



AP-1 in cell proliferation and survival

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A plethora of physiological and pathological stimuli induce and activate a group of DNA binding proteins that form AP-1 dimers. These proteins include the Jun, Fos and ATF subgroups of transcription factors. Recent studies using cells and mice deficient in individual AP-1 proteins have begun to shed light on their physiological functions in the control of cell proliferation, neoplastic transformation and apoptosis. Above all such studies have identified some of the target genes that mediate the effects of AP-1 proteins on cell proliferation and death. There is evidence that AP-1 proteins, mostly those that belong to the Jun group, control cell life and death through their ability to regulate the expression and function of cell cycle regulators such as Cyclin D1, p53, p21^{cip1/waf1}, p19^{ARF} and p16. Amongst the Jun proteins, c-Jun is unique in its ability to positively regulate cell proliferation through the repression of tumor suppressor gene expression and function, and induction of cyclin D1 transcription. These actions are antagonized by JunB, which upregulates tumor suppressor genes and represses cyclin D1. An especially important target for AP-1 effects on cell life and death is the tumor suppressor p53, whose expression as well as transcriptional activity, are modulated by AP-1 proteins. *Oncogene* (2001) 20, 2390–2400.

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Introduction

Although the AP-1 (activating protein-1) transcription factor was identified almost 15 years ago (Angel *et al.*, 1987; Lee *et al.*, 1987a,b) and retroviral homologs of some of its components were found even earlier (van Straaten *et al.*, 1983), the biological relevance and physiological functions of AP-1 and its components are still being elucidated. Recent progress has begun to illuminate how AP-1 transcription factors control cell proliferation, survival and death. In this review we focus on those biological effects of AP-1 that relate to control of cell proliferation and survival, and try to explain some of these processes in terms of target gene expression. We offer only brief discussion of the structural organization of AP-1 complexes or the mechanisms that regulate their activity as they were

extensively discussed previously (Angel and Karin, 1991; Karin, 1995).

The mammalian AP-1 proteins are homodimers and heterodimers composed of basic region-leucine zipper (bZIP) proteins that belong to the Jun (c-Jun, JunB and JunD), Fos (c-Fos, FosB, Fra-1 and Fra-2), Jun dimerization partners (JDP1 and JDP2) and the closely related activating transcription factors (ATF2, LRF1/ATF3 and B-ATF) subfamilies (reviewed by (Angel and Karin, 1991; Aronheim *et al.*, 1997; Karin *et al.*, 1997; Liebermann *et al.*, 1998; Wisdom, 1999)). In addition, some of the Maf proteins (v-Maf, c-Maf and Nrl) can heterodimerize with c-Jun or c-Fos (Nishizawa *et al.*, 1989; Swaroop *et al.*, 1992), whereas other Maf related proteins, including MafB, MafF, MafG and MafK, heterodimerize with c-Fos but not with c-Jun (Fujiwara *et al.*, 1993; Kataoka *et al.*, 1994, 1995). Jun proteins can form stable dimers that bind AP-1 DNA recognition elements (5'-TGAG/CTCA-3'), also known as TREs [phorbol 12-O-tetradecanoate-13-acetate (TPA) response elements] based on their ability to mediate transcriptional induction in response to the phorbol ester tumor promoter TPA (Angel *et al.*, 1987). Fos proteins do not form stable dimers but can bind DNA by forming heterodimers with Jun proteins that are more stable than Jun:Jun dimers (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988). ATF proteins, on the other hand, form homodimers as well as heterodimers with Jun proteins that preferentially bind to cAMP responsive elements (CRE, 5'-TGACGTCA-3'). The DNA binding affinities and transactivation capacities of the Jun proteins vary considerably, with c-Jun exhibiting the highest activation potential (Ryseck and Bravo, 1991). Heterodimerization with c-Fos further increases c-Jun's transcriptional capacity through formation of more stable dimers (Halazonetis *et al.*, 1988; Kouzarides and Ziff, 1988), while heterodimerization with JunB attenuates it (Chiu *et al.*, 1989; Schutte *et al.*, 1989a). Thus differential expression of AP-1 proteins in response to extracellular stimuli was suggested as one of the major mechanisms that modulate AP-1 activity (Chiu *et al.*, 1989). Indeed the c-Jun–JunB antagonism was recently confirmed to play an important role in determining the biological functions of AP-1 complexes containing these proteins (Bakiri *et al.*, 2000; Passegue and Wagner, 2000; Szabowski *et al.*, 2000). Phosphorylation of AP-1 proteins further modulates their activity and provides another route for extracellular stimuli to regulate AP-1 activity (Karin, 1995, 1998).

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The number of ancillary proteins found to interact with AP-1 components and regulate their transcriptional capacity is large and seems to be increasing (Karin *et al.*, 1997). Understanding the physiological significance of all of these interactions and the mechanisms by which they modulate AP-1 activity are major challenges for further research.

Regulation of AP-1 by physiological and pathophysiological stimuli

AP-1 was identified as a transcription factor that contributes both to basal gene expression (Lee *et al.*, 1987a), as well as TPA-inducible gene expression (Angel *et al.*, 1987). However, it was soon recognized that many other stimuli, most notably serum (Lamph *et al.*, 1988; Ryder and Nathans, 1988), growth factors (Quantin and Breathnach, 1988; Ryder and Nathans, 1988; Wu *et al.*, 1989) and oncoproteins, such as v-Src or Ha-Ras (Angel and Karin, 1991), are also potent inducers of AP-1 activity. Soon thereafter, AP-1 activity was found to be induced by tumor necrosis factor [TNF α ; (Brenner *et al.*, 1989)] and interleukin 1 [IL-1; (Goldgaber *et al.*, 1989; Muegge *et al.*, 1989)]. The findings that growth factors and tumor promoters induce AP-1 activity, and c-Jun and c-Fos are encoded by protooncogenes immediately suggested that AP-1 activity is likely to be important in growth control as well as play a key role in transformation. In addition, the response to proinflammatory cytokines (TNF α and IL-1), as well as the finding of AP-1 target genes such as collagenase (Angel *et al.*, 1987) and IL-2 (Muegge *et al.*, 1989; Serfling *et al.*, 1989), suggested that AP-1 is also likely to be involved in inflammation and innate immune response.

