The EMBO Journal Vol.18 No.6 pp.1559–1570, 1999

Regulation of Rb and E2F by signal transduction cascades: divergent effects of JNK1 and p38 kinases

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The E2F transcription factor plays a major role in cell cycle regulation, differentiation and apoptosis, but it is not clear how it is regulated by non-mitogenic signaling cascades. Here we report that two kinases involved in signal transduction have opposite effects on E2F function: the stress-induced kinase JNK1 inhibits E2F1 activity whereas the related p38 kinase reverses Rb-mediated repression of E2F1. JNK1 phosphorylates E2F1 *in vitro*, and co-transfection of JNK1 reduces the DNA binding activity of E2F1; treatment of cells with TNFα had a similar effect. Fas stimulation of Jurkat cells is known to induce p38 kinase and we find a pronounced increase in Rb phosphorylation within 30 min of Fas stimulation. Phosphorylation of Rb correlated with a dissociation of E2F and increased transcriptional activity. The inactivation of Rb by Fas was blocked by SB203580, a p38-specific inhibitor, as well as a dominant-negative p38 construct; cyclin-dependent kinase (cdk) inhibitors as well as dominant-negative cdks had no effect. These results suggest that Fas-mediated inactivation of Rb is mediated via the p38 kinase, independent of cdks. The Rb/E2F-mediated cell cycle regulatory pathway appears to be a normal target for non-mitogenic signaling cascades and could be involved in mediating the cellular effects of such signals.

Keywords: cell cycle/E2F/Fas/Rb phosphorylation/ transcription

Introduction

The E2F family of transcription factors play a major role in regulating a diverse array of cellular functions including proliferation, differentiation and apoptosis (Cobrinik, 1996; La Thangue, 1996; Nevins, 1998). E2F transcription factors have been shown to be the major downstream targets of the Rb family of proteins, and are necessary for the expression of many genes that are required for cell cycle progression (Nevins, 1992; Dyson, 1994; La Thangue, 1994, 1996). Rb exerts its growth regulatory functions at least in part by inhibiting the transcriptional activity of E2F (Chellappan *et al*., 1991; Helin *et al*., 1992; Kaelin *et al*., 1992; Qin *et al*., 1995). Rb binds to the activation domain of E2F and actively represses transcription from promoters with E2F sites, leading to a cell cycle arrest (Qin *et al*., 1995; Weintraub *et al*., 1995). Conversely, disruption of the Rb gene by deletion or mutation, or inactivation of the Rb protein by phosphorylation or interaction with viral oncoproteins cause the release of free, transcriptionally active E2F, thus permitting unrestricted cell proliferation (Chellappan *et al*., 1992; Nevins, 1993, 1994; Chellappan, 1994).

Overexpression of E2F1 can promote S-phase entry (Johnson, 1993; DeGregori *et al*., 1995) or apoptosis (Shan and Lee, 1994; Adams and Kaelin, 1996), while a dominant-negative E2F1 can induce cell cycle arrest (Hiebert *et al*., 1995). Apparently, E2F1 is the only E2F family member that can induce apoptosis in cells (DeGregori *et al*., 1997), either alone or in association with p53 (Hseih *et al*., 1997; Phillips *et al*., 1997; Kowalik *et al*., 1998). E2F1-mediated apoptosis can be efficiently blocked by the Rb protein (Hseih *et al*., 1997), suggesting that the proliferative as well as pro-apoptotic properties of E2F can be overcome by the Rb protein. Because of the profound effect of Rb on E2F function, the functional status of the Rb protein has considerable influence on E2F activity (Weinberg, 1995).

It has been established that the function of the Rb protein is regulated by phosphorylation mediated by cyclin-dependent kinases (cdks) during normal cell cycle progression (Weinberg, 1995). Cyclin D in association with cdk4 or cdk6 brings about the major inactivating phosphorylation of Rb during mid- to late-G_1 phase (Kato *et al*., 1993; Sherr, 1994). Although there are ~18 potential phosphorylation sites on the Rb protein, cdk4/6 has been shown to target four residues C-terminal to the pocket domain (Taya, 1997). In addition to cyclin D-associated kinases, cyclin E–cdk2 complexes have also been shown to modulate Rb function (Lundberg and Weinberg, 1998). In all cases, phosphorylation of Rb by cdks leads to a dissociation of E2F from Rb, resulting in an increased transcriptional activity. Furthermore, cyclin A and cdk2 have been shown to affect E2F activity directly or through DP1 (Krek *et al*., 1994, 1995; Xu *et al*., 1994).

Since Rb and E2F are vital regulators of G_1–S transition, they appear to be logical targets for multiple signaling cascades. Although Rb is inactivated by phosphorylation upon growth factor stimulation (Sherr, 1994; Weinberg, 1995), it is not clear how other signaling pathways affect Rb and E2F (Reddy, 1994). In this study, we examined whether kinases involved in specific non-proliferative pathways affect Rb and E2F function. We focused our attention on the effects of JNK1 (Hibi *et al*., 1993; Karin, 1994; Minden *et al*., 1994a) and the related kinase p38 (Lee *et al*., 1994) on Rb and E2F function, since these kinases are induced by a variety of non-mitogenic signals (Kyriakis and Avruch, 1996). JNK1 and p38 belong to

the MAP kinase family, members of which are rapidly induced in response to the appropriate signals (Rosette and Karin, 1996). JNK1 is induced in response to various stress stimuli, and p38 kinase is induced by cytokines and other agents such as the Fas receptor. Although both kinases are known to modulate the transcriptional activity of AP1 and to a certain extent ATF2 (Karin, 1994), information with respect to their effects on the components of the cell cycle machinery is scarce. As described below, we observe that JNK1 and p38 have effectively opposite effects on E2F activity: JNK1 could repress E2F activity directly, whereas the p38 kinase efficiently reversed Rb-mediated inhibition of E2F1 activity. Our results suggest that p38 kinase can inactivate Rb, probably by direct phosphorylation, independent of cyclins and cdks. We believe that these are novel mechanisms that are involved in the regulation of Rb and E2F, and appear to be vital links between cell-surface signaling and the cell cycle machinery.

Results

JNK1 and p38 can modulate E2F-mediated transcriptional activity

Transient transfection experiments were performed to examine the effect of JNK1 on E2F activity. E2F1 was co-transfected along with an E2–CAT reporter into Saos-2 human osteosarcoma cells, which are Rb-negative (Hiebert *et al*., 1992). As shown in Figure 1A, co-transfection of JNK1 along with its activator cdc42 totally blocked E2F1-mediated transcription (lane 3), but a kinase-deficient mutant of JNK1 did not (lane 4). JNK1 was as effective as Rb in repressing E2F1 activity, and this repression was observed in a variety of human cell lines (data not shown). Attempts were then made to examine whether agents that induce JNK1 had a similar effect on E2F1 activity; for this reason, Saos-2 cells transiently transfected with E2F1 and E2–CAT reporter were treated with tumor necrosis factor α (TNFα) for 48 h, since TNFα is known to be a strong inducer of JNK1 activity. TNFα treatment led to a total repression of E2F activity (Figure 1A, lane 5), comparable to co-transfection with JNK1, suggesting that overexpression of JNK1 or its induction by cytokine treatment had the same effect.

Next we examined whether JNK1 and TNFα had a similar effect on endogenous E2F activity. Transient transfection of higher amounts (12 μg) of an E2–CAT reporter showed a measurable degree of transcriptional activity (Figure 1B, lane 2); in contrast, transfection of an equal amount of a reporter that carried mutations in the E2F site exhibited no detectable transcriptional activity (Figure 1B, lane 1). This suggests that transcription from the E2–CAT is dependent on a functional E2F site; hence the observed transcription could be taken as a measure of endogenous E2F activity. Co-transfection of JNK1 with the E2–CAT reporter efficiently inhibited the transcription mediated by endogenous E2Fs (Figure 1B, lane 5). A similar effect was observed by treating the transfected cells with TNFα (lane 8). In contrast, JNK1 overexpression as well as TNFα treatment had only minor effects on AP1–CAT or c-fos–CAT vectors under the same conditions (Figure 1B, lanes 9 and 10). This experiment suggests that the repression of E2F activity by JNK1 is a specific event and is not due to a global repression of transcription. It should be noted that although TNFα induces apoptosis in certain cell lines (Fiers *et al*., 1995), Saos-2 cells remained viable (data not shown), suggesting that the reduction in E2F1 activity is not due to a reduction in the number of viable cells.

