c-Jun regulates cell cycle progression and apoptosis by distinct mechanisms

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c-Jun is a component of the transcription factor AP-1, which is activated by a wide variety of extracellular stimuli. The regulation of c-Jun is complex and involves both increases in the levels of c-Jun protein as well as phosphorylation of specific serines (63 and 73) by Jun N-terminal kinase (JNK). We have used fibroblasts derived from c-Jun null embryos to define the role of c-Jun in two separate processes: cell growth and apoptosis. We show that in fibroblasts, c-Jun is required for progression through the G_1 phase of the cell cycle; c-Jun-mediated G_1 progression occurs by a mechanism that involves direct transcriptional control of the cyclin D1 gene, establishing a molecular link between growth factor signaling and cell cycle regulators. In addition, c-Jun protects cells from UV-induced cell death and cooperates with NF-κB to prevent apoptosis induced by tumor necrosis factor alpha (TNFα). c-Jun mediated G_1 progression is independent of phosphorylation of serines 63/73; however, protection from apoptosis in response to UV, a potent inducer of JNK/SAP kinase activity, requires serines 63/73. The results reveal critical roles for c-Jun in two different cellular processes and show that different extracellular stimuli can target c-Jun by distinct biochemical mechanisms.
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Introduction

The c-Jun gene encodes a basic region-leucine zipper (bZIP) transcription factor implicated in many cellular processes. c-Jun regulates gene expression and cell function by participating in the formation of a variety of dimeric complexes that display high affinity sequence-specific DNA-binding activity. These include both Jun–Jun and Jun–Fos dimers, which recognize AP-1 sites (5′-TGAGTCA-3′), as well as Jun–ATF dimers, which bind cyclic AMP responsive element (CRE)-like sites (5′-TGAGCTCA-3′). Both c-Jun and its dimerization partners are subject to regulation by an incredibly diverse array of extracellular stimuli. Among these are peptide growth factors, pro-inflammatory cytokines, oxidative and other forms of cellular stress, and UV irradiation. Although the initial response to these stimuli is different, they all appear to regulate c-Jun and/or its dimerization partners by the generation of signals that converge to activate one of three families of MAP kinases: ERK, JNK (also known as SAPK) and p38 (Hill *et al.*, 1993; Marais *et al.*, 1993; Gille *et al.*, 1995; Whitmarsh *et al.*, 1995; Price *et al.*, 1996; Janknecht and Hunter, 1997; for a review see Treisman, 1996).

Functional data suggest that c-Jun is not merely a target for activation by many of the extracellular stimuli, but that it plays a role in mediating the cellular response. In the case of growth control, three lines of evidence suggest that the transcription factor AP-1, which is composed of Fos–Jun and Jun–Jun dimers, mediates cell proliferation in response to external growth signals in the form of peptide growth factors. First, studies carried out many years ago demonstrated that the proliferation of cells in response to growth factors is dependent on the synthesis of new mRNAs and new proteins, implying a critical role for growth factor signaling to the nucleus (Pardee, 1974; Brooks, 1977; for a review see Pardee, 1989). AP-1 proteins, including c-Fos and c-Jun, are prominent nuclear targets of growth factor induced signaling, making AP-1 a candidate nuclear effector of growth factor induced proliferation. Secondly, c-Jun was originally isolated as the cellular homolog of v-Jun, the oncogene in Avian Sarcoma Virus 17 (Maki *et al.*, 1987). Gain-of-function mutations in many intracellular components of the growth factor signaling cascade, including Src, Ras, Raf, Fos and Jun, induce similar transformed phenotypes in cell culture, implying that Fos and Jun proteins are important components of the growth factor signaling cascade. Thirdly, microinjection of antibodies directed against c-Jun results in a failure of progression from G_1 into S (Kovary and Bravo, 1991), suggesting that c-Jun is required for proliferation. Taken together, the data argue strongly that in fibroblasts, AP-1 is a critical component of the mitogenic response to growth factor signaling.

In addition to its identification as the cellular homolog of v-Jun, c-Jun was independently identified as the major component of AP-1 induced after UV irradiation (Angel *et al.*, 1987, 1988). Following exposure to UV, cells undergo a series of changes in gene expression that result in protection from UV-induced apoptosis and DNA damage. Part of this response includes changes in the expression of AP-1 family members, including c-Fos and c-Jun, and previous work has shown that fibroblasts harboring a null mutation at the c-Fos locus are hypersensitive to UV irradiation (Buscher *et al.*, 1988; Schreiber *et al.*, 1995). Activation of AP-1 following UV irradiation is due largely to signaling through JNK, a UV-activated MAP kinase that phosphorylates c-Jun within the activation domain, increasing its transcriptional regulatory properties (Devary *et al.*, 1991; Hibi *et al.*, 1993; Derijard *et al.*, 1994). In addition to augmenting the transcriptional regulatory properties of c-Jun, activation of JNK also

regulates two different c-Jun dimerization partners: the expression of Fos proteins is increased via activation of the Elk-1/SAP-1 family of transcription factors and ATF-2 is post-translationally activated by direct phosphorylation within its activation domain (Whitmarsh *et al.*, 1995, 1997; Janknecht and Hunter, 1997). The induction of c-Jun activity after UV irradiation, and the genetic evidence that a c-Jun dimerization partner, c-Fos, protects cells from UV-induced apoptosis, both suggest that c-Jun may play a role in the protective function of the UV response, although genetic evidence for such a role is lacking.

In addition to growth factor and UV signaling, c-Jun is strongly implicated in the cellular response to pro-inflammatory cytokines such as tumor necrosis factor-α (TNFα) and IL-1β (Brennan *et al.*, 1989). Signaling through the cytokine receptors results in prolonged induction of c-Jun expression and AP-1 activity (Brennan *et al.*, 1989). *In vivo*, TNFα mediates a diverse array of functions including septic shock, the functional activation of inflammatory cells (including macrophages, neutrophils and lymphocytes) and apoptosis of some cell types. Functional dissection of the TNF receptor (TNFR) has demonstrated that the recruitment of FADD to the receptor signaling complex is required for the induction of apoptosis, but not for JNK activation or c-Jun expression; in contrast, TRAF2 recruitment is required for JNK activation and c-Jun expression, but does not mediate cell death (Liu *et al.*, 1996; Song *et al.*, 1997). Thus, c-Jun does not appear to be an effector of TNFR-induced apoptosis. Whether c-Jun might function to attenuate TNFα-induced apoptosis is less clear. In cell culture, most cells are resistant to TNFα-induced killing but can be rendered sensitive by the addition of sub-lethal doses of protein synthesis inhibitors; this is commonly interpreted as revealing the existence of an anti-apoptotic response to TNFR activation that is dependent on changes in gene expression (van Antwerp *et al.*, 1996; C.Y.Wang *et al.*, 1996). As the increased expression of both Fos and Jun proteins by TNFα is blocked by the addition of protein synthesis inhibitors, AP-1 is a candidate mediator of the anti-apoptotic response to TNFR activation. Consistent with this hypothesis, targeted inactivation of TRAF2, which results in impaired activation of JNK following TNFα stimulation, results in enhanced TNFα-induced apoptosis (Yeh *et al.*, 1997). As JNK signaling activates many transcription factors, the specific role that c-Jun plays in TNFα-induced killing, if any, remains genetically undefined.