Several mechanisms account for stimulation of AP-1 activity by growth factors, proinflammatory cytokines and UV radiation (Karin, 1995). The most important mediator of the growth factor response is likely to be the ERK MAP kinase (MAPK) cascade, which through phosphorylation of ternary complex factors [TCFs; (Hill *et al.*, 1994)], causes induction of *fos* genes, whose products then heterodimerize with Jun proteins to form more stable AP-1 dimers. Through AP-1 sites in the *c-jun* promoter, these newly formed Jun:Fos heterodimers can lead to increased *c-jun* transcription (Angel *et al.*, 1988b). Erk activation may also contribute to *c-jun* induction through MEF2 proteins, another group of transcription factors that bind to the c-Jun promoter (Han and Prywes, 1995).

The responses to proinflammatory cytokines and UV radiation, on the other hand, are mostly dependent on two other MAPK cascades, JNK and p38 (Karin, 1995). In addition to phosphorylation of TCFs and *fos* gene induction, p38s contribute to *c-jun* gene induction through phosphorylation of MEF2c (Han *et al.*, 1997). A major role in *c-jun* induction by UV is played by the JNKs as they phosphorylate and enhance the transcriptional activity of two major players in *c-jun*

expression, c-Jun itself and ATF2 (Karin, 1995). In all of these cases the primary phosphorylation event results in potentiation of transactivation without exerting a considerable effect on DNA binding activity (Karin, 1995).

In addition to physiological stimuli AP-1 activity is induced by a variety of environmental stresses, most notably short wavelength UV radiation (Karin, 1998). Exposure of cultured cells to short wavelength UV light (UVC or UVB) results in induction of a number of immediate early genes, including *fos* and *jun* (Herrlich *et al.*, 1992; Holbrook and Fornace, 1991). In fact, the *c-jun* protooncogene is one of the most UV-responsive genes identified thus far (Devary *et al.*, 1991). UV exposure results in rapid and vigorous JNK activation (Hibi *et al.*, 1993) eventually leading to phosphorylation of c-Jun and ATF2, which enhances their transcriptional capacity (Gupta *et al.*, 1995; Karin, 1995). The binding of c-Jun:ATF2 heterodimers to divergent AP-1 sites within the *c-jun* promoter results in its transcriptional activation (Devary *et al.*, 1992; Herr *et al.*, 1994; Karin, 1995; van Dam *et al.*, 1993). The robustness of both UV-mediated JNK activation and *c-jun* induction, suggest that JNK and c-Jun may have evolved to mediate a physiologically important response to UV exposure. After all, the first organism that had adjusted to life out of water must have been able to deal with the increased risk of UV exposure, which was rather high in the early days of life on earth. Thus, in addition to being involved in mitogenic and proinflammatory responses, AP-1 factors (at least those containing c-Jun and ATF2) are likely to play an important role in the UV response (Herrlich *et al.*, 1997).

AP-1, transformation and growth regulation

Identification of c-Fos and c-Jun, the mammalian homologs of the retroviral oncoproteins v-Fos (van Straaten *et al.*, 1983) and v-Jun (Maki *et al.*, 1987), as components of AP-1 (Angel *et al.*, 1988a; Bohmann *et al.*, 1987; Chiu *et al.*, 1988; Rauscher *et al.*, 1988) immediately linked AP-1 to cellular growth control and neoplasia. This 'guilt by association' was reinforced by the knowledge that AP-1 activity is stimulated by phorbol ester tumor promoters and growth factors. At that time it was already known that deregulated overexpression of c-Fos was sufficient to induce transformation of immortalized rat fibroblasts (Miller *et al.*, 1984). Unlike most other oncogenes studied at the time, *c-fos* did not require any substantial changes in its coding region to unleash its oncogenic power. Overexpression of the normal protein was sufficient. Further experiments have shown that even a fragment of v-Fos composed mostly of its bZIP region (and therefore essentially identical in sequence and function to the equivalent portion of c-Fos) is sufficient for immortalization and transformation of chicken embryo fibroblasts (CEF) (Jenuwein and Muller, 1987; Yoshida *et al.*, 1989). Presumably by forming stable hetero-

dimers with c-Jun, the bZIP region of v-Fos increases the binding of c-Jun to target genes whose activation results in transformation. Given its avian retroviral origin it was not surprising that overexpression of v-jun gene was also found to transform CEF (Bos *et al.*, 1990).

Based on the c-Fos paradigm, subsequent experiments were aimed to compare the oncogenic ability of cellular Jun proteins to that of v-Jun. In non-immortalized rat embryo fibroblasts (REF) overexpression of mammalian or avian c-Jun is insufficient for induction of transformation (Schutte *et al.*, 1989a). In fact, overexpression of c-Fos does not transform REF either (Schutte *et al.*, 1989a). However, Ha-ras induced transformation of immortalized mouse fibroblasts requires c-Jun expression (Johnson *et al.*, 1996). These results are consistent with earlier studies indicating that c-jun can cooperate with Ha-ras in transformation of REF (Schutte *et al.*, 1989a,b). Presumably, expression of oncogenic Ha-ras results in activation of ERK and JNK, leading to elevated expression of Fos proteins and N-terminal phosphorylation of c-Jun, both of which increase the transcriptional activity of the latter (Binetruy *et al.*, 1991; Karin, 1995). Unlike CEF where v-jun is a much more potent oncogene than c-jun (Bos *et al.*, 1990), v-jun is considerably less potent than c-jun in cooperating with Ha-ras in transformation of REF (Alani *et al.*, 1991). These differences are consistent with the absence of JNK phosphorylation sites on v-Jun and its inability to respond to Ha-Ras expression with increased transactivation potential (Binetruy *et al.*, 1991; Hibi *et al.*, 1993). JunB and JunD are also much less potent than c-Jun in cooperation with Ha-Ras and their reduced transforming activity correlates with their lower transcriptional activity (Chiu *et al.*, 1989; Deng and Karin, 1993; Schutte *et al.*, 1989a; Vandel *et al.*, 1996). Furthermore, unlike c-Jun whose transactivation potential is highly responsive to JNK activation (Binetruy *et al.*, 1991), JunB and JunD are less responsive (Kallunki *et al.*, 1996). Coexpression of c-Fos further enhanced the transforming capacity of c-Jun, whereas JunB attenuated it (Schutte *et al.*, 1989a). These differences are consistent with higher DNA binding and transcriptional activity of c-Jun:c-Fos heterodimers in comparison to c-Jun:JunB heterodimers (Deng and Karin, 1993). Overexpression of c-Jun alone is sufficient for transformation of immortalized rodent fibroblasts, the Rat1a cell line, whereas overexpression of JunB does not transform these cells (Schutte *et al.*, 1989b). Furthermore overexpression of JunB in mouse fibroblasts significantly reduced transformation by v-ras or v-src (Passegue and Wagner, 2000). Overexpression of JunD in mouse fibroblasts also partially suppresses transformation by oncogenic Ha-ras (Pfarr *et al.*, 1994).