The JNK-related p38 kinase is induced by specific signals including cytokines (Kyriakis and Avruch, 1996), but it elicits different effects in the cell. For example, signaling from the Fas receptor is known to specifically induce the p38 kinase but not JNK1, and p38 kinase probably contributes to the apoptotic process (Juo *et al*., 1997; Lenczowski *et al*., 1997). Because of their functional differences, we examined the effect of p38 kinase on E2F1-mediated transcription in a co-transfection experiment. As shown in Figure 1C, p38 had no apparent effect on E2F1-mediated transcription (lane 4), but p38 could efficiently reverse Rb-mediated repression of E2F1 (lane 5). This result shows that JNK1 and p38 kinases have markedly different and effectively opposite effects on E2F1 activity. The effects of JNK1 and p38 could be observed even when cdc42 was not included in the transient transfections (Figure 1C).

JNK1 and p38 kinase have opposite effects on cell proliferation

Since E2F1 is a positive modulator of the cell cycle, we attempted to determine whether JNK1 has any effect on cell proliferation. The strategy was to transfect the human osteosarcoma cell line Saos-2 such that it would stably express a wild-type or kinase-inactive JNK1 kinase. The number of antibiotic-resistant colonies that survived after 14 days of selection was taken as a measure of the proliferative capacity of the transfected gene. As shown in Table I, transfection of a pSV-Neo vector resulted in ~200 colonies; however, upon co-transfection of two different amounts of wild-type JNK1 gene, the number of antibiotic-resistant colonies was reduced dramatically. In contrast, a kinase-inactive mutant of JNK1 had no discernible effects on cell proliferation, suggesting that the kinase activity is essential for the anti-proliferative effects. Interestingly, JNK1 was as effective as Rb in repressing colony formation in Saos-2 cells. To assess whether p38 kinase had any effect on the proliferation of Saos-2 cells, a similar co-transfection experiment was performed using cdc42 and p38 kinase. As shown in Table II, p38 kinase had no significant effect on the proliferation of Saos-2 cells, unlike JNK1.

Since p38 kinase was found to reverse Rb-mediated inhibition of E2F, we examined whether p38 could affect the anti-proliferative effects of Rb. In a stable co-transfection experiment (Table II), p38 could efficiently rescue Rb-mediated suppression of colony formation. The reversal was almost total, indicating p38 is as efficient as viral oncoproteins, such as adenovirus E1A or SV40 large T-antigen, in inactivating Rb. The results above point to a direct correlation between the effects of the two kinases on E2F activity and their ability to induce or restrict cell proliferation: JNK1, which inhibits E2F activity, abrogates cell proliferation whereas p38 kinase, which inactivates Rb, reverses Rb-mediated suppression of cell proliferation.

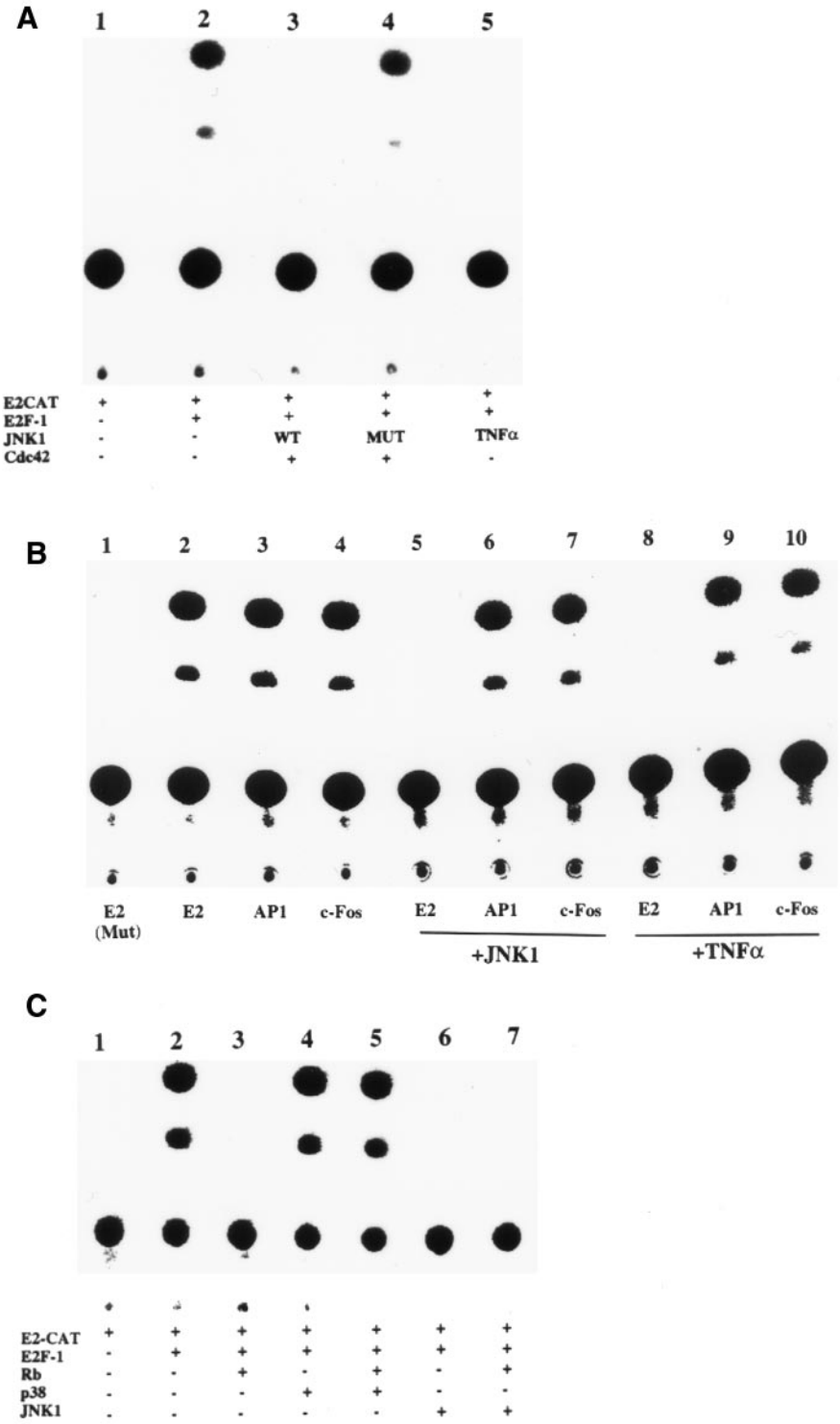

Fig. 1. Differential regulation of E2F function by JNK1 and p38 kinase. (**A**) Saos-2 cells were transiently transfected with 2 μg of E2–CAT reporter alone (lane 1) or along with pDCE2F1 (lane 2). Co-transfection of pSRα JNK1 along with pCMVcdc42 could repress E2F activity (lane 3) but pSRαJNK1-Mut could not (lane 4). Lane 5 shows Saos-2 cells treated with 10 ng/ml of TNFα for 48 h after transfecting with E2–CAT and E2F1. JNK1 co-transfection or TNFα treatment had no effect on a pSVβ-gal vector. (**B**) Saos-2 cells were transfected with 12 μg of E2–CAT vector with mutated E2F sites (lane 1) or intact E2F sites (lanes 2, 5 and 8), or 4 μg or an AP1–CAT (lanes 3, 6 and 9) or c-fos–CAT (lanes 4, 7 and 10). JNK1 was co-transfected in lanes 5–7, and cells were treated with TNFα after transfection in lanes 8–10. (**C**) A co-transfection experiment using pSRαp38 along with E2F1 (lane 4) or E2F1 and Rb (lane 5). JNK1 could repress E2F1-mediated transcription (lanes 6 and 7), whereas p38 reversed Rb-mediated repression of E2F (lane 5).