As noted above, c-Jun is regulated by many different extracellular stimuli, and circumstantial evidence suggests that it plays an important role in mediating the cellular response to many of these. This raises an unusual paradox: how can c-Jun play a role in regulating different cellular responses to such a diverse array of stimuli? To address this issue, we have utilized a genetic approach to define the role of c-Jun in regulating the proliferation of fibroblasts, as well as apoptotic response of fibroblasts to both UV irradiation and TNFα. We find that c-Jun is required for the progression of cells through the G_1 phase of the cell cycle by a mechanism that involves, in part, direct transcriptional control of the cyclin D1 gene. In addition, c-Jun protects cells from UV-induced apoptosis, and cooperates with NF-κB to protect cells from TNFα-induced cell death. Analysis of a panel of c-Jun mutant proteins shows that the ability of c-Jun to mediate G_1 progression can be separated from the ability to protect cells from UV-induced apoptosis: phosphorylation of c-Jun on serines 63 and 73 by JNK is required for the the UV protective effects, but not for the ability to promote cell proliferation. Thus, distinct biochemical mechanisms underlie the different cellular functions of c-Jun.

Results

Growth defects in c-Jun null cells

Embryos homozygous for a null mutation in the c-Jun gene die betwen E12.5 and E14.5 of development (Hilberg *et al.*, 1993; Johnson *et al.*, 1993). To analyze the role of c-Jun in mediating the cellular response to extracellular stimuli, we prepared mouse embryo fibroblasts (MEFs) from E11.5 embryos derived from a heterozygous intercross. At this point in time, c-Jun null embryos are indistinguishable from wild-type embryos.

The most striking feature of c-Jun null fibroblasts in cell culture is their proliferative defect. When fibroblasts passaged once outside the embryos were plated and then monitored for cell number over time, the c-Jun$^{-/-}$ cells exhibited a clear difference from wild type (Figure 1A), a result that is consistent with previous observations (Johnson *et al.*, 1993). Flow-cytometric analysis of the cells for DNA content showed that, compared with wild-type cells, there was an increase in the fraction of c-Jun null cells that were in the G_1 phase of the cell cycle and a decrease in cells in either S or G2/M (Figure 1B; data not shown). The arrest in G_1 is accompanied by a decrease in the fraction of cells that incorporate BrdU during a pulse labeling experiment (Figure 1C). The presence of a G_1 arrest in c-Jun$^{-/-}$ cells is consistent with the known induction of AP-1 immediately after growth factor addition.

Careful measurements of cell number showed that c-Jun null fibroblasts undergo between one and two rounds of cell division during the first 2–3 days after plating before undergoing the growth arrest that is shown in Figure 1A. To examine the changes in cell growth that occur during this early time period, we monitored the incorporation of BrdU following a pulse label at different time points after preparing fibroblasts. As shown in Figure 1C, the fraction of cells in S phase, as determined by BrdU incorporation, was similar between wild-type and c-Jun null cells at early time points (2 days). After 4 days in culture there was a precipitous decline in the number of c-Jun null cells that are in S phase (4.5 versus 22% for wild-type); this decline is even more pronounce after 7 days in culture (Figure 1C). During this time period, the cells undergo morphologic changes that are similar to those detected in senescent fibroblast cultures: they become enlarged, flat and spread out (Figure 1D). Despite multiple attempts, we have been unable to isolate 3T3-like cell lines from c-Jun null embryos. Thus the G_1 arrest of c-Jun$^{-/-}$ fibroblasts is associated with a defect in immortalization.

The observation that c-Jun$^{-/-}$ fibroblasts undergo between one and two rounds of division before undergoing growth arrest suggested that it would be possible to accomplish gene transfer into these cells by utilizing
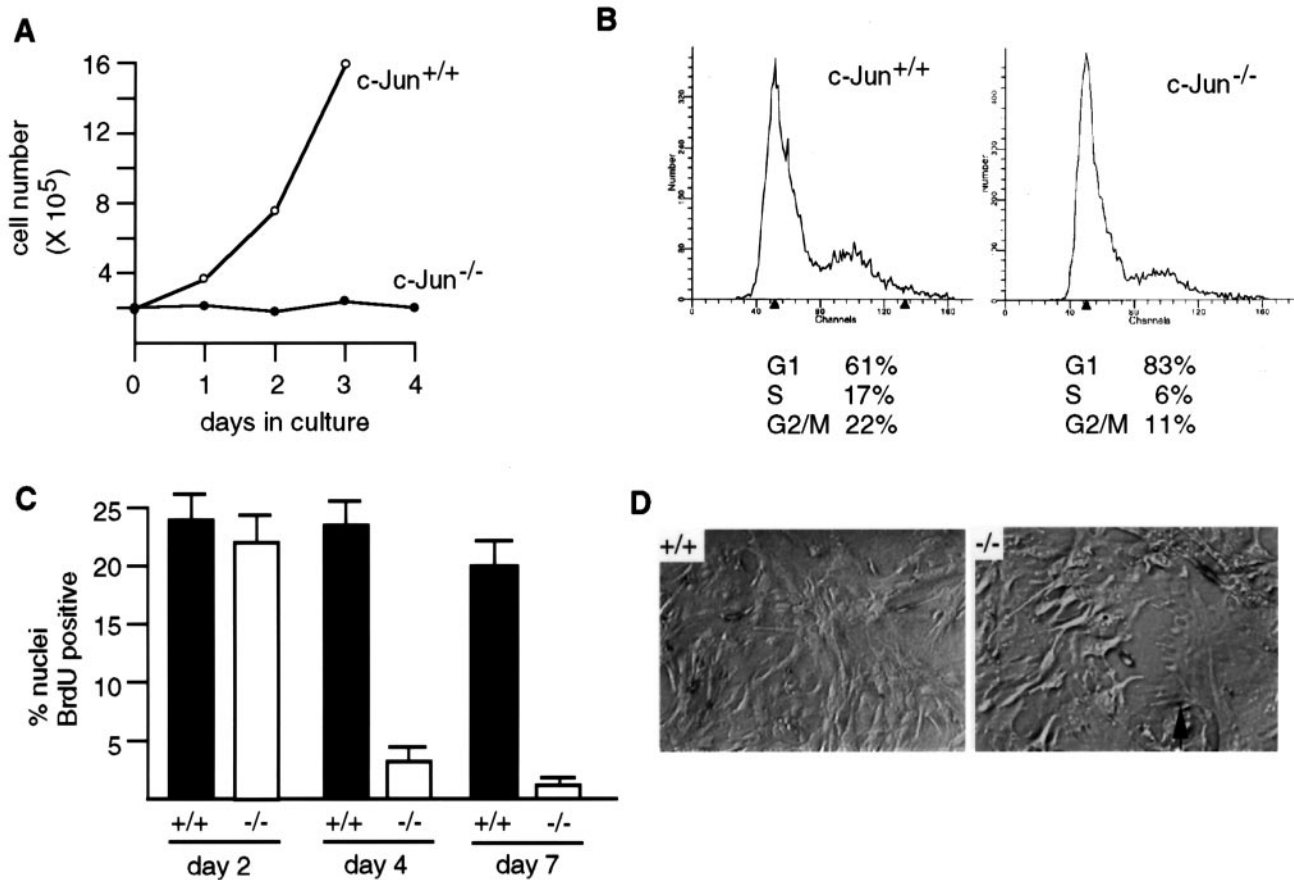
Fig. 1. Growth properties of c-Jun null fibroblasts. (**A**) Wild-type or c-Jun null fibroblasts were prepared by standard methods. Two days after explant from the embryo, the cells were plated at a density of 2×10^5 cells per 6 cm dishes, and the number of cells was determined daily. (**B**) Wild-type or c-Jun null cells were plated at a density of 2×10^6 cells per 10 cm dish two days after preparation. After an additional two days, the cells were fixed, stained with propidium iodide and analyzed by flow cytometry for DNA content. The fraction of cells with G_1, S and G_2/M DNA content are shown. (**C**) Two, 4 or 7 days after explant, cells were pulse labeled with BrdU for 1 h, and then analyzed for BrdU incorporation using anti-BrdU antibodies. Black bars indicate wild-type cells, and white bars indicate c-Jun$^{-/-}$ cells. (**D**) Photomicrographs of wild-type or c-Jun null fibroblasts after 5 days in culture. Note the larger size of the c-Jun null cells; the arrow indicates the large, spread, flattened morphology characteristic of c-Jun null cells after two to three passages through culture.