Whereas direct and positive correlation between transcriptional activation and DNA binding activities of Jun and Fos proteins and their transforming potential is seen in rodent fibroblasts, such a correlation has not been observed in avian fibroblasts. While v-Jun is a less potent transcriptional activator in

most mammalian cells (Angel and Karin, 1991), and does not respond to JNK (Hibi *et al.*, 1993), it is 15–25-fold more efficient than c-Jun in CEF transformation (Bos *et al.*, 1990). Furthermore, mutations that reduced the activation potential of c-Jun in both mammalian and avian cells increase its transforming potential in the latter (Havarstein *et al.*, 1992). At this point it is not fully clear why reduced transactivation potential results in higher transforming activity in avian cells. It is possible that beyond a certain threshold, AP-1 activates the expression of growth inhibitory on cell death genes (see below). This threshold may be much lower in avian cells than in rodent cells. Furthermore, by providing survival and growth promoting signals, Ha-Ras may suppress the effect of c-Jun-induced growth inhibitors or activators of apoptosis such as TGF β (Kim *et al.*, 1990) or Fas ligand (Kasibhatla *et al.*, 1998).

Deregulated expression of Jun and Fos proteins can induce transformation *in-vivo*. Transgenic mice expressing c-Fos developed bone lesions, as early as 4 weeks after birth, that progressed into bone tumors in 15% of the animals by 9–10 months of age (Rüther *et al.*, 1987, 1989). Conversely the development of malignant skin tumors following TPA treatment in mice carrying a v-H-ras transgene, is inhibited in a c-fos^{-/-} genetic background (Saez *et al.*, 1995). Similar inhibition of malignant transformation is observed in mice expressing a dominant negative c-Jun transgene subjected to a two stage skin carcinogenesis protocol (Young *et al.*, 1999).

The transforming activity of c-Jun and c-Fos suggests that AP-1 complexes containing these components are involved in stimulation of cell proliferation. However, transformation or tumor promotion may also result from decreased cell death. More direct evidence for the involvement of certain AP-1 complexes in stimulation of cell proliferation was initially derived from experiments in which expression or function of AP-1 components was blocked via antisense RNA or antibody microinjection. Antisense oligonucleotides specific for c-fos or c-jun were found to inhibit the proliferation of mouse fibroblasts and erythroleukemia cells, respectively (Holt *et al.*, 1986; Nishikura and Murray, 1987; Smith and Prochownik, 1992). Microinjection of antibodies against Fos and Jun proteins demonstrated that AP-1 activity is required for serum stimulated cell cycle re-entry of serum starved cells (Kovary and Bravo, 1991; Riabowol *et al.*, 1988). AP-1 activity may also be required for cell cycle progression in serum fed cells (Kovary and Bravo, 1991). Microinjection of antibodies to any of the Fos proteins only partially inhibited S phase entry suggesting that none of them is individually essential for cell cycle progression. Indeed, a cocktail of all Fos antibodies was very efficient in inhibiting cell cycle progression (Kovary and Bravo, 1991). Curiously, antibodies against any of the three Jun proteins were sufficient to inhibit cell cycle progression (Kovary and Bravo, 1991). As discussed below, these results are inconsistent with more recent analysis based on gene disruption

experiments that points out a stimulatory function for c-Jun and JunD and an inhibitory function for JunB. The experimental design used in these experiments did not provide data about cell cycle transition points that may depend on AP-1 activity, with the exception of the G0/G1 interphase. Although many results suggest that c-Jun is required for transit beyond the G1/S interphase (Schreiber *et al.*, 1999; Smith and Prochownik, 1992), a recent study suggests that c-Jun might also have a role in allowing UV irradiated cells to re-enter the cell cycle from the G2/M interphase (Shaulian *et al.*, 2000). Interestingly, c-Jun expression levels do not significantly change during the cell cycle, but the protein undergoes transient N-terminal phosphorylation as cells proceed from G2 to M that persists until the cells complete mitosis (Bakiri *et al.*, 2000). It is not clear however, whether these changes result in cell cycle dependent regulation of AP-1 transcriptional activity.

Generation of mice and cells deficient in individual AP-1 proteins presents a more rigorous way to study their physiological functions than antisense RNA or antibody microinjection. Consistent with the inability of anti-c-Fos antibodies to inhibit cell cycle progression, fibroblasts and ES cells lacking c-Fos proliferate normally (Brüsselbach *et al.*, 1995). These results suggest that other Fos proteins might compensate for the absence of c-Fos. Indeed, fibroblasts deficient in both c-Fos and FosB exhibit reduced proliferation and *c-fos*^{-/-} *fosB*^{-/-} mice are about 30% smaller than wt littermates or the corresponding single mutants (Brown *et al.*, 1998). c-Fos-deficient fibroblasts are also partially defective in re-entering the cell cycle after exposure to UV radiation (Schreiber *et al.*, 1995).