JNK1 can phosphorylate E2F1 and inhibit its DNA-binding activity

Attempts were made to elucidate the mechanisms by which JNK1 regulates E2F activity. First, the ability of ^{35}S-labeled JNK1 to bind to glutathione *S*-transferase (GST) fusions of E2F1, DP1 and DP2 was analyzed *in vitro*. JNK1 could bind to DP1 as well as c-Jun very efficiently, but there was no binding to E2F1 or DP2

Table I. Suppression of colony formation by JNK1

Vectors transfected	No. of colonies	
	Experiment 1	Experiment 2
pSV-Neo (2 μg)	215	195
pBabe-Puro (2 μg)	198	209
pSV-Neo + pCMV-JNK1WT (1 μg)	35	39
pSV-Neo + pCMV-JNK1WT (2 μg)	22	16
pSV-Neo + pCMV-JNK1Mut (1 μg)	189	179
pSV-Neo + pCMV-JNK1Mut (2 μg)	212	215
pBabe-Puro + Rb (2 μg)	45	42

Approximately 10 000 Saos-2 cells were stably transfected with the indicated vectors. After selection in 40 μg/ml of G418 or 1 μg/ml of puromycin for 14 days, colonies of >20 cells were counted.

Table II. p38 can reverse Rb-mediated suppression of colony formation

Vectors transfected	No. of colonies	
	Experiment 1	Experiment 2
pSV-Neo (2 μg)	209	221
pBabe-Puro (2 μg)	215	229
pSVNeo + pBabePuro	211	225
pBabe-Puro + Rb (2 μg)	31	43
pSRαp38 (2 μg) + pCMV-cdc42 (2 μg)	164	161
pSRαp38 (4 μg) + pCMV-cdc42 (2 μg)	215	159
Puro + Rb + p38 (2 μg) + Cdc42 (2 μg)	208	191
Puro + Rb + p38 (4 μg) + Cdc42 (2 μg)	199	195

Transfections were done as in Table I using the indicated vectors. pBabe-Puro was co-transfected with Rb in the indicated lanes. pCMV-cdc42 vector had a Neomycin resistance marker. Cells transfected with Rb and p38 were subjected to double selection.

under the same conditions (Figure 2A, left panel). We next examined whether JNK1 associates with DP1 *in vivo*. This question was addressed by performing an immunoprecipitation–Western blot experiment. U937 whole-cell extracts (100 μg) were immunoprecipitated with antibodies to c-Myc (as control) or to DP1; a Western blot analysis of the immune complexes could detect JNK1 only in the DP1 immunoprecipitate (Figure 2A, right panel). This indicates that DP1 can associate with JNK1 in mammalian cells, and this interaction can be detected without overexpressing any component. Since JNK1 was found to bind to DP1, we examined the ability of JNK1 to phosphorylate E2F1 or DP1 *in vitro*. Lysates from NIĤ3T3 cells transiently transfected with expression vectors for HA-JNK and cdc42 were immunoprecipitated with an anti-HA antibody, and *in vitro* kinase reactions were performed (Minden *et al*., 1994a,b, 1995; Lenczowski *et al*., 1997) using GST–E2F1 or GST–c-Jun as a substrate. JNK1 could phosphorylate E2F1 efficiently but there was no detectable phosphorylation of DP1 (data not shown). Nevertheless, E2F1 phosphorylation was enhanced in the presence of DP1 (Figure 2B). Extracts from untransfected cells possessed no kinase activity. It appears possible that JNK1 tethers to DP1 and phosphorylates E2F1 in a fashion analogous to AP1, where it tethers to one component and phosphorylates other constituents (Kallunki *et al*., 1996).

Since JNK1 was found to associate with components of the E2F complex and phosphorylate E2F1 *in vitro*, we examined whether JNK1 can be found in association with E2Fs *in vivo*. E2F1 to -5 were expressed in U937 cells, as well as DP1, as detected using a direct Western blot analysis (Figure 2C, lower panel). A co-immunoprecipitation experiment was used to determine whether JNK1 is associated with all E2Fs; whole-cell extracts from the human pro-monocytic U937 cells were immunoprecipitated with antibodies against the five different E2Fs as well as DP1, and the presence of JNK1 in the immunoprecipitates examined by Western blot analysis. As shown in Figure 2C (upper panel), JNK1 could be detected in the immunoprecipitates of all five E2Fs as well as DP1, but not in a control immunoprecipitate where an anti-c-Myc antibody was used. Thus, JNK1 can be detected in association with E2F family members without overexpressing any component. Since JNK1 could not bind to E2F1 *in vitro*, the association of JNK1 with the different E2Fs *in vivo* is probably indirect and via its interaction with DP1.

If indeed JNK1 is regulating E2F function through the mediation of DP1, it should be able to affect the transcriptional activity of all the E2Fs, since DP1 binds to all transcriptionally active E2F family members. A transient transfection experiment was conducted in Saos-2 cells to verify this possibility. As shown in Figure 2D, E2F1 to -5 could induce transcription from the E2–CAT vector; co-transfection of JNK1 efficiently repressed the transcriptional activity of the five E2F family members.

Experiments were designed to elucidate precisely how JNK1 represses E2F-mediated transcription. As a first step, we examined whether an increase in the levels or activity of JNK1 affects the DNA-binding activity of E2F using an electrophoretic mobility shift assay (EMSA). Saos-2 cells were transiently transfected with E2F1 alone or with JNK1; alternately, E2F1-transfected cells were treated with TNFα, and the DNA-binding activity of E2F1 present in the whole-cell extracts was assessed by EMSA (Figure 2E). There was increased E2F binding activity in extracts prepared from cells after transfecting with E2F1 (lane 2) compared with untransfected control cells (lane 1). This band was abolished by an E2F1 monoclonal antibody, confirming its identity (Figure 2E, right panel). It was found that co-transfection of JNK1 could significantly reduce the DNA binding activity of transfected E2F1 (Figure 2E, compare lanes 2 and 3) to levels comparable to those found in untransfected control cells. A similar reduction was also observed in the cells treated with TNFα (lane 4). The DNA-binding activity of E2F in these cells correlates well with transcriptional activity observed (Figure 1A). A Western blot analysis of the extracts shows that the levels of E2F1 in cells transfected with JNK1 do not change (Figure 2F, lanes 2–4, which correspond to the EMSA lanes above), suggesting that the reduced DNA binding activity is due to protein modification rather than a reduction in the level of E2F1. It may be concluded that JNK1 inhibits the transcriptional activity of E2F1 by repressing its DNA binding activity, probably through phosphorylation.

p38 kinase mediates Fas-induced inactivation of Rb

Attempts were made to study further the p38-mediated regulation of Rb and E2F. Since p38 kinase is stimulated