recombinant retroviruses. When combined with the rapid selection afforded by the antibiotic puromycin, this resulted in cultures of transduced cells 3–4 days after infection. Infection of c-Jun$^{-/-}$ cells with a retrovirus directing expression of c-Jun resulted in the expression of c-Jun protein in the transduced cells at levels that were similar to those observed in wild-type cells (Figure 2A). The re-introduction of c-Jun led to a nearly complete rescue of BrdU incorporation at early time points after infection, as well as to the generation of continuously growing cell lines that were derived from c-Jun null embryos (Figure 2B; data not shown). This suggests that both the proliferative defect at early time points and the immortalization defect at latter times are the result of loss of c-Jun function in the fibroblasts, and are not a consequence of metabolic or non-specific abnormalitites *in utero*.

Cell cycle defects in c-Jun null cells

To begin to define the molecular basis of the G_1-arrest phenotype, we examined the expression of markers of G_1 progression in wild-type and c-Jun null cells. The Rb protein is phosphorylated and functionally inactivated during mid-late G_1, and this phosphorylation results in reduced mobility on SDS–PAGE. We detected only the

most rapidly migrating form of Rb protein in extracts from c-Jun null cells (Figure 3A), suggesting that c-Jun null cells are arrested in early G_1. To determine whether the failure to functionally inactivate Rb is the cause of the G_1 arrest, we attempted to isolate c-Jun/Rb double-mutant embryos. However, no c-Jun/Rb double-mutant embryos were isolated (83 embryos analyzed from a double heterozygous intercross) between E9.5 and E11.5, a time when both single mutants are viable. This result suggests that c-Jun and RB interact genetically, resulting in embryonic lethality before E9.5.

As a second method of testing the role of Rb in mediating the growth arrest of c-Jun$^{-/-}$ cells, we infected cells with a recombinant retrovirus that directs the expression of the adenovirus 12S E1A protein, which binds to and functionally inactivates proteins of the Rb family (Whyte *et al.*, 1988). The expression of E1A in c-Jun mutant cells restored the levels of BrdU incorporation to levels similar to those detected in wild-type cells. Furthermore, expression of E1A prevented the morphologic changes that otherwise develop and allowed the isolation of continuously growing cell lines. In addition to inactivating proteins of the Rb family, E1A also binds to and inactivates proteins of the CBP/p300 family of
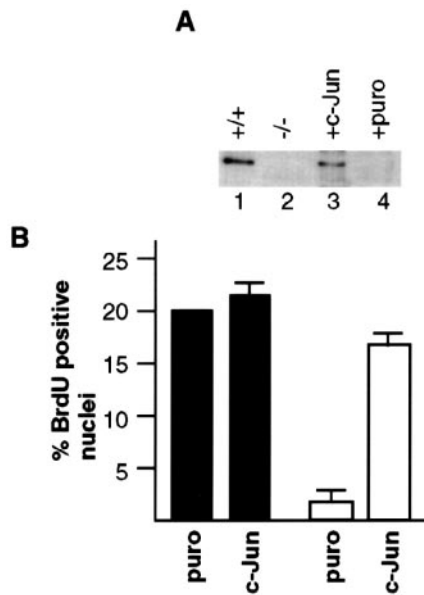
A

B

Fig. 2. The growth defect of c-Jun null fibroblasts is rescued by c-Jun expression. (**A**) Wild-type and c-Jun null fibroblasts were infected with recombinant retroviruses directing the expression of wild-type c-Jun or the puromycin resistance gene only. Following selection of transduced cells, nuclear extracts were prepared and c-Jun protein was detected by immunoblotting. The samples are: lane 1, c-Jun$^{+/+}$; lane 2, c-Jun$^{-/-}$; lane 3, c-Jun$^{-/-}$ infected with c-Jun retrovirus; lane 4, c-Jun$^{-/-}$ infected with control virus. (**B**) Wild-type and c-Jun$^{-/-}$ cells transduced with the indicated retroviruses were analyzed for incorporation of BrdU following a pulse label as before.

A

B

Fig. 3. c-Jun null fibroblasts are arrested in early G_1. (**A**) Nuclear extracts from either wild-type or c-Jun null fibroblasts were separated by SDS–PAGE and transferred to nylon membranes. Rb protein was detected by immunoblotting with anti-Rb antibodies. (**B**) Wild-type or c-Jun null cells were infected with recombinant retroviruses directing the expression of either wild-type or mutant adenovirus E1A proteins. After the selection of infected cells, cells were monitored for the incorporation of BrdU as described above. Black bars indicate wild-type cells and white bars designate c-Jun null cells.

proteins, which function as transcriptional coactivators for AP-1 proteins (Arias *et al*., 1994; Bannister *et al*., 1995). The analysis of E1A mutant proteins showed that it is the ability to inactivate the Rb family of proteins that is required for the rescue of BrdU incorporation in c-Jun null cells: E1A Δ2-28, which does not interact with CBP/p300, fully rescues growth, while E1A Δ121–127, which does not interact with Rb family proteins, does not (Stein *et al*., 1990; Wang *et al*., 1993). These data suggest that it is the failure to functionally inactivate proteins of the Rb family that is responsible for the growth arrest of c-Jun null cells.