By contrast to cells deficient in individual Fos proteins, fibroblasts lacking a single Jun protein, with the exception of JunB, exhibit significantly altered growth properties. The most severe defects are exhibited by *c-jun*^{-/-} fibroblasts, which can be passed only once or twice in culture before they exhibit a pseudo-senescent phenotype and their cell cycle transit time increases dramatically (Johnson *et al.*, 1993; Schreiber *et al.*, 1999; Wisdom *et al.*, 1999). Re-introduction of c-Jun into such cells results in increased proliferation, indicating that the proliferation defect is indeed due to the absence of c-Jun. A less severe proliferation defect is exhibited by fibroblasts that express a variant of c-Jun in which the JNK phosphoacceptor sites were replaced by alanines (*c-Jun*^{A63/73}) (Behrens *et al.*, 1999). Although *c-Jun*^{A63/73} mice develop normally and are viable, unlike *c-jun* deficient mice which die *in utero* (Hilberg *et al.*, 1993; Johnson *et al.*, 1993), they are smaller than wt mice (Behrens *et al.*, 1999). A proliferation defect is also exhibited by immortalized *c-jun*^{-/-} fibroblasts and again, this defect can be rescued by re-introduction of c-Jun (Schreiber *et al.*, 1999). Immortalized fibroblasts lacking *c-jun* also exhibit defective cell cycle re-entry after UV-irradiation (Shaulian *et al.*, 2000). Unlike c-Jun expressing cells, the *c-jun*^{-/-} fibroblasts undergo a prolonged cell cycle arrest after UV

exposure and exhibit a senescent phenotype (E. Shaulian and M. Karin unpublished data).

A correlation between decreased proliferative potential and reduced body weight was also observed in *junD*^{-/-} mice (Thepot *et al.*, 2000; Weitzman *et al.*, 2000). *junD*^{-/-} MEFs exhibit a proliferation defect that is not as severe as that of *c-jun*^{-/-} cells (Weitzman *et al.*, 2000). However, by contrast to c-Jun-deficient fibroblasts, immortalized JunD-deficient fibroblasts show increased proliferation and are more sensitive to p53-dependent apoptosis after UV exposure (Weitzman *et al.*, 2000). In view of the striking resemblance of the behavior of *junD*^{-/-} immortalized fibroblasts to that of c-Jun overexpressing cells (Shaulian *et al.*, 2000), it is possible that immortalized *junD*^{-/-} cells express much higher levels of c-Jun than wt cells, to compensate for the absence of JunD.

Disruption of the *junB* gene does not change the proliferation rates of embryonic stem cells (ES) or fibroblasts (Schorpp-Kistner *et al.*, 1999). Re-introduction of a *junB* transgene into the *junB*^{-/-} background rescues JunB-deficient mice from early embryonic lethality, which is caused by defective placentation (Passegue *et al.*, 2001; Schorpp-Kistner *et al.*, 1999). However, specific extinction of the transgene expression in the myeloid lineage resulted in progressive myeloid hyperplasia with over-production of granulocytic progenitors (Passegue *et al.*, 2001). This hyperproliferative disease progresses to blast crisis, resembling human chronic myelogenous leukemia. Most importantly, these findings afforded the specific analysis of JunB function in granulocytic progenitors. Apparently, the loss of JunB expression is directly responsible for the hyperproliferative phenotype of the granulocytic progenitors as re-expression of JunB fully reverts the hyperproliferative phenotype and induces formation of terminally differentiated granulocytes (Passegue *et al.*, 2001). These results indicate that JunB is a negative regulator of cell proliferation. Correspondingly, fibroblasts from transgenic JunB mice that express 5–10-fold more JunB than normal, exhibit reduced proliferative capacity (Passegue and Wagner, 2000). In addition, expression of endogenous JunB increases in normal MEFs as the cells get closer to entering senescence (Passegue and Wagner, 2000). Immortalized JunB transgenic fibroblasts also proliferate slower than normal fibroblasts due to an extended G1 phase (Passegue and Wagner, 2000). In addition, the growth inhibitory cytokine TGFβ is a more potent inducer of *junB* than *c-jun* transcription (Laiho *et al.*, 1991). It would be of interest to examine whether JunB contributes to the growth inhibitory activity of TGFβ.

Recently it was demonstrated that the antagonistic effects of c-Jun and JunB on cell proliferation are not exclusively cell autonomous. Significant differences were found in the proliferation and differentiation of primary human keratinocytes grown on wt, *c-jun*^{-/-} or *junB*^{-/-} mouse fibroblasts (Szabowski *et al.*, 2000). Whereas culturing on wt mouse fibroblasts results in proper differentiation of human keratinocytes leading to formation of stratified epithelium that looks like

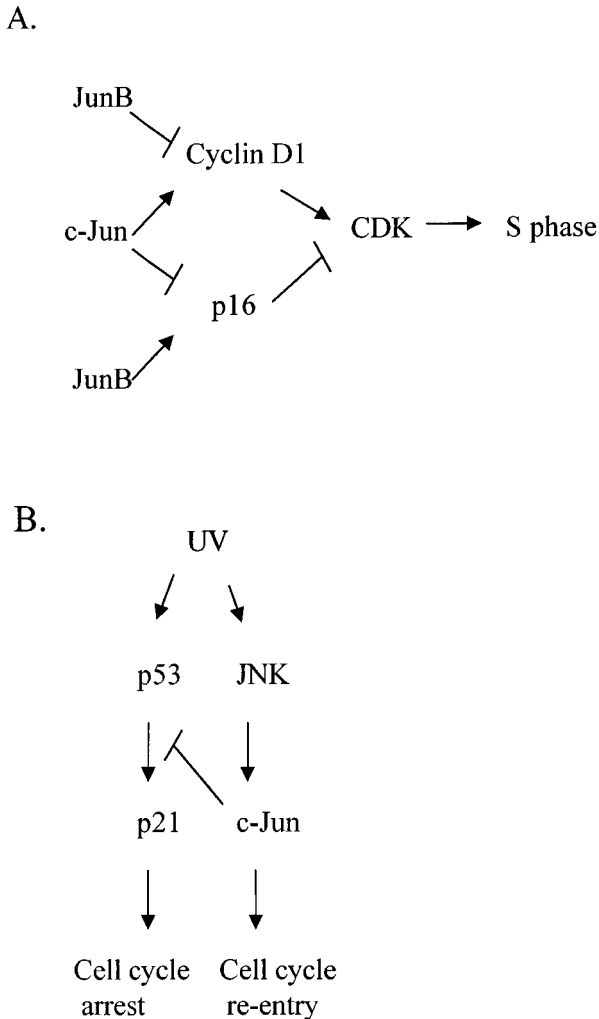


Figure 1 c-Jun and JunB have antagonistic effects on expression of target genes controlling cell proliferation (a), and the exit from UV induced cell cycle arrest (b)