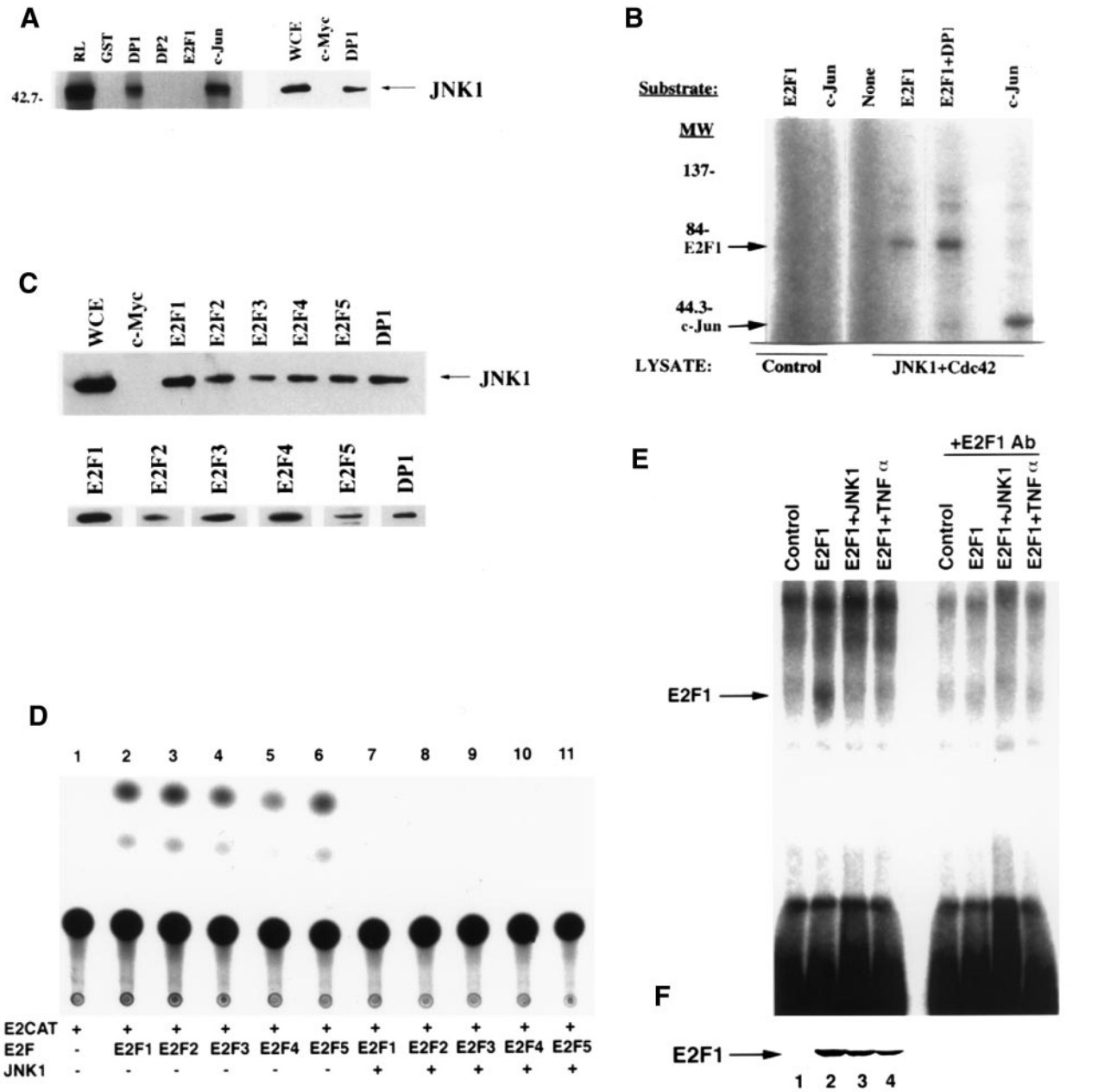

Fig. 2. Mechanism of JNK1-mediated repression of E2F1 activity. (**A**) The binding of ^{35}S-labeled JNK1 to unprimed GST, or GST fusions of DP1, DP2, E2F1 and c-Jun was assessed *in vitro* (left panel). RL is one-fifth of the JNK1 used for binding. JNK1 could bind to DP1 as well as c-Jun, which was the positive control. (Right panel) U937 whole-cell extract (100 μg) was immunoprecipitated with antibodies to c-Myc (as control) and DP1. The immunoprecipitates along with the whole-cell extract were probed for the presence of JNK1 by Western blots. (**B**) An *in vitro* kinase assay for JNK1. Lysates from untransfected 3T3 cells or those transfected with JNK1+cdc42 were immunoprecipitated with an anti-HA antibody, and *in vitro* kinase reactions were performed using 10 μg of GST–c-jun or 20 μg of GST-E2F1. The combined lane had 10 μg each of E2F1 and DP1. Lane marked 'None' had no substrate. (**C**) JNK1 can be detected in association with E2Fs. Whole-cell extracts from U937 cells were immunoprecipitated with antibodies to different E2Fs and DP1 or a c-myc antibody as control, and the precipitates Western-blotted with anti-JNK1 antibody (upper panel). Levels of the different E2Fs and DP1 in U937 extracts is shown in the lower panel. (**D**) JNK1 can inhibit the transcriptional activity of all the five E2Fs. A transient transfection experiment was performed on Saos-2 cells using 2 μg of E2Fs 1, 2 and 3, or 6 μg of E2Fs 4 and 5 (lanes 2–6). Co-transfection of 2 μg of JNK1 inhibits their transcriptional activity (lanes 7–11). (**E**) JNK1 inhibits DNA binding activity of E2F1. Saos-2 cells were transfected with E2–CAT (lane 1) along with E2F1 (Lanes 2–4). JNK1 was co-transfected in lane 3, or cells were treated with TNFα (lane 4). The DNA binding activity of E2F1 is reduced to the levels of untransfected cells (lane 1) when JNK1 is co-transfected or induced by TNFα (lanes 3 and 4). Equivalent amounts of the same extracts were treated with 2 μl of an E2F1 antibody prior to the shift reaction (right panel). (**F**) The same whole-cell extracts were Western blotted to examine the levels of E2F1 protein.

by Fas-mediated signaling, we examined whether Fas receptor activation had any effect on E2F activity. In transient transfection experiments on the human T-cell line Jurkat, Rb could totally repress E2F1-mediated transcription (Figure 3A, lane 3); stimulation of cells transfected with Rb and E2F1 with 50 ng/ml of anti-Fas antibody led to the complete reversal of Rb-mediated repression of E2F1 (Figure 3A, lane 4). This reversal occurred within 30 min and Fas stimulation for longer periods had no further effects. Thus, co-transfection of p38 kinase or its

