The retinoblastoma gene family members pRB and p107 coactivate the AP-1-dependent mouse tissue factor promoter in fibroblasts

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Serum-stimulation of quiescent mouse fibroblasts results in transcriptional activation of tissue factor (TF), the cellular initiator of blood coagulation. This requires the rapid entry of c-Fos into specific AP-1 DNA-binding complexes and can be strongly inhibited by the adenovirus E1A 12S gene product. In this study, we utilized a panel of E1A mutants deficient in cellular protein binding to analyse the molecular basis for E1A inhibition of a minimal, c-Fos-dependent TF promoter/reporter construct in mouse AKR-2B fibroblasts. Mutations which impaired binding of the retinoblastoma tumor suppressor protein family members pRB, p107, and p130 relieved E1A-mediated inhibition of transcription in response to serum-stimulation or c-Fos overexpression. Inhibition was restricted to the G₀ to G₁ transition, consistent with the specificity of E1A for hypophosphorylated forms of RB proteins. Although E1A mutants deficient in CBP/p300 binding retained the ability to inhibit TF transcription, deletion of the amino-terminal portion of the CBP/p300 interaction domain was required to permit rescue of TF promoter activity by coexpression of pRB. Moreover, ectopic p107 could effectively substitute for pRB in relieving E1A-mediated repression. In primary mouse embryo fibroblasts, activity of the minimal AP-1-dependent TF promoter was suppressed in *Rb*^{-/-} cells compared to parallel *Rb*^{+/-} and *Rb*^{+/+} transfectants. Ectopic expression of either pRB or p107 markedly enhanced TF promoter activity in *Rb*^{-/-} fibroblasts. Collectively, these data imply that pRB and p107 can cooperate with c-Fos to activate TF gene transcription in fibroblasts and suggest a requirement for another, as yet unidentified, E1A-binding protein. *Oncogene* (2000) 19, 3352–3362.

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Introduction

Tissue factor (TF) is a 46 kDa transmembrane glycoprotein that serves as a cell-surface receptor and essential cofactor for plasma coagulation factor VII/VIIa. Although the primary function of TF in the adult is to initiate blood coagulation (reviewed in Edgington *et al.*, 1991), recent studies suggest that TF may contribute to blood vessel maturation in the developing embryo (Bugge *et al.*, 1996; Carmeliet *et*

al., 1996), to maintenance of the placental labyrinth during gestation (Erlich *et al.*, 1999), and to tumor angiogenesis (Contrino *et al.*, 1996). Tissue factor has also been implicated as a determinant of metastatic potential in melanoma cells (Mueller *et al.*, 1992; Bromberg *et al.*, 1995) and expression of TF in the stromal compartment of breast carcinomas has been shown to correlate with progression to invasive cancer (Contrino *et al.*, 1996; Vrana *et al.*, 1996). Thus, it is important to understand how TF gene expression is regulated in both normal and neoplastic cell types.

Earlier studies in our laboratory established that the TF gene in mouse fibroblasts is serum-inducible (Felts *et al.*, 1995) and that this effect can be strongly potentiated by costimulation with transforming growth factor type β 1 (Felts *et al.*, 1997). Two AP-1 DNA-binding motifs located between –200 and –220, relative to the start site of transcription, were found to be both necessary and sufficient for transcriptional activation in response to serum stimulation, or to serum stimulation in the presence of TGF- β 1. Because the effect of serum and TGF- β 1 was synergistic rather than additive, these observations implied that the AP-1 DNA-binding sites served to integrate components of the serum and TGF- β signaling pathways in a cooperative fashion. A variety of experiments, including the use of fibroblasts derived from c-fos knockout mice, demonstrated that heterodimeric AP-1 DNA-binding complexes specifically containing c-Fos were required for transcriptional activation of the TF gene in response to serum and for synergistic activation in response to a combination of serum and TGF- β 1 (Felts *et al.*, 1997). However, when c-Fos was overexpressed in stimulated cells, transcriptional synergism was progressively squelched, pointing to the existence of a c-Fos-interacting protein(s) which may function as a specific coactivator(s).