normal epidermis, cultivation on *c-jun*^{-/-} fibroblasts results in formation of an aberrant epithelium containing fewer cell layers and a reduced number of proliferating cells in the basal layer. By contrast, cultivation on *junB*^{-/-} fibroblasts resulted in hyperproliferation and formation of an epithelium with an increased number of cell layers (Szabowski *et al.*, 2000). These different effects of c-Jun and JunB were attributed to the secretion of cytokines such as keratinocyte growth factor (KGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), which are underproduced by *c-jun*^{-/-} fibroblasts and overproduced by *junB*^{-/-} fibroblasts (Szabowski *et al.*, 2000). This *in vitro* skin formation model provides a primary example for the antagonistic effects of c-Jun and JunB, confirming the initial model which suggested transcriptional antagonism between the proteins and was based on transient transfection experiments (Chiu *et al.*, 1988). Data discussed below demonstrate that the model is also valid for the genes coding for p16 and

cyclin D1 (Bakiri *et al.*, 2000; Passegue and Wagner, 2000). In summary, the analysis of gene targeted animals and cell cultures indicate that c-Fos and c-Jun are growth promoting components of AP-1, whereas JunB is a negative regulator of cell proliferation. The role of JunD in the control of cell proliferation is more complex, being growth promoting under some conditions and inhibitory under other conditions.

AP-1 in apoptosis and survival

AP-1 transcription factors have also been implicated in the control of cell death and survival. The involvement of growth promoting proteins in apoptosis has been previously demonstrated for c-Myc (Evan *et al.*, 1992) and E2F1 (Qin *et al.*, 1994; Wu and Levine, 1994). However, among growth regulators AP-1 is somewhat unusual because in addition to being responsive to growth factors it is also upregulated by genotoxic stresses, such as UV or alkylating agents, that cause growth arrest and/or cell death. Furthermore, the consequence of AP-1 activation seems to be cell type specific. While it may promote apoptosis in some cell types, it is required for the survival of others. Of course, given the many different forms of AP-1, its exact functions are likely to be dependent on its composition post translational modifications and presence of interacting factors.

The clearest data regarding the involvement of AP-1 in induction of apoptosis comes from studies on the nervous system. Examination of brains of *c-fos-lacZ* transgenic mice after administration of kainic acid, a potent activator of glutamate receptors, revealed that continuous c-Fos expression precedes and correlates with neuronal cell death (Smeyne *et al.*, 1993). Further studies using PC12 pheochromocytoma cells and cultured neuronal cells showed that inhibition of c-Jun activity with a dominant negative mutant that lacks the c-Jun N-terminal activation domain or by neutralizing antibodies, can protect the cells from apoptosis induced by withdrawal of nerve growth factor (NGF) or chronic depolarization (Estus *et al.*, 1994; Ham *et al.*, 1995; Le-Niculescu *et al.*, 1999; Xia *et al.*, 1995). Ectopic expression of c-Jun or c-Fos can induce apoptosis in sympathetic neurons as well as in mouse fibroblasts, Syrian hamster embryo (SHE) cells and a human colorectal carcinoma (RKO) cell line (Bossy-Wetzel *et al.*, 1997; Ham *et al.*, 1995; Preston *et al.*, 1996). However, it is important to realize that the levels of c-Jun and c-Fos achieved in such experiments are very high and are probably of little physiological relevance. Indeed, c-Jun and c-Fos can be induced by physiological or pathological stimuli that not necessarily lead to neuronal apoptosis (Herdegen *et al.*, 1998). The JNK pathway has also been linked to induction of apoptosis in neuronal cells (Le-Niculescu *et al.*, 1999; Xia *et al.*, 1995). Expression of the phosphorylation deficient c-Jun^{A63/73} mutant blocked apoptosis induced by NGF withdrawal in cultured neuronal cells as

effectively as treatment with various JNK inhibitors (Le-Niculescu *et al.*, 1999; Watson *et al.*, 1998). It seems that c-Jun is an important mediator of proapoptotic effects of persistent JNK activation. Indeed, both AP-1 activation and kainate induced apoptosis are reduced in the hippocampus of *Jnk3*^{-/-} mice (Yang *et al.*, 1997). Most importantly, resistance to kainate induced cytotoxicity was also observed in *c-jun*^{A63/73} knock-in mice (Behrens *et al.*, 1999), thereby providing strong genetic support for the role of N-terminal c-Jun phosphorylation in this apoptotic pathway. Nevertheless, it should be emphasized that there are many physiological and pathological conditions that lead to JNK activation and c-Jun N-terminal phosphorylation in the CNS that do not result in apoptosis (Kenney and Kocsis, 1998; Xu *et al.*, 1997; Herdegen *et al.*, 1998).

Another setting in which AP-1 proteins may be involved in induction of apoptosis is after exposure of cells to genotoxic stress. An early study demonstrated that the dominant negative c-Jun mutant can reduce apoptosis in human monoblastic leukemia cells after exposure to various DNA damaging agents (Verheij *et al.*, 1996). Both *c-jun*^{-/-} fibroblasts and *Jnk1*^{-/-} *Jnk2*^{-/-} double mutant mouse fibroblasts, were found to be resistant to apoptosis induced by UVC radiation (Shaulian *et al.*, 2000; Tournier *et al.*, 2000). *c-jun*^{-/-} fibroblasts are also resistant to the apoptotic effects of alkylating agents (Kolbus *et al.*, 2000). However, as shall be discussed below the normal physiological function of c-Jun or JNK even in the context of a stress response is not necessarily the induction of apoptosis.