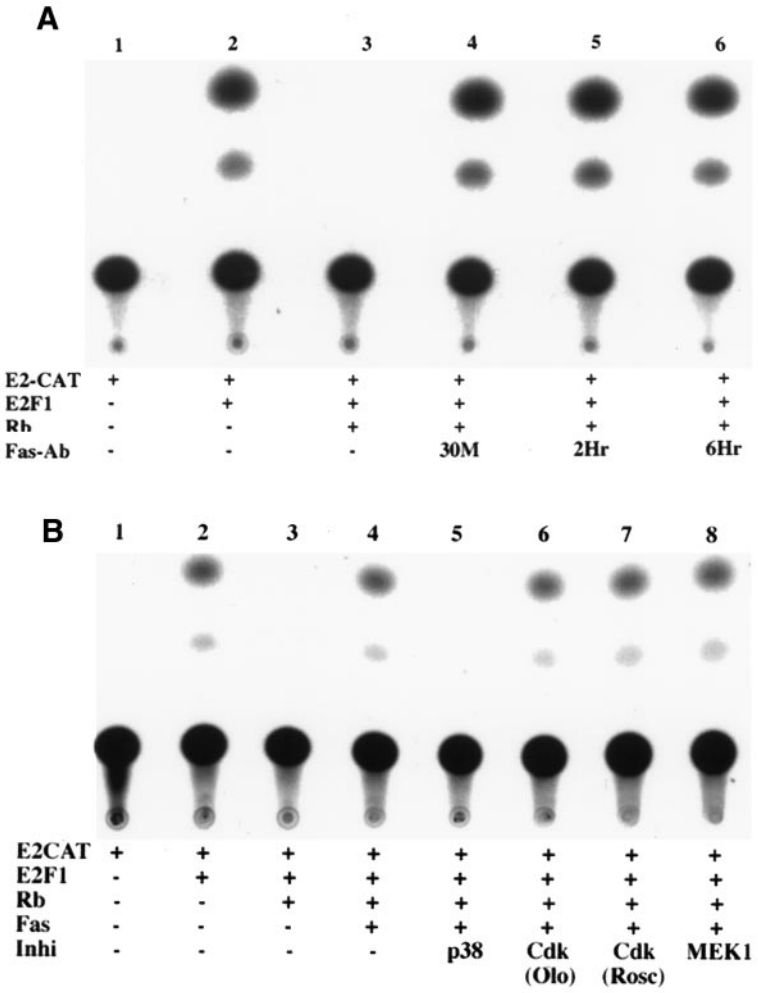

Fig. 3. Reversal of Rb function by stimulation of Fas receptor. (**A**) Jurkat cells were transfected with E2–CAT and E2F1 (Lanes 1 and 2); E2F1-mediated induction of transcription is blocked by co-transfecting Rb (lane 3). Stimulating the transfected cells with 50 ng/ml of anti-Fas antibody reverses Rb-mediated inhibition of E2F within 30 min (lanes 4–6). (**B**) A similar co-transfection experiment where the cells were stimulated with an anti-Fas antibody for 2 h (lanes 4); stimulation was done in the presence of chemical inhbitors of p38 (10 μM of SB203580; lane 5) cdks (olomoucine, 200 μM and roscovitine, 50 μM; lanes 6 and 7) and MEK1 (PD98059, 100 μM; lane 8).

induction by the appropriate agent could prevent Rb-mediated repression of E2F activity. It is a possibility that Fas-induced apoptotic pathways may lead to an inactivation of Rb function, since it has been demonstrated that Rb has anti-apoptotic properties.

To examine whether the inactivation of Rb by Fas involved the p38 kinase, Jurkat cells transiently transfected with Rb and E2F1 were stimulated with a Fas antibody in the presence or absence of the p38 inhibitor SB203580, a pyridinyl imidazole inhibitor specific to p38 (Kumar *et al*., 1997; Young *et al*., 1997). It was observed that the p38 inhibitor could totally prevent the inactivation of Rb (Figure 3B), suggesting that the abrogation of Rb function by the Fas pathway occurs through the mediation of the p38 kinase. Interestingly, two different cdk inhibitors, olomoucine and roscovitine, had no effect on the Fas-mediated inactivation of Rb, suggesting that the p38-mediated reversal of Rb function may not involve cdks (Figure 3B). Furthermore, a specific inhibitor of MEK1 kinase which can block the MAP kinase pathway had no effect on Fas-mediated inactivation of Rb. In conclusion, this set of experiments indicate that p38 kinase is the major and probably the exclusive intermediary between Fas signaling and Rb inactivation.

Fas-induced phosphorylation of Rb correlates with E2F activation

Since release of E2F from Rb leads to E2F transcriptional activation (Chellappan *et al*., 1991; Hiebert *et al*., 1992), we examined whether the association of E2F with Rb changes upon Fas stimulation. Extracts from Jurkat cells treated with an anti-Fas antibody for different periods of time were immunoprecipitated with an anti-Rb antibody. The bound proteins were recovered on protein A–Sepharose beads, dissociated with deoxycholate, and the levels of E2F assessed by EMSA (Chellappan *et al*., 1991). As shown in Figure 4A, there was a considerable amount of E2F associated with Rb in extracts from untreated cells (lane 2). In contrast to the untreated Jurkat cells, there was very little E2F bound to Rb after 30 min of Fas stimulation; there was no detectable E2F associated with Rb after 2 h (Figure 4A, lanes 3–6). Thus, the kinetics of dissociation of E2F from Rb correlates very well with its transcriptional activation, as described earlier

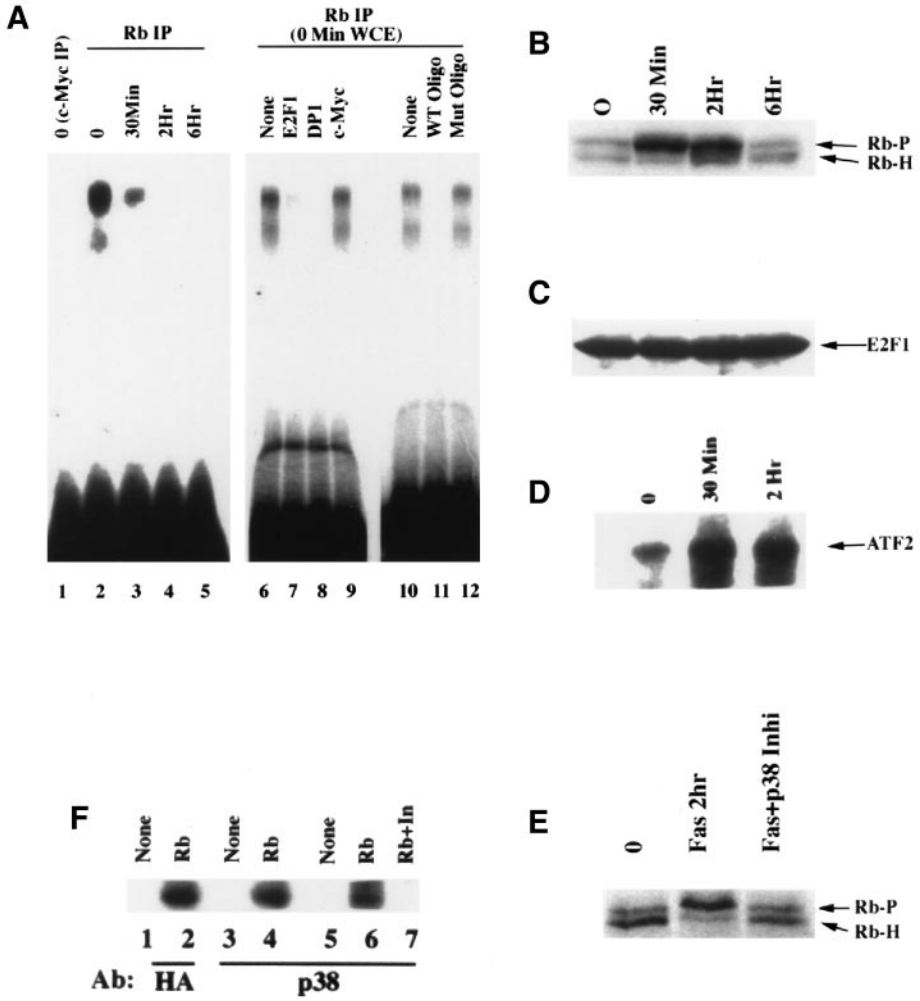

Fig. 4. Modulation of Rb function by Fas receptor. (**A**) Whole-cell extracts from untreated Jurkat cells (0 h) or those treated with anti-Fas antibody were immunoprecipitated with an anti-Rb antibody (lanes 2–5) or a c-myc antibody (t_0) as control (lane 1). The presence of E2F in the immunoprecipitates was assessed by EMSA. There is a marked reduction in the amount of E2F associated with Rb upon Fas stimulation, and there is no E2F associated with Rb after 2 h (lanes 3–5). The specificity of the immunoprecipitated band was established by treating the eluate from the Rb IP of the untreated extract with antibodies to E2F1, DP1 or c-Myc (as control) prior to the mobility shift analysis (lanes 6–9). The immunoprecipitate eluate was incubated with an oligonucleotide carrying a wild-type E2F binding site or one with a mutated site in lanes 11 and 12. (**B**) The status of the Rb protein in the same extracts (as in lanes 2–5) was assessed by Western blotting. Rb-P and RB-H indicates the phosphorylated and hypo-phosphorylated forms of Rb. (**C**) The levels of E2F1 remain constant upon Fas stimulation as seen by Western blotting. (**D**) The kinase activity of p38 in the same extracts was measured *in vitro* using immunoprecipitated p38 and 10 μg of GST–ATF2 as substrate. (**E**) A Western blot analysis of the Rb protein after 2 h of Fas stimulation of Jurkat cells in the presence or absence of the inhibitor SB203580. (**F**) *In vitro* kinase assays using Rb protein as a substrate. 3T3 cells were transfected with pSRαp38 and immunoprecipitated with an anti-HA antibody (lanes 1 and 2), or Fas-stimulated Jurkat extracts were immunoprecipitated with an anti-p38 antibody (lanes 3–7). *In vitro* kinase reactions were performed in the absence of substrate (lanes 1, 3 and 5), or in the presence of 4 μg of Rb (lanes 2, 4, 6 and 7). The reaction in lane 7 had 10 μM of SB203580.