CREB binding protein (CBP) and the closely related coactivator p300 (collectively referred to as CBP/p300) have been shown to cooperate with a variety of transcriptional activating proteins to regulate specific gene transcription (Kwok *et al.*, 1994; Lundblad *et al.*, 1995; Arany *et al.*, 1994; Arias *et al.*, 1994). In particular, Bannister and Kouzarides (1995) showed that CBP directly interacted with c-Fos *in vitro* and could stimulate GAL4-Fos transactivation *in vivo*. Because CBP has also been implicated in TGF- β -dependent transcription (Topper *et al.*, 1998; Shen *et al.*, 1998), CBP/p300 was an attractive candidate to mediate c-Fos-dependent transcription of TF. To study the involvement of CBP/p300 in regulating TF transcription, we exploited the ability of the adenovirus E1A 12S oncoprotein to inhibit TF

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promoter activity. The E1A 12S gene product has been shown to repress AP-1-dependent transcription, at least partly by sequestering CBP/p300 through direct protein-protein interactions (Bannister and Kouzarides, 1995 and references therein). However, an amino-terminal deletion mutant deficient in CBP/p300 binding (Barbeau *et al.*, 1994) retained the ability to inhibit TF promoter activity, and coexpression of CBP/p300 did not rescue promoter activity from inhibition by wild-type E1A. These observations strongly suggested that an E1A-binding protein(s) other than CBP/p300 was required to coactivate c-Fos-dependent transcription of the TF gene. This factor was provisionally termed CAF for coactivator of Fos (Felts *et al.*, 1997).

In the present study, we utilized a panel of E1A mutants deficient in their ability to bind and sequester cellular proteins other than CBP/p300 to identify candidates for CAF. We find that mutations which eliminate E1A interaction with the retinoblastoma tumor suppressor family of proteins also relieve E1A-mediated inhibition of TF promoter activity. These proteins include the founding member, pRB, and two related proteins, p107 and p130. Although the best characterized function of these proteins is the restriction of cell cycle progression via sequestration of the E2F family of transcription factors (reviewed by Mulligan and Jacks, 1998), pRB has been shown to interact with several other transcription factors to positively regulate gene transcription in a manner characteristic of a coactivator (Singh *et al.*, 1995; Shao *et al.*, 1995; Chen *et al.*, 1996a,b). Our data demonstrate that pRB and p107 cooperate with c-Fos to activate TF gene transcription in mouse fibroblasts. To our knowledge, functional cooperation between members of the RB tumor suppressor family and the c-Fos proto-oncogene to

activate specific gene transcription has not been previously described.

Results

Construction of a panel of E1A 12S mutants deficient in cellular protein binding

The adenovirus E1A 12S gene product has been shown to bind a number of cellular proteins, including the transcriptional coactivators CBP/p300, the retinoblastoma tumor suppressor protein pRB, and the pRB-related pocket proteins p107 and p130 (Wang *et al.*, 1993; Bishopric *et al.*, 1997, and references therein). Specific E1A amino acids required for binding of these proteins have been mapped to the N-terminus and to two highly conserved regions, CR1 and CR2, and mutants deficient in binding have been described (Wang *et al.*, 1993, and references therein). Based on these data, we constructed a panel of E1A deletion and point mutants specifically defective in the binding of CBP/p300, pRB, p107 and p130. The binding sites for these proteins have been shown to be bipartite and non-contiguous as illustrated schematically in Figure 1. Thus, CBP/p300 interacts with the N-terminus of the E1A protein, and with the C-terminal portion of CR1. RB proteins exhibit some heterogeneity in binding, depending on identity, but tend to interact strongly with the N-terminal portion of CR2, and less avidly with the N-terminal portion of CR1.

To independently verify the binding specificity of these constructs, CMV-expression vectors encoding wild-type and mutant proteins were transfected into AKR-2B fibroblasts and whole cell extracts were immunoprecipitated using an E1A monoclonal antibody (M73) directed against an unaltered C-terminal

Figure 1 Structure of E1A 12S wild-type and mutant proteins. (a) Representation of the 243 amino acid residue E1A protein encoded by 12S mRNA showing conserved regions 1 and 2, CR1 and CR2. (b) E1A domains involved in binding to CBP/p300, p130, p107, and pRB proteins. Dashed brackets indicate regions of reduced affinity relative to solid brackets. (c) Structure of E1A deletion and point mutants used in this study. Straight lines between open boxes represent deleted regions while arrows indicate mutated amino acid residues

epitope (Harlow *et al.*, 1985). Expression of E1A proteins was assessed by Western blotting using the same antibody, while cellular proteins co-immunoprecipitating with E1A were analysed by Western blotting of dissociated immunoprecipitates using antibodies specific for CBP, p300, pRB, p107 and p130. As shown in Figure 2 (bottom panel) all wild-type and mutant E1A proteins were expressed, albeit at differing levels. In general, mutants defective in binding of one or more of the RB proteins such as dCR2, CG124, and YH47/CG124, were expressed at considerably higher levels than either wild-type E1A, or mutants deficient in CBP/p300 binding (d2-36, d60-80, RG2). Such differences may simply be due to a disparity in protein stability among the E1A mutants. Alternatively, the CMV promoter used to drive E1A expression may be subject to differential feedback regulation by mutant E1A proteins, particularly those defective in pRB binding. Such a mechanism is plausible given that (1) the CMV promoter contains multiple AP-1 *cis*-elements (Prestridge, 1991; Reddy *et al.*, 1995 and references therein) and (2) such elements have been shown to be targets of pRB regulation in the context of other promoters (Robbins *et al.*, 1990; Nead *et al.*, 1998; Nishitani *et al.*, 1999, and this study). Irrespective, all mutants displayed the expected specificity as shown in the upper panels of Figure 2.