In contrast to apoptosis induced by survival factor withdrawal or genotoxic stress, AP-1 does not promote apoptosis during normal development. *In vivo* observations suggest that AP-1 proteins may in fact have a protective role during embryonic development, as c-Jun deficient embryos exhibit massive increase in liver cell apoptosis (Eferl *et al.*, 1999), which may be the cause of their lethality (Hilberg *et al.*, 1993). Embryos deficient in both JNK1 and JNK2 exhibit an open neuronal tube, a result of dysregulated neuronal apoptosis (Kuan *et al.*, 1999). However, reduced apoptosis was observed only in certain areas of the developing brain, while increased apoptosis was observed in other regions (Kuan *et al.*, 1999; Sabapathy *et al.*, 1999). *In vitro* studies demonstrated that JNK activation is not required for TNF-induced apoptosis (Liu *et al.*, 1996), and other experiments suggested that JNK kinase 1 (JNKK1, SEK1 or MKK4) has a protective role in Fas induced apoptosis (Nishina *et al.*, 1997), and that TNF induced JNK activation may be protective (Natoli *et al.*, 1997).

A key question is how does AP-1 mediate its proapoptotic effects in those cases where it is positively involved in the process? One possibility is that AP-1 directly activates the transcription of genes, whose products can trigger apoptosis. Indeed, several reports suggest that AP-1 induces expression of the Fas-ligand (FasL) gene (Kasibhatla *et al.*, 1998; Kolbus *et al.*,

2000; Le-Niculescu *et al.*, 1999). However there is no clear explanation why some activators of AP-1 lead to FasL induction while others do not. Another possibility is that AP-1 has a homeostatic function that reacts to changes in growth and environmental conditions to adjust the gene expression profile in a way that allows the cell to adapt to the new environment. However, after excessive environmental stress, a conflict may arise between the AP-1 regulated program and other stress activated gene expression programs that may cause a catastrophe that culminates in cell death. For instance, exposure to UV radiation, as other genotoxic stresses, results in stabilization of p53 and induction of p53 target genes, such as *p21*^{cip1/waf1} whose product leads to growth arrest (Vogelstein *et al.*, 2000; Vousden, 2000). However, UV exposure also results in JNK activation and c-Jun induction (Devary *et al.*, 1991; Hibi *et al.*, 1993). Prolonged expression of c-Jun overcomes the growth inhibitory effect of p53 by downregulating *p21*^{cip1/waf1} transcription, thereby promoting p53-mediated apoptosis or a mitotic catastrophe (Shaulian *et al.*, 2000; Figure 1). Several observations support this model. The first is the finding that only persistent, but not transient, JNK activation is linked to apoptosis (Chen *et al.*, 1996). Persistent JNK activation in cells exposed to moderate to high levels of UVC (12–40 J/m²) can lead to c-Jun induction lasting 24 h or longer (Shaulian *et al.*, 2000). The proapoptotic role of p53 is well established and supported by analysis of *p53*^{-/-} mice (Lowe *et al.*, 1993). However, after low to moderate levels of genotoxic damage p53 induction is more likely to cause growth arrest (Lane, 1992), and thus endow the damaged cells with some protection and time for damage repair (Bissonnette and Hunting, 1998; Sheikh *et al.*, 1997). Indeed, *p21*^{-/-} cells, which can not undergo p53 dependent growth arrest are more susceptible to apoptosis induced by DNA damaging agents than wt cells (Waldman *et al.*, 1996). Thus by repressing *p21*^{cip1/waf1} transcription, elevated c-Jun expression favors the proapoptotic function of p53 (Shaulian *et al.*, 2000). The interaction between AP-1 and p53 is reciprocal, as downregulation of p53 reduced the proapoptotic effects of c-Fos in RKO cells (Preston *et al.*, 1996).

Nevertheless, the two modes of action discussed above are not mutually exclusive and multiple effects of AP-1 might be expected. In conclusion, AP-1 activity seems to be regulated by many stimuli and in turn activate different gene expression programs. While in some cases AP-1 activation promotes cell proliferation, in other cases it may increase cell survival and under extreme conditions it actually contributes to cell death.

AP-1 regulated genes involved in growth regulation and apoptosis

The analysis of cells and mice deficient in individual AP-1 proteins has also resulted in the identification of physiologically relevant target genes, some of which are

differentially regulated in a manner that depends on the composition of AP-1 (Table 1 and Figure 1). The following discussion is focused on target genes that may be involved in proliferation and apoptotic functions of AP-1.

Cyclin D proteins are regulators of G1 to S phase transition that bind to the CDK4 cyclin dependent kinase and increase its kinase activity, thereby causing the phosphorylation and inactivation of the retinoblastoma (Rb) tumor suppressor protein (reviewed by Sherr, 1996). The human cyclin D1 gene (*CycD1*) regulatory sequences contain two AP-1 binding sites (Albanese *et al.*, 1995; Herber *et al.*, 1994). Several AP-1 proteins including c-Jun, JunB, c-Fos and ATFs, were shown to bind these sites and suggested to regulate *CycD1* transcription (Bakiri *et al.*, 2000; Beier *et al.*, 1999; Brown *et al.*, 1998; Wisdom *et al.*, 1999). As predicted from its proliferative effects, c-Jun was found to induce *CycD1* transcription in transient transfection assays (Albanese *et al.*, 1995; Bakiri *et al.*, 2000; Herber *et al.*, 1994; Wisdom *et al.*, 1999). Phosphomimicking mutations that replace serines 63 and 73 of c-Jun with negatively charged residues further increased the extent of *CycD1* induction, whereas substitution of these serines with leucines decreased the ability of c-Jun to activate *CycD1* transcription (Bakiri *et al.*, 2000). By contrast, but in perfect correlation with its biological role as a negative growth inhibitor, JunB inhibits *CycD1* transcription in a dose-dependent manner (Bakiri *et al.*, 2000). Although *c-jun*^{-/-} MEFs express less cyclin D1 and cyclin D3 than wt counterparts, no significant differences in cyclin D1 expression were detected between immortalized wt and *c-jun*^{-/-} fibroblasts (Schreiber *et al.*, 1999; Wisdom *et al.*, 1999). c-Fos or FosB cooperate with c-Jun to activate the *CycD1* gene, as the induction of cyclin D1 expression by serum is more efficient in wt cells than in *c-fos*^{-/-} *fosB*^{-/-} double mutant cells (Brown *et al.*, 1998). Curiously, in addition to promotion of cell proliferation, cyclin D1 was reported to induce cell death when overexpressed in certain cell types (Freeman *et al.*, 1994; Kranenburg *et al.*, 1996).