(Figure 3A). The specificity of the observed band in the Rb immunoprecipitation was confirmed by adding antibodies to E2F1, DP1 and c-Myc to the EMSA; while E2F1 and DP1 antibodies could abolish the immunoprecipitated band, the control c-Myc antibody had no effect (Figure 4A, lanes 7–9). Further, an oligonucleotide carrying wild-type E2F binding sites could compete with the binding of the observed band, whereas an oligonucleotide carrying a point mutation in the E2F binding site could not compete with the binding (Figure 4A, lanes 10–12).

A Western blot analysis of the extracts from Fas-stimulated cells showed a marked increase in the level of Rb phosphorylation within 30 min of Fas stimulation (Figure 4B, lane 2) and this phosphorylation probably caused the dissociation of E2F. This conclusion is supported by the fact that there was no change in the levels of the E2F protein during this period (Figure 4C). There appeared to be a decrease in the levels of the Rb protein after 6 h, probably by proteolysis, as has been reported previously (Janicke *et al*., 1996; Dou *et al*., 1997).

Although Rb phosphorylation is brought about by cyclin D/cdk4 kinase during normal cell cycle progression (Sherr, 1994), it is possible that other kinases can phosphorylate Rb; indeed, Rb has multiple sites that can be potentially phosphorylated by different kinases (Lees *et al*., 1991). The fast kinetics of the Rb phosphorylation, along with the observations that Fas actually represses cdk activity (Oguri *et al*., 1995; De *et al*., 1997), raise the possibility that p38 is inactivating Rb directly. To verify this possibil-

ity, we first examined whether the phosphorylation of Rb correlates with an increase in p38 activity. p38 kinase was immunoprecipitated from the same extracts and used in an *in vitro* kinase assay using GST–ATF2 as a substrate (Xia *et al*., 1995; Whitmarsh and Davis, 1996). There is a 2- to 3-fold increase in the p38 kinase activity within 30 min of Fas antibody stimulation which correlates with the phosphorylation of Rb (Figure 4D). We then stimulated Jurkat cells with anti-Fas-antibody in the presence of the p38 inhibitor SB203580 (Figure 4E). Fas-mediated phosphorylation of Rb was effectively blocked by the p38 inhibitor, and the hypo-phosphorylated form of Rb persisted, suggesting that p38 is directly involved in the process. Attempts were then made to evaluate whether p38 can phosphorylate Rb *in vitro*. First, HA-p38 was transfected into 3T3 cells, immunoprecipitated with an anti-HA antibody and was used for an *in vitro* kinase assay using Rb as substrate (Figure 4F, lanes 1 and 2). Similarly, p38 was immunoprecipitated from Fas-stimulated Jurkat cells using an anti-p38 antibody (Figure 4F, lanes 3 and 4); in both the cases, p38 could effectively phosphorylate Rb. When the *in vitro* kinase reaction was performed in the presence of SB203580, there was a total inhibition of Rb phosphorylation (Figure 4E, lanes 5–7), suggesting that the effects seen were mediated by p38. Furthermore, SB203580 could block Fas-mediated dissociation of E2F from Rb (data not shown), suggesting that the observed functional effects involved the p38 kinase. These results when viewed in conjunction with the fast kinetics of Rb inactivation by Fas suggest that Rb is probably a direct downstream target of p38 kinase.

p38-mediated inactivation of Rb is cdk independent

Although the data so far suggested a direct role for p38 in inactivating Rb, the possibility exists that p38 is activating cdks which in turn inactivate Rb. Additional experiments were conducted to verify this possibility. It had been observed earlier that Fas-stimulation of cells does not increase cdk activity; furthermore, in certain cases there is a repression of cdk activity. As a first step, we attempted to determine whether Fas stimulation induced cdk activities. Jurkat cells were treated with an anti-Fas antibody for 2 h and lysates were prepared. Antibodies to cyclin D and cyclin E were used to immunoprecipitate the associated cdk activities. An *in vitro* kinase assay using Rb protein or histone H1 as substrates showed that there was no increase in the kinase activities associated with either cyclin D or cyclin E upon Fas stimulation (Figure 5A, lanes 2). Since the p38 inhibitor SB203580 could block Fas-mediated phosphorylation of Rb, a similar *in vitro* kinase assay was performed to see whether the p38 inhibitor had any effect on the cdk activities. As shown in Figure 5A, lane 3, SB203580 had no effect on cdk activities, suggesting that the inhibition of Rb phosphorylation is not due to a reduction in the kinase activity associated with cyclins D and E. To confirm that the immunoprecipitations bring down the specific cdks, immunoprecipitates of cyclin D and cyclin E were probed for the presence of cdk4 or cdk2 (Figure 5B). cdk4 and cdk2 could be detected in the cyclin D and cyclin E immunoprecipitates, respectively. As a control for specificity, the immunoprecipitates were probed with an anti-ERK2 antibody; there was no ERK2 associated with either cyclin. This suggested that the kinase activity we observe in the cyclin IPs is due to the appropriate cdk (Figure 5B, lower panel). Given this scenario, it maybe concluded that increase in Rb phosphorylation upon Fas stimulation is brought about by a kinase(s) other than those associated with cyclin D and E, probably p38.

The above results were verified in two functional assays. In the first experiment, attempts were made to determine whether dominant-negative forms of cdk2, cdk4 and cdk6 could block Fas-/p38-mediated reversal of Rb function. The logic of this experiment was that if cdks are indeed involved in the inactivation of Rb, negation of the endogenous cdk activity by overexpressing dominant-negative proteins should prevent Fas-mediated reversal of Rb function (Figure 5C). It had been demonstrated that the dominant-negative cdks could block the inactivation of Rb by cyclins D and E. We confirmed that overexpression of a combination of dominant-negative cdk4 and cdk6 could block cyclin D-mediated inactivation of Rb in Jurkat cells (Figure 5C, lane 5). Similarly, overexpression of a dominant-negative cdk2 could inhibit cyclin E-mediated reversal of Rb function (lane 7). In contrast, co-transfection of a combination of dominant-negative cdk4 and cdk6, or cdk2 alone had no effect on the Fas-mediated reversal of Rb function (Figure 5C, lanes 8–10). These results again illustrate that the Fas/p38 mediated phosphorylation and inactivation of Rb occurs through pathways independent of cyclins D and E and the kinases associated with them.

The involvement of p38 kinase in Fas-mediated inactivation of Rb was verified in a similar transient transfection experiment (Figure 5D). Jurkat cells transiently transfected with E2CAT, E2F1 and Rb were stimulated with Fas antibody in the presence of increasing amounts of a dominant-negative p38 kinase. As shown in Figure 5D (lanes 5–8), the dominant-negative p38 kinase construct could block Fas-mediated reversal of Rb function in a dose-dependent manner. This experiment confirms that Fas-mediated inactivation of Rb occurs through the mediation of the p38 kinase, independent of cyclin D and cyclin E associated kinase activities.

