that c-Jun induces an apoptotic death program that can be inhibited by Bcl-2 and involves the activation of the ICE/CED-3-related cysteine proteases. Hence, c-Jun increases the repertoire of gene products that are capable of stimulating apoptotic cell death. The system described here will serve as a valid tool to dissect further the molecular mechanism by which c-Jun is triggering apoptosis and to identify the critical downstream targets.

Materials and methods

Reagents

17-β-estradiol was purchased from Sigma. Stock solutions were prepared in ethanol (2 mM) and stored, protected against light, at 20°C The tripeptide inhibitor benzyloxycarbonyl-Val-Ala-Asp (*O*-methyl)-fluoromethylketon [z-Val-Ala-Asp-(*O*-ME)-CH2F; z-VAD-fmk] was purchased from Kamiya Biomedicals (Seattle). Stock solutions were prepared in dimethylsulfoxide (DMSO; 20 mM) and stored in aliquots at –80°C. Monoclonal antibodies against human Bcl-2 protein were purchased from DAKO (monoclonal 124). Anti-fodrin monoclonal antibody 1622 was purchased from Chemicon International Inc. (Temecula, CA). The ApopTag kit was purchased from Oncor. The MTT assay kit was purchased from Boehringer Mannheim; hygromycin B was purchased from Calbiochem. Dulbecco's modified Eagle's medium (DMEM), without phenol red, and G418 were purchased from GIBCO.

Construction of expression vectors

The plasmid AH119 (Ryseck *et al.*, 1988), containing the mouse c-*jun* cDNA, was digested with the restriction enzyme *Psp*1406I, blunt ended and re-digested with *Sal*I. The 1386 bp S*al*I–*Psp*1406I fragment containing the c-*jun* sequence was then cloned into the *Sal*I and the blunted *Bam*HI sites of the vector pUC19-HE14. The pUC19-HE14 plasmid was constructed by digesting the PMV-7-myc-ER plasmid (Eilers *et al.*, 1989) with *Bam*HI and *Eco*RI. The resulting 954 bp fragment, containing a part of the human ER gene HE14 (1204–2158 bp fragment), was cloned into the *Bam*HI and *Eco*RI sites of pUC19. The resulting pUC19-c-jun-HE14 plasmid was digested with *Eco*RI and the 2316 bp fragment, containing the c-*jun–ER* fusion gene, was isolated and cloned into the *Eco*RI site of the retroviral vector PMV-7.

Generation of cell lines and culture conditions

NIH 3T3 cells were cultured in DMEM plus 10% bovine calf serum containing penicillin and streptomycin. To create the NIH 3T3/c-jun–ER cell lines, NIH 3T3 cells were seeded at a density of 1×10^6 cells per 10 cm dish in DMEM without phenol red and with 10% activated charcoal-treated bovine calf serum. Ten μg of expression vector PMV-7 or the PMV-7-c-jun–ER were used for transfection with the calcium phosphate DNA precipitation method and selected with G418 (500 μg/ml). Individual G418-resistant colonies were isolated 2–3 weeks later and expanded into cell lines. To generate the c-jun–ER/bcl-2 cell lines, c-jun–ER fibroblasts (clone 3) were transfected with 10 μg of plasmid RSV-tk-hygromycin or plasmid RSV-tk-hygromycin-hbcl-2 (provided by David Vaux) as described above. Transfected cells were selected with 50 μg/ml of hygromycin B. After 3 weeks of selection, individual hygromycin-resistant colonies were isolated and expanded into cell lines.

Assays for apoptosis

To detect DNA fragmentation *in situ*, cells were seeded into Nunc plastic chamber slides in DMEM containing 0.75% calf serum and in the presence or absence of 2 μM β-estradiol for different time periods. The medium was then aspirated and cells were fixed in ice-cold methanol for 10 min. Fixed slides were allowed to air dry. After rehydration with phosphate-buffered saline (PBS) for 5 min, the slides were treated with the ApopTag *in situ* apoptosis detection kit, following the instructions of the manufacturer. After the final wash steps, nuclei with fragmented DNA were visualized by treatment with a solution of 0.25 mg/ml of diaminobenzidine, 3 mg/ml of nickel sulfate and 0.003% H_2O_2. The substrate reaction was stopped after 6–10 min by rinsing the slides in H_2O for 5 min. Slides were mounted with cytifluor and examined with a Zeiss Axiophot microscope. To analyze DNA for nucleosomal size fragmentation, adherent and non-adherent cells were collected. Cell pellets were resuspended in 1 ml of ice-cold buffer A [150 mM NaCl, 10 mM Tris–HCl pH 7.4, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT)] and NP-40 was added to a final concentration of 0.5%. After incubation for 30 min on ice, nuclei were pelleted by centrifugation at 13 000 r.p.m. for 10 min and resuspended in ice-cold buffer B (350 mM NaCl, 10 mM Tris–HCl pH 7.4, 2 mM MgCl, 1 mM DTT). After 15 min on ice, nuclei were collected by centrifugation. The supernatants were transferred to a new Eppendorf tube and extracted with phenol/chloro-

form. After ethanol precipitation, DNA pellets were resuspended in 20 µl of TE buffer and separated on a 1.2% TBE–agarose gel.