A pivotal question is whether cyclin D1 overexpression can substitute for c-Jun expression? In other words, is cyclin D1 the major mediator of the growth promoting effect of c-Jun-containing AP-1 complexes? To answer this question Wisdom and coworkers transduced cyclin D1 or c-Jun into *c-jun*^{-/-} MEFs and compared the ability of the two proteins to restore DNA synthesis in these growth-impaired cells (Wisdom

et al., 1999). It was found that cyclin D1 overexpression restored only 25–30% of the DNA synthesis induced by c-Jun. Therefore, other c-Jun target genes are likely to be involved in its growth promoting activity.

Another important c-Jun target gene that had recently surfaced is *p53* (Schreiber *et al.*, 1999; Shaulian *et al.*, 2000). However, whereas cyclin D1 is a positive growth regulator, *p53* is a negative growth regulator (Vogelstein *et al.*, 2000; Vousden, 2000). So how does c-Jun promote cell proliferation via *p53*? The answer is that c-Jun is a negative regulator of both *p53* expression and its ability to activate target gene transcription (Schreiber *et al.*, 1999; Shaulian *et al.*, 2000). The basal level of *p53* expression is higher in *c-jun*^{-/-} fibroblasts than in wt counterparts and stable expression of c-Jun in *c-jun*^{-/-} cells reduces the level of *p53* expression (Schreiber *et al.*, 1999). Further investigation revealed that the effect of c-Jun on *p53* expression is likely to be direct and exerted through a variant AP-1 site in the *p53* promoter (Schreiber *et al.*, 1999). Curiously, while c-Jun is a potent activator of genes that contain AP-1 sites, when bound to the variant AP-1 site within the *p53* promoter it represses rather than activates transcription. Most importantly, the removal of *p53* from *c-jun*^{-/-} cells (achieved by crossing *c-jun*^{+/-} and *p53*^{+/-} mice) completely suppresses their proliferative defect (Schreiber *et al.*, 1999).

The relationship between c-Jun and *p53* is quite extensive and goes well beyond the negative regulation of the *p53* promoter by c-Jun. Despite the difference in basal *p53* expression levels, fibroblasts that either express or lack c-Jun contain the same amounts of *p53* after exposure to UV radiation or other genotoxic stresses (Schreiber *et al.*, 1999; Shaulian *et al.*, 2000). Nevertheless, *c-jun*^{-/-} cells express higher levels of *p53* regulated gene products, especially *p21*^{cip1/waf1}, after UV exposure, whereas cells that express c-Jun constitutively express very low levels of *p53* target genes (Shaulian *et al.*, 2000). Because of the prolonged expression of *p21*^{cip1/waf1}, *c-jun*^{-/-} fibroblasts are extremely sensitive to *p53* induced growth arrest after UV exposure. The majority of *c-jun*^{-/-} cells undergo growth arrest rather than return to the cell cycle within 24–48 h, as wt cells do. Conversely, cells that express c-Jun constitutively fail to express sufficient levels of *p21*^{cip1/waf1} and therefore do not undergo *p53*-mediated growth arrest after UV exposure (Shaulian *et al.*, 2000). Although the mechanism by which c-Jun represses *p53*-mediated gene induction is not fully understood, this repressive activity of c-Jun seems to account for most of its growth regulatory functions in UV irradiated cells (Shaulian *et al.*, 2000). Most importantly, these findings strongly suggest that the major function of *c-jun* induction in UV irradiated cells is to repress *p53*-mediated *p21*^{cip1/waf1} induction and thereby allow growth arrested cells to re-enter the cell cycle. As JNK activity is particularly responsive to UV radiation and necessary for *c-jun* induction, the JNKs are also important components of this regulatory loop, that lies at the core of the mammalian UV response.

Table 1 The effect of Jun proteins on AP-1 target genes involved in cell proliferation and apoptosis

Gene	<i>c-Jun</i>	<i>JunB</i>	<i>JunD</i>
<i>Cyclin D1</i>	Up	Down	–
<i>p16</i>	Down	Up	–
<i>p19^{Arf}</i>	–	–	Down
<i>p53</i> and <i>p21</i> ^{cip1/waf1}	Down	Down	–
<i>FasL</i>	Up	–	–

Although constitutive expression of c-Jun at levels equivalent to those achieved after UV exposure reduces p53 and p21^{cip1/waf1} expression (Schreiber *et al.*, 1999; Shaulian *et al.*, 2000), transient overexpression of c-Jun was shown to increase p53 expression (Fuchs *et al.*, 1998). However, the later response most likely represents a non-specific cellular stress response caused by the vast overexpression of almost any transcription factor.