Discussion

The central role for Rb in maintaining the integrity of the R-point has been well documented, and the Rb-mediated inhibition of G_1–S transition involves a downregulation of E2F activity (Beijersbergen and Bernards, 1996). Although it has been suggested that Rb molecules that cannot bind to E2F can prevent cell cycle progression (Sellers *et al*., 1998), it is very likely that under normal circumstances Rb exerts its growth regulatory function at least in part by inhibiting the activity of E2F. The finding that many different signaling cascades can target either of these molecules or their interaction suggests that the Rb–E2F interaction is a very convenient and sensitive modulator of cell proliferation. Indeed, complexes containing the E2F transcription factor are known to be altered in response to a wide array of signals ranging from cytokines, growth factors, cyclic AMP, TPA and other agents that can induce DNA damage or differentiation (Chellappan, 1994; Corbeil *et al*., 1995; Ikeda *et al*.,

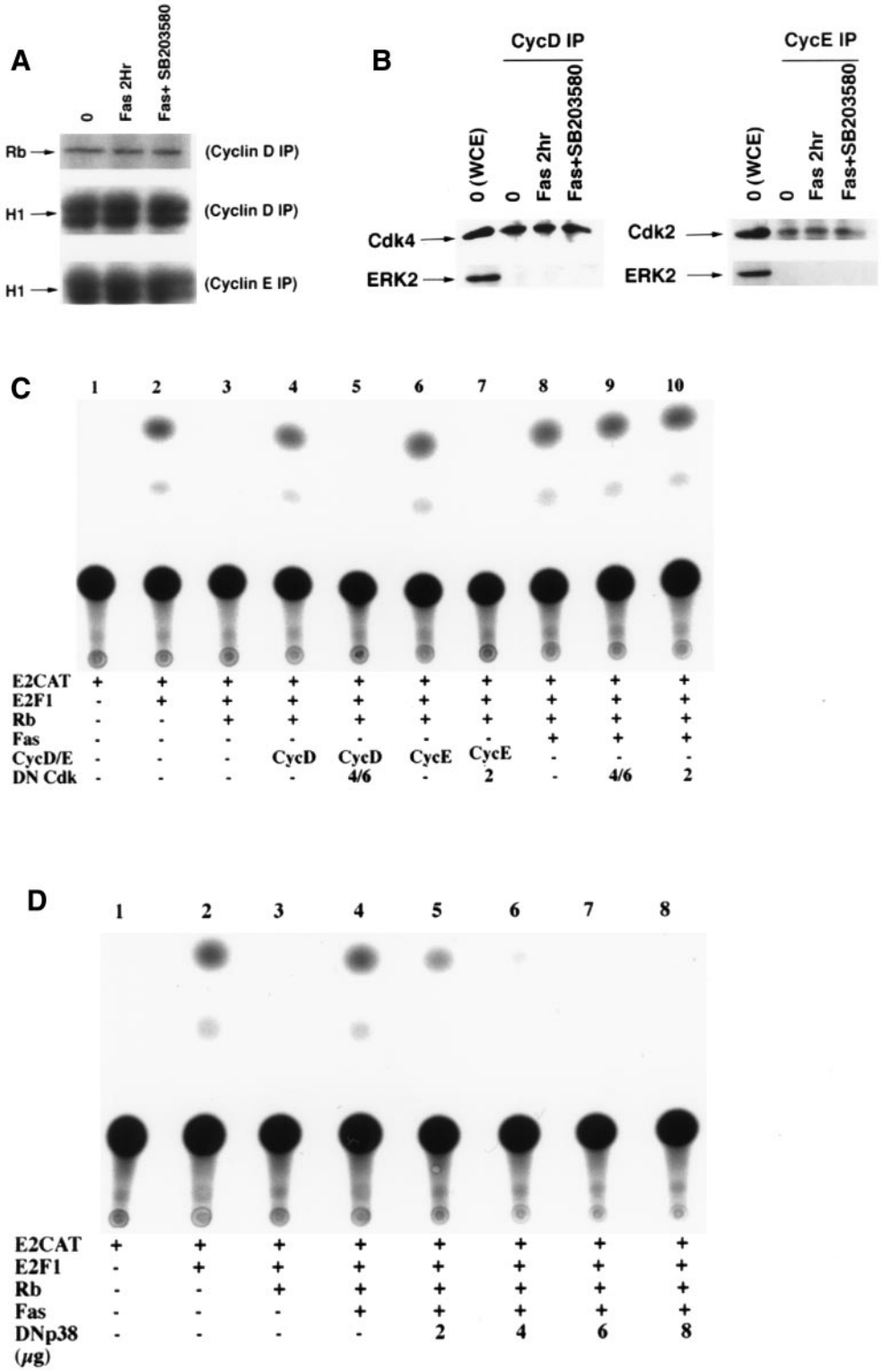

Fig. 5. Stimulation of the Fas receptor does not induce cyclin D- and E-dependent kinase activity. (**A**) *In vitro* kinase assays were performed on extracts from untreated Jurkat cells (lane 1), cells stimulated with anti-Fas antibody for 2 h (lane 2) or treated with anti-Fas in the presence of 10 μM p38 inhibitor SB203580. A cyclin D antibody (upper and middle panels) or a cyclin E antibody (lower panel) was used for immunoprecipitations, using Rb or histone H1 as subtrates. (**B**) Presence of cdks in the cyclin immunoprecipitation was assessed by a Western blot of the untreated whole-cell extract or the cyclin D- and E-immunoprecipitated from the three extracts. cdk4 and cdk2 were present in cyclin D and cyclin E immunoprecipitations, but ERK2 could not be detected. (**C**) Transient transfection of cyclin D (lane 4) or cyclin E (lane 6) could reverse Rb-mediated repression of E2F1 activity in Jurkat cells; this could be blocked by a combination of dominant-negative cdk4 and cdk6 (lane 5) or dominant-negative cdk2 (lane 7). Fas stimulation for 2 h could reverse Rb mediated repression (lane 8) and this was not affected by dominant-negative cdk4/6 (lane 9) or cdk2 (lane 10). (**D**) A similar co-transfection experiment where Fast stimulation for 2 h (lane 4) was conducted in the presence of increasing amounts of co-transfected dominant-negative p38 kinase (lanes 5–8). Fas-mediated inactivation of Rb function was effectively blocked by the dominant-negative p38 in a dosage-dependent manner.

1996). The results presented in this paper identify kinases that are induced by such non-mitogenic pathways and can modulate E2F activity directly or through the Rb protein.

The finding that JNK1 can target E2F1 may be significant in the context that the proliferative status of the cells may have to be adjusted in response to stress situations where JNK1 is induced. For example, it may be imagined that DNA damage might require the cell to attenuate cell cycle progression to make the necessary repairs to its genome. Repression of E2F activity would be an efficient way to pause the cell cycle in a timely fashion, especially if the stress occurs after Rb has been inactivated by cdks. Our results indicate that JNK1 is capable of phosphorylating E2F1 directly. Regulation of E2F1 activity by phosphorylation mediated by cdks has been demonstrated previously. Those studies indicated that the ability of E2F1 to bind to Rb as well as to induce transcription was modulated by phosphorylation; similarly, DP1 itself has been shown to be a target for regulatory phosphorylation events. Supporting these findings, a role for the phosphatase PP2A in dephosphorylating and activating E2F1 and DP1 has been proposed (Altiok *et al*., 1997). This was based on the observation that a reduction in the DNA-binding and transcriptional activities of E2F1 correlated with a decreased expression of PP2A in adipogenic cells responding to the PPARγ nuclear receptor. In this system, an increase in E2F phosphorylation correlated with a reduced DNA-binding activity, as we are observing with JNK1. Given these findings, we believe that it is quite plausible for kinases responding to stress signals to modulate E2F function directly by modifying its DNA binding properties. Interestingly, JNK1 was found to directly bind to the dimerization partner of E2F and not to E2F1 under similar conditions. The ability of JNK1 to interact with one partner of a dimeric transcription factor while modifying the properties of the other partner is manifest in the case of E2F also, as has been reported previously for AP1. Additional experiments are in progress to identify the potential JNK1 phosphorylation sites on E2F1.