Flow cytometric analysis

Cells were trypsinized and collected by centrifugation at 2000 r.p.m. for 5 min. The cell pellets were then resuspended in 300 µl of PBS and 5 ml of ice-cold 75% ethanol was added drop-wise under agitation to the cell suspension and stored at –20°C until further use. The cells were then collected by centrifugation at 2000 r.p.m. and the cell pellets were washed twice with PBS and then resuspended in PBS containing 50 µg/ml propidium iodide (Sigma) and 50 µg/ml DNase-free RNase A (Boehringer Mannheim). The cell suspension was incubated for 15 min on ice and protected against light and then analyzed with an Epics XL Flow cytometer (Coulter). Propidium iodide fluorescence of individual cells was plotted against cell size and cell numbers using the WinMDI Software (2.0.3).

Viability assay

Cells were seeded in triplicate at a concentration of 1×10^4 cells per well of a 96-well microtiter dish and treated for various periods of time with 2 µM β-estradiol. The number of live cells was determined by the MTT assay and used as recommended by the manufacturer. When z-VAD-fmk inhibitors were used, the c-jun–ER cells were seeded in DMEM containing 0.5% calf serum, pre-treated for 4 h with the tripeptide inhibitor followed by treatment with β-estradiol. After 68 h, the surviving cells were trypsinized and live cell numbers were determined by trypan blue exclusion.

Immunoblotting

To analyze the expression of the c-Jun protein, immunoblotting was done as previously described in Ham *et al.* (1995). To test the expression of the human Bcl-2 protein, cell pellets were resuspended in lysis buffer (142.5 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES pH 7.2, 1 mM EGTA, 0.2% NP-40, 0.2 mM phenylmethylsulfonyl fluoride, 0.1% aprotinin, 0.7 mg/ml pepstatin, 1 mg/ml leupetin) and incubated for 30 min on ice. Cellular debris was removed by centrifugation for 10 min at 13 000 r.p.m., and supernatants were transferred to new Eppendorf tubes. Fifty µg of cytoplasmic extracts were loaded into each lane of a 10% SDS–PAGE gel and blotted after electrophoresis to Hybond-ECL nitrocellulose membrane (0.45 µm) (Amersham) at 150 V and 4°C for 2 h in transfer buffer (20 mM Tris, 150 mM glycine, 20% methanol). Unspecific binding was blocked by incubation in 3% bovine serum albumin, 3% non-fat milk powder, 0.05% Tween-20 in PBS for 1 h at room temperature. Bcl-2-specific antibodies (DAKO) were diluted 1:60 in PBS, 3% non-fat milk powder and 0.05% Tween-20. After overnight incubation at 4°C, filters were washed three times with PBS, 3% non-fat milk and 0.05% Tween-20. As secondary antibody, a horseradish peroxidase (HRP)-coupled donkey anti-rabbit (Amersham) antibody was incubated at a dilution of 1:4000 for 30 min to 1 h. The filters were then washed extensively (four times, for 5 min) with PBS, 3% non-fat milk and 0.05% Tween-20, and the specific protein complexes were identified using the ECL-Western blotting detection system (Amersham). When α-fodrin protein expression was analyzed, 20 µg cytosolic extracts were separated by 8% SDS–PAGE. The protein blots were probed with the monoclonal anti-fodrin antibody at a dilution of 1:2000. The rest of the procedure was done as described above.

Measurement of DNA synthesis

To measure DNA synthesis, cells were seeded at a density of 1×10^4 cells/well in a 96-well microtiter dish in DMEM (without phenol red) and 0.5% calf serum. After 3 days of growth arrest, triplicate cultures were stimulated every hour with either 2 µM β-estradiol or 15% calf serum for a total of 32 h. One hour before harvest, cells were treated with a pulse of 0.1 µCi of [^{3}H]thymidine per microtiter well and subsequently lysed by freezing at –20°C. Cell lysates were then transferred to filters using a cell harvester, washed three times with H_2O and counted with a scintillation counter.

Transient transfection assay and X-gal staining

Cells were seeded at a density of 5×10^5 cells/60 mm dish in DMEM 10% bovine calf serum. After overnight culture, 7.5 µg of c-*jun* expression vector (PMV-7-c-jun–ER; RSV-c-jun; RSV-c-junΔ131–220; RSV-c-junΔ169; RSV-c-junΔ196; RSV-c-junΔ238–311) and 1.5 µg of a β-galactosidase expression vector (RSV-β-gal) were co-transfected using the calcium phosphate method. After 3 h of transient transfection, cells were rinsed twice with PBS, and DMEM containing 0.75% bovine calf serum was added. To identify β-galactosidase enzyme activity, cells were fixed after 24 h of transient transfection with 1% glutaraldehyde in PBS for 5 min at room temperature, washed twice with PBS (for 5 min) and stained with X-gal solution [0.5% mg/ml 5-chromo-4-chloro-3-indoxyl β-galactosidase, 3 mM $K_3Fe(CN)_6$, 3 mM $K_4Fe(CN)_6\cdot3H_2O$, 1 mM $MgCl_2$, 10 mM KCl, 0.1% Triton X-100 in PBS] at 37°C for 12–24 h. Photographs of stained cells were taken with an Axiovert Zeiss microscope and Hoffman optics.

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