JunD also interacts with the p53 pathway (Weitzman *et al.*, 2000). Expression of p19^{Arf} is upregulated in *junD*^{-/-} MEFs, suggesting that it is negatively regulated by JunD. However, no increase in p53 expression was found in these cells and the mechanism of p19^{Arf} regulation by JunD is yet to be defined (Weitzman *et al.*, 2000). p19^{Arf} acts as a sensor for hyper-proliferative signals generated by oncogenes, such as *Myc*, *E1A*, *Ha-ras* and *E2F-1* (Palmero *et al.*, 1998; reviewed by Sherr, 1998). Once p19^{Arf} is upregulated it interacts with Mdm2 to prevent it from associating with p53 (Sherr, 1998). As the binding of Mdm2 induces degradation and transactivational silencing of p53, the upregulation of p19^{Arf} causes accumulation of transcriptionally active p53 (Sherr, 1998). p19^{Arf} can also inhibit cell proliferation independently of p53 (Weber *et al.*, 2000). Interestingly, the *INK4a* locus is a transcription unit shared by the *p19^{Arf}* and *p16* genes (Quelle *et al.*, 1995) and recent results suggests that JunB regulates *p16* expression (Passegue and Wagner, 2000). High levels of p16 were detected in mouse fibroblasts that express a *junB* transgene (Passegue and Wagner, 2000). The growth inhibitory activity of JunB is dependent in part on p16, as JunB does not inhibit the proliferation of *INK4a*^{-/-} fibroblasts, which are p16 deficient (Passegue and Wagner, 2000). Furthermore, the *p16* promoter contains three AP-1 binding sites and is activated in cells that express JunB (Passegue and Wagner, 2000). Interestingly, c-Jun downregulates *p16* transcription in transient transfection experiments (Passegue and Wagner, 2000), suggesting that *p16* is another target that explains the growth promoting activity of c-Jun. The notion that c-Jun acts as a trans-repressor of tumor suppressor genes to stimulate cell proliferation is very intriguing and deserves further investigation.

In addition to regulation of genes whose products are closely connected to cell cycle and growth control, AP-1 proteins also regulate the expression of genes whose connection to cell cycle is far from being obvious. For instance, c-Fos was recently found to regulate the gene coding for the enzyme 5-methylcytosine transferase, which is responsible for methylation of cytosines that occur within CpG sequences on DNA (Bakin and Curran, 1999). Induction of this enzyme is required for c-Fos induced transformation of immortalized fibroblasts (Bakin and Curran, 1999). Thus altered DNA methylation, perhaps leading to the repression of tumor suppressor genes, is likely to contribute to the transforming capacity of c-Fos.

Another AP-1 regulated gene coding for FasL was suggested to provide an explanation to the pro-

apoptotic capacity of c-Jun. FasL expression is induced by DNA damaging agents including topoisomerase II inhibitors and UV irradiation, agents that activate JNK (Kasibhatla *et al.*, 1998). The *FasL* promoter can be directly activated by c-Jun and c-Fos in transient transfection experiments, through a single AP-1 binding site (Kasibhatla *et al.*, 1998). Additional support for the role of AP-1 in *FasL* induction came from studies showing that *c-jun*^{-/-} fibroblasts, that are relatively resistant to apoptosis induced by UV radiation and alkylating agents, are also impaired in *FasL* expression (Kolbus *et al.*, 2000). The regulation of *FasL* gene expression, however, is likely to be complex as many other stimuli which activate JNK, for instance IL-1 and TNF α , do not result in fibroblast cell death. It is likely that additional pathways need to be activated along with AP-1 in response to genotoxic damage to result in *FasL* induction. Furthermore, *FasL* induction may contribute only in part to genotoxic damage-induced apoptosis, as this process seems to occur with almost normal efficiency in mice that are deficient in either Fas or FADD/MORT1 (Newton and Strasser, 2000; Yeh *et al.*, 1998), both of which are essential mediators of FasL action. As discussed above, another target that mediates the pro-apoptotic activity of c-Jun in UV irradiated cells is p53. By repressing p53-mediated *p21^{cip1/waf1}* induction, high levels of c-Jun prevent UV induced growth arrest and channel most of p53 activity towards the induction of apoptosis (Shaulian *et al.*, 2000). Correspondingly, *c-jun*^{-/-} and *Jnk1*^{-/-} *Jnk2*^{-/-} cells (which are defective in c-Jun induction) are resistant to UV induced apoptosis but retain full sensitivity to other pro-apoptotic stimuli (Shaulian *et al.*, 2000; Tournier *et al.*, 2000).

Conclusions

The discovery of AP-1 as a transcription factor that mediates responsiveness to phorbol ester tumor promoters, and is composed of the cellular homologs of the Jun and Fos oncoproteins, immediately implied its involvement in growth control and oncogenesis. Initially, it was expected that after receiving growth promoting signals AP-1 caused the activation of genes whose products stimulate S phase entry and cell cycle progression. Although further research had provided a great deal of information about the growth promoting signal transduction pathways that control AP-1 activity, positively regulated AP-1 target genes that mediate cell cycle progression were never identified. Now we show that this initial hypothesis was overly simplistic and that pro-mitogenic AP-1 complexes, especially those that contain c-Jun, seems to accomplish their growth promoting function through repression of tumor suppressor genes, such as *p53*, *p21^{cip1/waf1}* and *p16*. It has also become clear that many of the components of AP-1, especially the c-Jun proteins, are not functionally equivalent. As outlined above strong genetic and biochemical evidence support an antagonistic function for c-Jun and JunB in growth control.

Thus, while c-Jun represses the *p16* tumor suppressor gene, JunB activates it. The molecular mechanisms responsible for these fundamental differences in the transcriptional activities of c-Jun and JunB, first observed more than a dozen years ago, deserve further investigation. It is also not clear whether all AP-1 target genes are conversely regulated by the two proteins, as it seems that JunB is simply a less potent activator of certain promoters that are positively regulated by c-Jun.

Part of the problem in assigning a specific and well defined biological function to AP-1, is the large diversity of biological responses regulated by this heterogenous transcription factor. As amply discussed above, in addition to its growth promoting functions AP-1 is also involved in the activation of programmed cell death. Again, the molecular mechanisms that divert the activity of AP-1 from promotion of cell proliferation to induction of apoptosis needs to be further defined. Nevertheless, it seems that AP-1 factors contribute to the activation of the apoptotic program only after extreme conditions, such as potent excitotoxic stimulation of hippocampal neurons, or exposure to rather high levels of UV radiation. While some of the proapoptotic activity of AP-1 is due to transcriptional activation of apoptotic mediator genes, such as *FasL*, a substantial contribution to this biological activity as well, seems to derive from transcriptional repression. For instance, the repression of *p21^{cip1/waf1}* by c-Jun in UV irradiated cells promotes their p53-dependent apoptosis.

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