The physiological mode of inactivating the growth suppressive function of Rb is by phosphorylation. The cyclin D/cdk4 complex has Rb kinase activity *in vitro*, and is believed to be a major Rb kinase activity *in vivo* (Kato *et al*., 1993; Matsushime *et al*., 1994). c-Jun$^{-/-}$ cells exhibit reduced expression of cyclin D1 and D3 compared with c-Jun$^{+/+}$ cells, while the level of cdk4 protein was similar in the two cell types (Figure 4A). Consistent with the reduction of cyclin D1 proteins levels, cyclin D1 associated kinase activity, using glutathione *S*-transferase (GST)–Rb as a substrate, was reduced in c-Jun null cells. Immunoblotting experiments showed that there was no difference in the expression of the cdk inhibitor proteins p16, p21 and p27. RNA blotting showed that the levels of cyclin D1 mRNA were reduced in mutant cells to a level similar to that observed for the protein (~4.5-fold) (Figure 4A). The results suggest that cyclin D1 is regulated at the transcriptional level in a c-Jun-dependent manner, a result which is consistent with the identification of cyclin D1 as a gene that is induced by growth factors in a manner
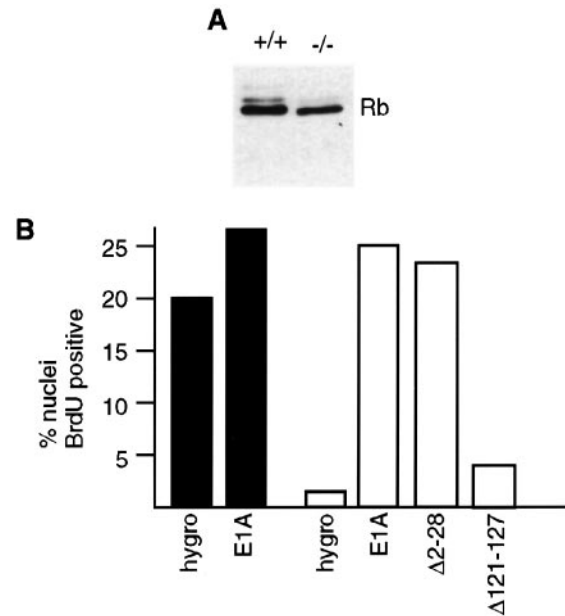
that requires prior protein synthesis (Matsushime *et al*., 1991; Won *et al*., 1992).

To define the mechanisms by which cyclin D1 mRNA expression is regulated by c-Jun, we generated a cyclin D1-luciferase (D1-luc) reporter harboring 1.2 kb of upstream sequence from the human cyclin D1 gene (Herber *et al*., 1994). Following transient transfection, this reporter was expressed ~5-fold more efficiently in wild-type cells than in c-Jun null cells, a result consistent with the relative cyclin D1 mRNA levels in the two cell types (Figure 4B). The D1 promoter contains a single consensus AP-1 site at –840; mutation of this site resulted in a decrease in the expression of the D1-luc reporter in wild-type cells, while the expression in c-Jun$^{-/-}$ cells was unaffected. Consistent with this result, co-transfection of a c-Jun expression plasmid into c-Jun$^{-/-}$ cells significantly increased expression of the D1-luc reporter (Figure 4B). Thus, the AP-1 site at –840 bp appears to mediate the effects of c-Jun on cyclin D1 expression.

To determine whether the ectopic expression of cyclin D1 can restore cell cycle progression in c-Jun$^{-/-}$ cells, cells were infected with a retrovirus that directs cyclin D1 expression. The cyclin D1 retrovirus elicits an increase in BrdU labeling from 1.5–5.5% (Figure 4C). We conclude that cyclin D1 is a direct target of c-Jun activity in embryonic fibroblasts, and that the low levels of cyclin D1 are in part, but not entirely, responsible for the cell cycle defect in c-Jun$^{-/-}$ cells.

Apoptosis in c-Jun mutant cells

In addition to growth factors, UV irradiation is a potent activator of c-Jun expression. In addition, UV activates the JNK signaling pathway, which results in the phos-
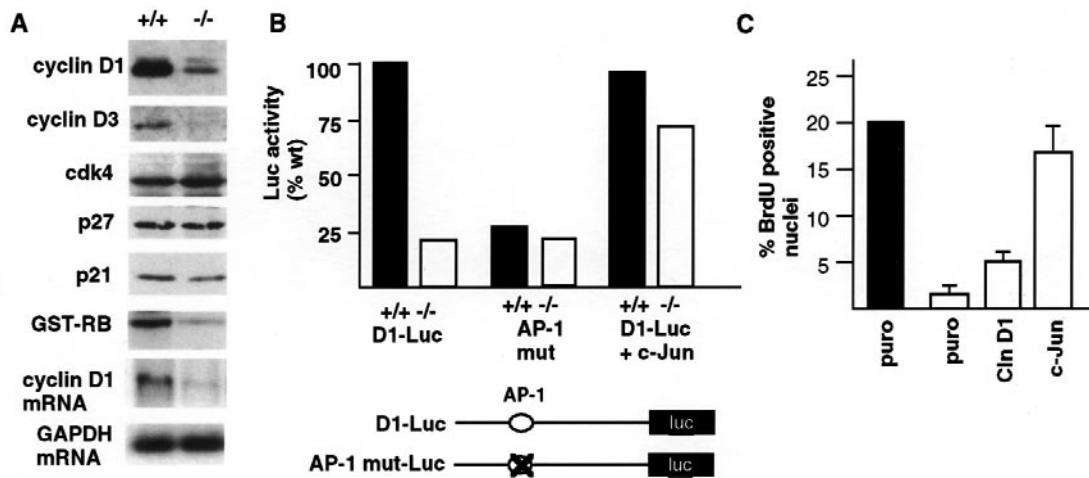
Fig. 4. Cyclin D expression is reduced in c-Jun null cells. (**A**) After growth in culture for 5 days, whole-cell extracts or total RNA was prepared. The levels of cyclin D1, cyclin D3, cdk4, p27 and p21 proteins were determined by immunoblotting; cyclin D1-associated kinase activity, using GST–Rb as a substrate, was determined by immune complex assay; and cyclin D1 and GAPDH mRNA levels were determined by Northern blotting. Similar results were obtained from extracts of cells after 7 or 10 days in culture. (**B**) c-Jun activates expression from the cyclin D1 promoter. A reporter construct harboring 1.2 kb of cyclin D1 upstream sequence fused to the firefly luciferase gene (D1-luc), or a mutant of D1-luc in which the AP-1 site at –840 was mutated (AP-1 mut), was transfected into wild-type or c-Jun null cells, along with a control plasmid directing β-galactosidase expression (pCH110), and where indicated, a c-Jun expression plasmid, as described in Materials and methods. Forty hours after transfection, the β-galactosidase and luciferase activities were determined. (**C**) Cyclin D1 expression partially rescues the proliferative defect of c-Jun null cells. Wild-type (black bars) or c-Jun null (white bars) fibroblasts were infected with recombinant retroviruses directing the expression of the indicated genes. Following selection of infected cells, BrdU incorporation was determined after pulse labeling as before.