Our observation that p38 kinase can inactivate Rb *in vivo* is intriguing, although it is known that Rb can be phosphorylated *in vitro* by a variety of kinases (Lees *et al*., 1991). Rb has been shown to be involved in modulating the cell cycle in response to non-mitogenic signals, and p38 kinase appears to be one such kinase that can target Rb in response to such signals. We believe that our studies describe for the first time a kinase outside the cdk family regulating Rb function in an *in vivo* situation. One salient observation is that the growth regulatory properties of JNK1 and p38 appear tightly linked to their effects on E2F activity. JNK1, which can repress E2F activity, restricts cell proliferation as efficiently as Rb. In contrast, p38 kinase can reverse Rb-mediated growth suppression effectively, despite not having a direct effect on cell proliferation by itself. This emphasizes further the central role of the Rb–E2F pathway in regulating cell proliferation, and underlines how agents that can affect their activity brings about effects on proliferation that parallels their regulation of E2F. The situation may be complicated by the fact that many extracellular signals can induce JNK1 as well as p38; it may be imagined that the response of the cell could depend on other factors such as the relative amount of free E2F present, as well as the cell cycle point at which it encounters the signal.

The finding that Fas-mediated signaling can affect Rb phosphorylation is novel and intriguing. Fas mediated signaling has been demonstrated to induce proteolytic cleavage of Rb by caspases. In our experiments, the Rb levels remain high until at least 2 h post Fas stimulation, and the overall Rb level reduces within 6 h. This could be due to the cleavage and release of a 5 kDa fragment from Rb as reported previously (Chen *et al*., 1997). As described earlier, the functional inactivation of Rb correlates perfectly with release of free E2F. The kinetics of these events suggests that they occur very early in the Fas signaling pathway. Although a role of caspases in inactivating Rb at later points during the apoptotic process has been established, we believe that the inactivation of Rb by phosphorylation precedes that event.

The role of p38 kinase in mediating Fas-mediated apoptosis is not yet clear. While it has been demonstrated that inducers of p38 kinase can augment Fas-mediated apoptosis, inhibitors of p38 kinase have been generally inefficient at blocking the apoptotic process. We find that SB203580 generally results in a 10–15% reduction in apoptotic cells (data not shown). This supports the idea that although p38 kinase could be playing a role in mediating Fas-induced apoptosis, its activation alone is not sufficient to induce apoptosis. The studies on Fas-induced signaling also reveals that p38 kinase can inactivate Rb directly, independent of cdks. Furthermore, we find that cdks are not activated at the time points when Rb is phosphorylated. Similarly, we also observe a Fas-mediated reversal of Rb function in serum-starved cells (data not shown), where the cdk activity is expected to be very low. It appears reasonable to speculate that inactivation of Rb and stimulation of E2F plays a role in Fas signaling and may facilitate Fas-induced apoptosis, based on the earlier general observations that overexpression of E2F1 induces apoptosis (Qin *et al*., 1994; Hseih *et al*., 1997), whereas Rb is anti-apoptotic.

In conclusion, we believe that our results show Rb and E2F to be regulated by various signaling cascades, often independent of cyclins and cdks. Further characterization of the actual mechanisms involved in the inactivation would throw light on how precisely the cell cycle machinery responds to various extracellular stimuli.

Materials and methods

Transfections

Saos-2 osteosarcoma cells were transfected by calcium phosphate precipitation method and Jurkat cells were electroporated using a Bio-Rad Gene Pulser. Cells were harvested after 72 h and assays for CAT and β-galactosidase performed using standard protocols. A pSV-βgal vector (2 μg) was included in all transient transfections as internal control. Constructs pDCE2F1, pE2CAT and pSVRb (Zhang and Chellappan, 1995), as well as pSRαJNK1, cdc42 and p38 have been described previously (Minden *et al*., 1994a,b, 1995). Two micrograms of E2CAT, E2F1 and Rb were used in all transfections unless mentioned otherwise. The dominant-negative p38 construct was a kind gift of Dr JiaHuai Han, Scripps Research Institute, La Jolla, CA. The kinase inhibitors were added at the time of Fas stimulation at the following concentrations: p38 inhibitor SB203580, 10 μM; cdk inhibitors olomoucine, 200 μM, and roscovitine, 50 μM; MEK inhibitor PD98059, 100 μM. Stable transfections were performed on 35 mm dishes, each having 10 000 cells, and selection in 1 μg/ml of puromycin, 40 μg/ml neomycin or

both commenced after 48 h. Colonies having ⩾20 cells were counted after 14 days.

In vitro binding assays

GST fusion proteins of DP1, E2F1 and DP2 as well as GST–cJ carrying a c-jun fragment were prepared using standard protocols as described previously (Zhang and Chellappan, 1995). pSRα-JNK1 vector was digested with *Bgl*III and transcribed *in vitro* using SP6, and JNK1 protein was synthesized in rabbit reticulocyte lysate in the presence of [^{35}S]methionine. An aliquot of 8 µl of the synthesized polypeptide was incubated with beads carrying equal amount of GST fusion proteins in 200 µl of a buffer containing [20 mM Tris pH 7.5, 50 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Nonidet P-40 (NP-40), 3 mg/ml bovine serum albumin (BSA)] at 4°C for 2 h. The beads were washed six times and eluted with 10 mM glutathione. Eluates were separated on a 10% SDS–polyacrylamide gel and visualized by autoradiography. The protein amount in control input lane is one-fifth of the total used in binding assay.

Immunoprecipitations, Western blots and kinase assays

Antibodies to E2F1-5, JNK1, p38 kinase, ERK2 and cyclins D and E were obtained from Santa Cruz Biotechnologies; anti-Fas antibody (CH11) was from MBL; anti-HA antibody from Boehringer Mannheim; anti-Rb and anti-c-Myc antibody from Oncogene Science-Calbiochem. Whole-cell extracts prepared by hypotonic shock (Chellappan *et al*., 1991) were used for immunoprecipitations, EMSAs, Western blots and *in vitro* kinase assays. For immunoprecipitations, 50–200 µg of whole-cell extracts were treated with 5 µl of the appropriate primary antibody in a volume of 100 µl at 4°C for 1 h. Three milligrams of protein A–Sepharose or protein G–Sepharose in 100 µl volume was then added and incubated for additional 1 h. The binding was performed in a buffer containing 20 mM HEPES pH 7.9, 40 mM KCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM NaF, 0.1 mM Na_3VO_4, 0.5% NP-40 and 3 mg/ml BSA. The beads were washed six times, boiled in 20 µl of SDS sample buffer and proteins separated on 8 or 10% polyacrylamide gels. After semi-dry transfer to supported nitrocellulose membrane the proteins were detected using an ECL system from Amersham. EMSA following immunoprecipitation was performed as described previously (Chellappan *et al*., 1991). For JNK1 kinase assays, 3T3 cells were transfected with HA-JNK1 and cdc42, and immunoprecipitation was performed using an anti-HA antibody under stringent conditions in a buffer containing 20 mM Tris pH 7.5, 1% Triton X-100, 0.5% deoxycholate and 0.5 M $LiCl_2$. The beads were washed six times in the same buffer and a kinase assay was performed as described previously (Minden *et al*., 1994b). An HA antibody was used to immunoprecipitate p38 from pSRαHA-p38-transfected 3T3 cells for kinase assays on Rb protein, or p38 was immunoprecipitated from Fas-stimulated Jurkat cells using an anti-p38 antibody and used for kinase assay reactions on GST–ATF2 or on 4 µg of bacterially produced human Rb protein (QED Biotechnologies, CA).

Acknowledgements

We thank Drs Kathryn Calame, Stephen Goff and Lloyd Greene for helpful comments. This work was supported by a grant from the NCI (CA63136). S.P.C. is a recipient of the Irma-Hirschl Trust Research Award.

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Received September 14, 1998; revised November 23, 1998; accepted January 28, 1999