phorylation of c-Jun on serines 63 and 73 within its activation domain, increasing its ability to effect transcriptional activation of target genes. One important biologic consequence of exposure of cells to UV irradiation is the induction of apoptosis. To define the role of c-Jun in the cellular response to UV, wild-type and c-Jun null cells were exposed to different doses of UV, and the fraction of cells with apoptotic morphology was determined. A higher fraction of c-Jun null cells underwent apoptosis in response to UV than wild-type cells; the difference was apparent at several different doses of UV treatment and several different time points (Figure 5A and B). DNA end-labeling (TUNEL) assays revealed that the cell death monitored by morphologic changes was in fact apoptosis, and confirmed the increased sensitivity of c-Jun^{−/−} cells to UV-induced apoptosis (Figure 5D). This result is consistent with previous data showing that c-Fos also protects cells from UV-induced apoptosis. Whether other aspects of the mammalian UV response, such as cell cycle arrest or increased DNA repair, are affected in c-Jun mutant cells remains to be determined.

Like UV irradiation, the cytokine TNFα induces c-Jun expression and phosphorylation, JNK/SAPK signaling and, in some cell types, apoptosis. The cellular response to TNFα appears to be controlled by at least two distinct signals: a pro-apoptotic signal that is mediated by the recruitment of TRADD and FADD to the TNFR signaling complex, and anti-apoptotic signals generated by the recruitment of TRAF2 to the TNFR signaling complex (Chinnaiyan et al., 1995; Hsu et al., 1995, 1996; Liu et al., 1996). TRAF2 recruitment results in the activation of two transcription factors, NF-κB and AP-1 (Liu et al., 1996; Song et al., 1997). As NF-κB activation has been shown to block TNF-induced apoptosis (Beg and Baltimore, 1996; Liu et al., 1996; van Antwerp et al., 1996; C.Y.Wang et al., 1996), we considered the possibility that AP-1 activation might also affect the cellular response

to TNFα. Therefore, we measured the fraction of apoptotic cells after treatment of wild-type and c-Jun null cells with TNFα. As shown in Figure 6A, TNFα treatment did not induce significant levels of apoptosis in either wild-type or c-Jun null cells.

Because neither wild-type nor c-Jun mutant fibroblasts were sensitive to TNFα-induced apoptosis, we attempted to create a sensitized genetic background that might reveal a role for c-Jun in apoptotic signaling by TNFα. Consistent with previously published data, we found that inhibition of NF-κB activity (by transduction with a retrovirus expressing ΔN-IκB, a dominant-negative IκB that is not degraded in response to TNFα signaling) resulted in a marked increase in TNF-induced apoptosis (Beg and Baltimore, 1996; Liu et al., 1996; van Antwerp et al., 1996; C.Y.Wang et al., 1996). Furthermore, apoptosis was enhanced in the presence of simultaneous inactivation of c-Jun and NF-κB, with the fraction of apoptotic cells >80% at 24 h, versus 40% in c-Jun^{+/+} cells expressing ΔN-IκB (Figure 6B). Once again, TUNEL assays confirmed that the increased TNFα-induced killing in c-Jun^{−/−} cells was due to apoptosis (Figure 6C). Therefore, in addition to protecting cells from UV-induced apoptosis, c-Jun cooperates with NF-κB to protect cells from TNFα-induced apoptosis. Although we have no insight into the biochemical basis of the cooperation, it is interesting to note that AP-1 and NF-κB have been shown to cooperate in transcriptional activation by a mechanism involving direct physical interaction (Stein et al., 1993).

Separation of the proliferative and anti-apoptotic functions of c-Jun

The results described above provide strong genetic evidence that in fibroblasts, c-Jun mediates cell proliferation and protects cells from UV-induced apoptosis. Are the biochemical mechanisms used to provide these two functions the same or different? To address this issue, we
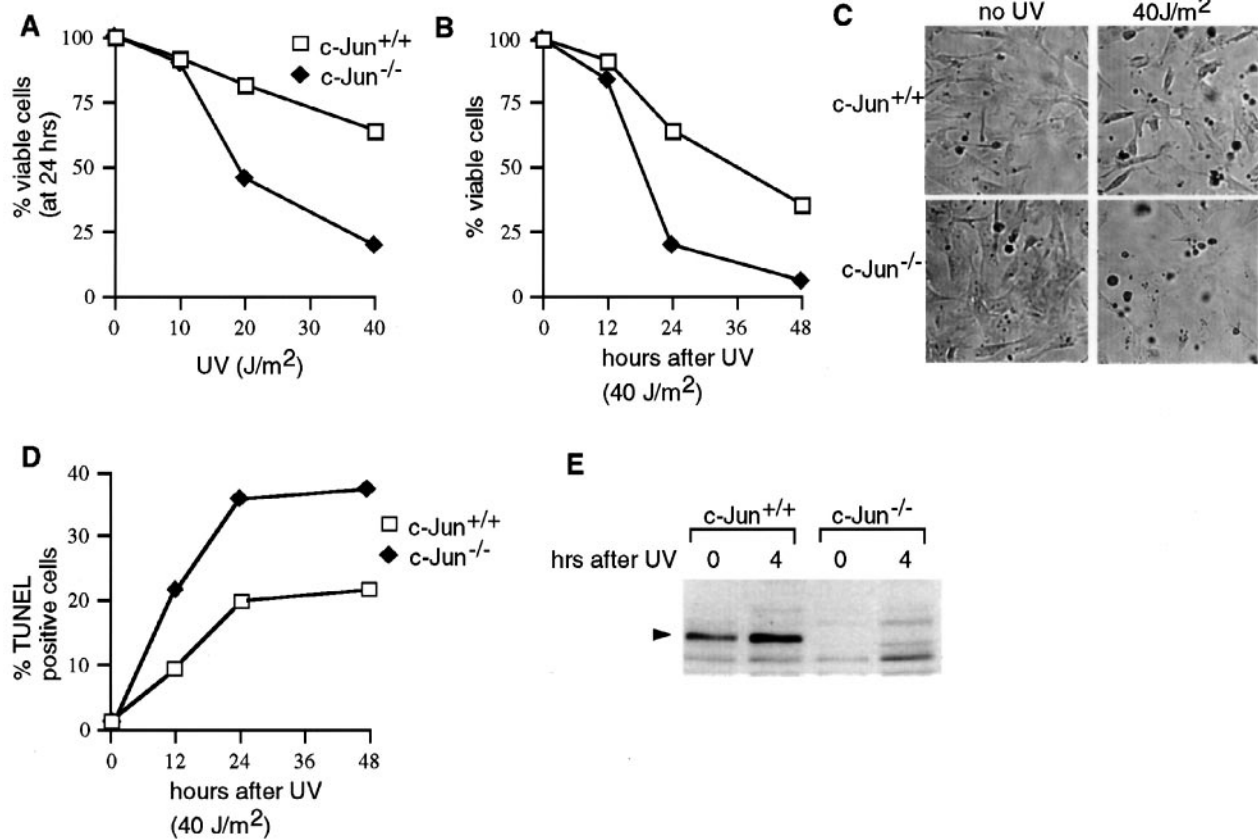
Fig. 5. c-Jun null cells show increased sensitivity to UV-induced apoptosis. (**A**) Wild-type or c-Jun null fibroblasts were exposed to the indicated doses of UV irradiation (254 nm), and the fraction of apoptotic cells was determined by counting 24 h after exposure. (**B**) Wild-type or c-Jun null fibroblasts were exposed to 40 J/m^2, and the fraction of apoptotic cells was counted at the indicated times. (**C**) Phase-contrast photomicrographs of wild-type and c-Jun null fibroblasts before or 24 h after 40 J/m^2 UV. (**D**) Wild-type or c-Jun null fibroblasts were exposed to 40 J/m^2. At the indicated times, cells were fixed and the presence of fragmented DNA was determined by TUNEL staining as described in Materials and methods. (**E**) Nuclear extracts of wild-type or c-Jun null cells before or 4 h after 40 J/m^2 of UV irradiation were separated by SDS–PAGE transferred to nylon membrane. c-Jun protein was detected by immunoblotting with c-Jun specific antisera. c-Jun protein is indicated by the arrowhead. Note the increased expression of c-Jun after exposure to UV in wild-type cells.

analyzed the ability of different c-Jun mutant proteins to provide these functions in c-Jun mutant cells.

Transduction of c-Jun$^{-/-}$ cells transduced with a retrovirus that directs expression of wild-type c-Jun results in nearly complete rescue of both functions: BrdU incorporation and UV-induced apoptosis both occur at levels similar to those detected in wild-type cells (Figure 7A and B). Both functions are dependent on the ability of c-Jun to bind DNA, as the expression of a c-Jun mutant protein that can not bind DNA (Δ284–286) does not rescue either the growth arrest or the UV sensitivity of c-Jun null cells (Figure 7A; data not shown).

The levels of c-Jun protein are increased in response to activation of either the ERK or JNK pathway, but only JNK activation results in the phosphorylation of c-Jun on serines 63 and 73. Mutation of serines 63 and 73 to alanine (S63/73A) results in a c-Jun protein that supports wild-type levels of BrdU incorporation, and the generation of immortalized cell lines from c-Jun$^{-/-}$ embryos (Figure 7A; data not shown). However, cells expressing c-Jun S63/73A display sensitivity to UV-induced apoptosis that is essentially identical to the parental c-Jun$^{-/-}$ cells (Figure 7B). Thus, the c-Jun S63/73A mutant is able to support cell proliferation at levels similar to wild-type, but is

completely inactive with regard to protection of cells from UV-induced apoptosis. The requirement for phosphorylatable residues at serines 63 and 73 to support protection from UV-induced apoptosis, but not cell growth, is consistent with the fact that high levels of JNK activity are present after UV irradiation, but not in cells growing in culture.

Discussion

c-Jun is regulated by a wide variety of extracellular stimuli and is implicated in the cellular responses to many of these stimuli. We have genetically defined a role for c-Jun in two processes in which it has long been implicated: cell cycle progression and the UV response. We have shown that c-Jun is required for progression through G$_1$ in fibroblasts; this is due in part to the ability of c-Jun to directly regulate cyclin D1 expression. In addition, inactivation of c-Jun results in increased apoptosis in response to UV irradiation; furthermore, c-Jun synergizes with NK-κB to protect fibroblasts from TNFα-induced apoptosis. By analyzing the ability of c-Jun mutant proteins to rescue these properties, we have shown that the proliferative function of c-Jun does not require
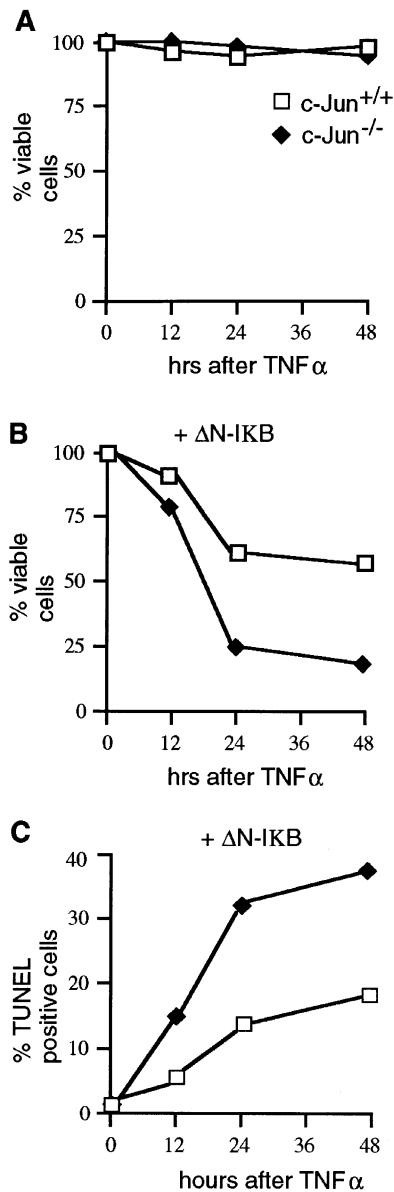
Fig. 6. c-Jun cooperates with NF-κB to prevent TNFα-induced apoptosis. (**A**) Wild-type and c-Jun$^{-/-}$ fibroblasts were treated with recombinant TNFα (10 ng/ml) and the fraction of surviving cells was determined at the indicated times by counting. (**B**) Wild-type and c-Jun$^{-/-}$ fibroblasts were transduced with a recombinant retrovirus directing the expression of ΔN-IκB. Following selection of transduced cells, the cells were grown in non-selective media for 24 h before treatment with TNFα. The fraction of apoptotic cells was determined at the indicated times. (**C**) Wild-type and c-Jun$^{-/-}$ cells expressing ΔN-IκB were treated with TNFα for the indicated periods of time. Cells were then fixed and the presence of DNA fragmentation was determined by TUNEL assay as described in Materials and methods.

phosphorylation by JNK, while the UV-protective effect does. The results reveal roles for c-Jun in cellular processes in which it is implicated and provide data on the mechanisms used to couple different extracellular stimuli to cellular responses.

c-Jun control of proliferation

Ever since its identification as the cellular homolog of v-Jun, a role for c-Jun in controlling cellular proliferation has been hypothesized. Our data demonstrate that c-Jun
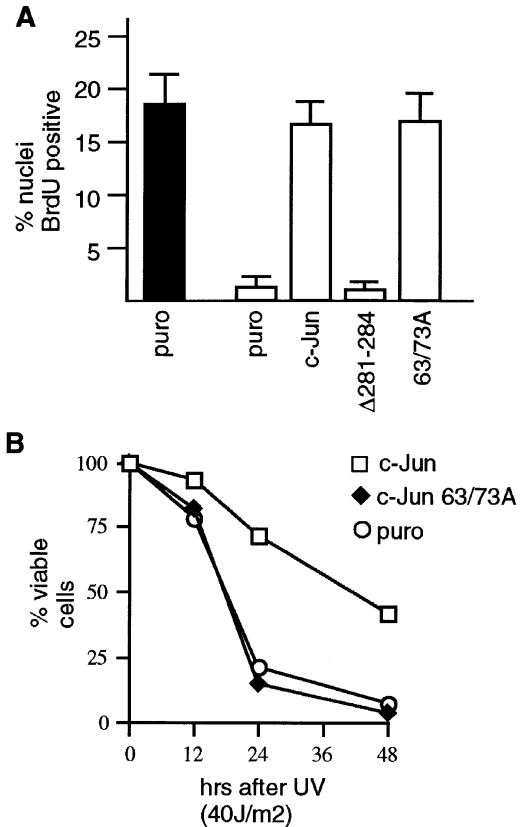

Fig. 7. Phosphorylation of c-Jun on serines 63 and 73 mediates protection from UV-induced apoptosis but not proliferation. (**A**) c-Jun null cells were transduced with recombinant retroviruses directing the expression of c-Jun or the indicated c-Jun mutant proteins. Cells were analyzed for incorporation of BrdU following a pulse label as described above. (**B**) Fibroblasts expressing the indicated c-Jun mutant proteins were generated by retroviral infection. After selection of infected cells, cells were exposed to 40 J/m^2 UV, and the fraction of surviving cells was determined at the indicated times.

is required for fibroblast proliferation in cell culture. The essential role of c-Jun for fibroblast proliferation is not observed in all cell types: specifically, c-Jun$^{-/-}$ embryonic stem cells grow normally in culture (Hilberg and Wagner, 1992). This raises an important question: is c-Jun required for fibroblast proliferation *in vivo*? One possibility is that c-Jun null embryos die because of failure of proliferation of one or more cell types. Contrary to this is the observation that in chimeric mice, c-Jun null cells contribute to most tissues in the animal, including tissues that contain cell types derived from fibroblasts, suggesting that the proliferative defect is not the cause of the embryonic lethality of c-Jun null embryos (Hilberg *et al.*, 1993).

It is not clear how the proliferative functions that c-Jun supplies *in vitro* are supplied *in vivo*. It is possible is that either cell–cell or cell–matrix contacts provide a function in the animal that is supplied by c-Jun in tissue culture. One model would be that cell–cell or cell–matrix interactions induce the expression of either JunB or JunD, and that in the absence of these signals a requirement for c-Jun is unmasked in cell culture. Alternatively, the proliferative function supplied by c-Jun in cell culture may not be supplied by AP-1 proteins *in vivo*.

The demonstration that cyclin D1 is a direct target of c-Jun transcriptional activation provides a molecular link

between growth factor signaling and changes in cell cycle proteins that regulate G_1 progression. This result is consistent with previous data showing that activation of other components of the growth factor signaling cascade, such as Ras and Raf, act to induce proliferation, in part, by increasing expression of the D-type cyclins (Liu *et al.*, 1995; Peeper *et al.*, 1997, Sewing *et al.*, 1997; Woods *et al.*, 1997). Furthermore, the ability of cyclin D1 to partially rescue the proliferative defect of c-Jun mutant cells suggests that cyclin D1 is likely to be an important target of c-Jun action. The partial rescue by ectopic cyclin D1 expression also implies the existence of other c-Jun targets important for cell cycle progression. Cyclin D3 is a candidate for such a second target gene; while there is no clear evidence of functional distinction between cyclins D1 and D3, it is possible that they target cdk4, the catalytic subunit, to distinct substrates, and therefore might have non-redundant functions. In this regard, it is interesting to note that the cyclin D3 gene contains AP-1 sites in its upstream region (Z.Wang *et al.*, 1996). In addition, functional inactivation of Rb requires the action of both cyclin D/cdk4 and cyclin E/cdk2 complexes, and so the cyclin E–cdk2 complex is another target potential target of c-Jun action.

c-Jun regulation of apoptosis

In addition to its identification as the cellular homolog of v-Jun, c-Jun was also identified as a UV-activated DNA-binding protein specific for the AP-1 site in the collagenase gene (Angel *et al.*, 1987). Here we have shown that c-Jun mutant fibroblasts show enhanced sensitivity to UV-induced apoptosis, and thus demonstrate that c-Jun protects cells from UV damage. The ability of c-Jun to protect cells from apoptosis is not specific to UV; in the setting of NF-κB inactivation, c-Jun protects cells from TNFα-induced apoptosis. The molecular mechanisms by which c-Jun protects cells from apoptosis are unclear. This effect appears to be context dependent, as c-Jun overexpession has been shown to induce apoptosis during growth factor deprivation (Bossy-Wetzel *et al.*, 1997). One possibility is that c-Jun might participate in a checkpoint function, mediating a growth arrest function that permits repair of damaged DNA. Against this is the observation that growth arrested c-Jun null cells show increased sensitivity to UV-induced apoptosis. A second possiblity is that c-Jun induces the expression of genes that block apoptosis. This would be functionally analogous to the mechanism by which NF-κB protects cells from TNFα-induced apoptosis, which involves transcriptional activation of anti-apoptotic gene cIAP2 (Chu *et al.*, 1997). Further studies will be required to define the molecular basis of the anti-apoptotic functions, as well as the basis of the cooperation with NF-κB.

Signal integration and the selection of biological responses

Our genetic evidence indicates that c-Jun plays an essential role in both normal fibroblast proliferation and in the UV response. The identity of the DNA binding complexes that mediate these activities is left unresolved, as c-Jun has several different dimerization partners. The proliferative defect observed in c-Jun$^{-/-}$ fibroblasts is in many respects similar to the defect described in c-Fos$^{-/-}$ FosB$^{-/-}$

fibroblasts, in which defective G_1 progression is also associated with decreased transcription of the cyclin D1 gene (Brown *et al.*, 1998). This phenotypic correspondence suggests, but does not prove, that it is heterodimeric Fos–Jun complexes that are required for cell cycle progression. In the case of protection from UV-induced apoptosis, both c-Fos (via activation of Ternary Complex Factors) and ATF2 are targets of UV-induced JNK activation, and genetic analysis shows that UV-induced apoptosis is increased in c-Fos$^{-/-}$ fibroblasts (Schreiber *et al.*, 1995). Thus, the relative roles that Fos and ATF proteins play as c-Jun dimerization partners to effect these processes remains to be determined. The existence of c-Jun mutants with selective dimerization specificities provides one possible method to address this problem (van Dam *et al.*, 1998).

c-Jun is subject to multiple forms of regulation in response to a diverse array of extracellular stimuli. How specificity is achieved in terms of the cellular response to these stimuli has been unclear. Some of the specificity presumably lies in the selective induction of other transcription factors by these different stimuli. In addition, our data provide evidence that some cellular functions of c-Jun (e.g. protection from UV-induced apoptosis) require phosphorylation by JNK, while others (e.g. G_1 progression) do not. The biochemical basis by which this specificity is determined awaits the molecular identification of additional c-Jun target genes.

Materials and methods

Cells

E11.5 embryos were dissected and minced in 0.05% trypsin to generate single cell suspensions; the cells from a single embryo were typically plated in one 10 cm dish. DNA was extracted from the remaining carcass and used to genotype the embryo by PCR using previously described primer pairs (Johnson *et al.*, 1993). Two days after plating, cells were split 1:2 (~10^6 cells per 10 cm dish) and used for experiments. For retroviral infections, cells were plated at 6×10^5 cells per 6 cm dish. One day after plating, cells were infected twice at 12 h intervals with 5 ml of retroviral supernatants (titre ~10^6/ml) in polybrene (4 μg/ml). Twenty-four hours after the last infection, selection was initiated with puromycin (1.5 μg/ml) or hygromycin (200 μg/ml).

Viruses

Retroviral supernatants were prepared by transfecting 10 cm dishes of BOSC23 cells with proviral DNA as described previously (Johnson *et al.*, 1996). cDNAs encoding E1A or E1A-mutants, were cloned into the retroviral vector pWZL Hygro (Serrano *et al.*, 1997); cDNAs encoding mouse cyclin D1, ΔN-IκBα, c-Jun and c-Jun mutants, were cloned in the retroviral vector pBabepuro. Forty-eight and 60 h after transfection, supernatants were filtered and used to infect primary fibroblasts in 4 μg/ml of polybrene.

Cell cycle analysis

Fibroblasts cultures were fixed in ethanol after 4 and 7 days in culture. Cells (10^6) were stained with propidium iodide and analyzed by flow cytometry. Similar patterns were obtained from cells that had been in culture for either 4 or 7 days, although the decrease in c-Jun$^{-/-}$ S phase cells was more pronounced at 7 days (3%).

To measure BrdU incorporation, cells were seeded on glass coverslips 24 h before labeling for 1 h with 50 mM BrdU followed by fixation in acid-ethanol. BrdU detection was by immunostaining with the Cell Proliferation kit from Amersham, used according to the manufacturer's specifications. For each coverslip, the fraction of BrdU positive nuclei was determined by counting a total of 300 cells; the values represent the average of measurements obtained using fibroblasts from four different embryos of each genotype.

Protein detection

For immunoblots, either whole-cell (cyclin D1, cyclin D3, cdk4, p21, p27 and p16) or nuclear extracts (Rb and p53) were prepared from cells of the indicated genotypes. Following SDS–PAGE, immunoblots were performed using the following antibodies: Rb (PharMingen), cdk4 (Santa Cruz), cyclin D1 (Santa Cruz), cyclin D3 (Santa Cruz), p27 (Santa Cruz) and p21 (a gift from J.Pietenpol). Detection was by chemiluminescence.

Measurement of cyclin D1-associated kinase activity was carried out according to previously described methods, with minor variations (Matsushime *et al.*, 1994). Briefly, cyclin D1 immune complexes were prepared from 600 µg of whole-cell extract prepared as described previously and incubated with 1 µg of GST–Rb in the presence of kinase buffer (20 mM MgCl$_2$, 50 mM Tris pH 7.5, 20 µM ATP, 10 µCi [γ-^{32}P]ATP). After 20 min the reaction was terminated by addition of 2× Laemmli sample buffer, boiled and separated by SDS–PAGE before autoradiography.

RNA measurements were carried out as previously described (Wisdom *et al.*, 1992).

Reporter gene assays

A 1.2 kb fragment of the human cyclin D1 gene (Herber *et al.*, 1994) was obtained by PCR and cloned into the plasmid pGL3 to generate the reporter –D1-luc. Site directed mutagenesis was used to mutate the AP-1 site at –840 (5′-TGAGTCA-3′ to 5′-TCCGTGG-3′) to generate AP-1 mut-luc. Wild-type or mutant cells in 6 cm dishes were transfected with 1.0 µg of luciferase reporter, 1.0 µg of a control β-galactosidase reporter (pCH110, Pharmacia) and 0.5 µg of a c-Jun expression vector or the empty expression (pBabe puro-c-Jun) vector using Lipofectamine (Gibco-BRL). Forty hours after transfection, luciferase and β-galactosidase activities were determined. Results represent the averages of three experiments using fibroblasts prepared from different embryos.

UV- and TNFα-induced killing experiments

Cells were plated in 12-well plates at 2×10^4 cells per well for 24 h before irradiation with the indicated dose of UV (254 nm) or recombinant mouse TNFα (10 ng/ml). At different times, the fraction of cells with apoptotic morphology was determined by counting 200 cells. Results represent the average of three separate experiments using fibroblasts prepared from different embryos. In the indicated experiments, fibroblasts were transduced with a retrovirus that directs expression of ΔN-IκBα (a gift from Dean Ballard). Following selection of transduced cells, cells were grown in the absence of puromycin for 24 h before treatment with TNFα. Immunoblotting with anti-IκB antibodies (a gift from Dean Ballard) confirmed that transduced cells expressed predominantly the truncated ΔN-IκBα.

In addition to scoring the number of apoptotic cells morphologically, apoptosis was scored by DNA end labeling (TUNEL) assays. At various time points after either UV or TNF treatment, cells were fixed in formaldehyde and TUNEL assays were performed using the DNA Fragementation Detection Kit from Oncogene Research. The fraction of TUNEL positive cells was determined by counting 200 cells of each type. Results are the average of two separate experiments using cells derived from separate embryos.

c-Jun rescue experiments

Cells of the indicated genotypes (c-Jun$^{+/+}$ or c-Jun$^{-/-}$) were transduced with recombinant retroviruses directing the expression of the indicated c-Jun protein. After selection of transduced cells with puromycin, the fraction of cells in S phase was determined by measuring BrdU incorporation, and the sensitivity of the cells to UV-induced killing was determined as described above. The results represent the averages of two experiments performed on two separate preparations of fibroblasts of each genotype.

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