Figure 2 Ligand-binding specificity of wild-type and E1A mutant proteins expressed in AKR-2B fibroblasts. AKR-2B cells were transfected with 10 μ g wild-type or mutant E1A expression vectors, rendered quiescent, then restimulated with 20% FBS plus 5 ng/ml TGF- β 1 for 5 h. E1A protein was immunoprecipitated from whole cell lysates with E1A monoclonal antibody, M73. Co-immunoprecipitated cellular proteins, p300, CBP, p130, p107, pRB were detected by Western blotting using antibodies described in Materials and methods. (Bottom panel) Expression of wild-type and mutant E1A proteins were analysed by Western blotting of total lysed cell protein (2.8% of IP input per lane) with M73

E1A mutations that impair binding of pRB, p107 and p130 relieve inhibition of TF promoter activity

To test the effects of E1A mutations on TF promoter activity, AKR-2B fibroblasts were cotransfected with AP1-TF60CAT and the wild-type and mutant E1A expression vectors illustrated in Figure 1. AP1-TF60 consists of the TF AP-1 elements linked to the mouse TF promoter deleted to -60 and containing only the TATA box as a recognizable sequence motif (Felts *et al.*, 1995). Previous work established that the AP-1 elements are both necessary and sufficient to confer inducibility in response to serum or synergistic inducibility in response to serum plus TGF- β 1 (Felts *et al.*, 1995, 1997).

Following transfection, fibroblasts were rendered quiescent by incubation in serum-free medium for 48 h, then restimulated for 5 h by the addition of fresh medium containing 20% fetal bovine serum, or the same containing 5 ng/ml TGF- β 1. Lysates were prepared and assayed for CAT protein by ELISA and for E1A oncoprotein by immunoblotting. Because E1A mutants are expressed at different levels in AKR-2B fibroblasts (Figure 2), CAT protein values were normalized for E1A expression as quantified by densitometry of wild-type and mutant E1A protein bands detected on Western blots. As shown in Figure 3, wild-type E1A effectively repressed promoter activity in response to either serum plus TGF- β 1 (a) or serum alone (b) when compared to control cells cotransfected with empty expression vector. As previously observed (Felts *et al.*, 1997), mutants deficient in CBP/p300 binding retained the ability to repress promoter activity under both sets of conditions. These included the deletion mutants d2-36 and d60-80, as well as the point mutant RG2. In contrast, mutants deficient in binding of pRB, p107, and p130 were generally ineffective, or less effective than wild-type E1A in repressing promoter activity. These included the deletion mutant dCR2 and the double-point mutant YH47/CG124 which are deficient in the binding of all three RB proteins, and the double mutant d2-36/CG124 which additionally lacks the ability to bind CBP/p300. The single-point mutant CG124 appeared to completely relieve repression in serum-stimulated cells (b) but was considerably less effective in cells additionally stimulated with TGF- β 1 (a). This pattern was also observed, albeit to a lesser degree, with all mutants deficient in RB protein binding.

E1A-mediated inhibition of promoter activity resulting from c-Fos overexpression requires intact RB binding domains

Overexpression of c-Fos in the presence of JunD largely abrogates the requirement for serum in stimulating AP1-TF60 promoter activity, while overexpression of JunD alone or in combination with JunB has no effect (Felts *et al.*, 1995). To study the requirement for RB proteins under these conditions, fibroblasts were cotransfected with AP1-TF60 and with expression vectors encoding c-Fos, JunD, and either wild-type E1A, or mutants specifically deficient in their ability to bind RB family members. Cells were rendered quiescent by incubation in serum-free media, then assayed for CAT protein without further

Figure 3 Effect of wild-type and mutant E1A proteins on AP-1-dependent tissue factor promoter activity in AKR-2B fibroblasts. AKR-2B cells were cotransfected with 5 μ g API-TF60CAT reporter along with 0.5 μ g pCIE1Awt or mutant expression vectors and 0.5 μ g pCMV β . Empty pCI vector was used to maintain a total of 10 μ g DNA per transfection. Control cells were cotransfected with 5 μ g API-TF60CAT, 0.5 μ g pCMV β , and 4.5 μ g pCI vector. After being rendered quiescent, transfectants were stimulated for 5 h with 20% FBS plus 5 ng/ml TGF- β 1 (a), or 20% FBS alone (b). Cell lysates were assayed for total protein and for CAT and β -galactosidase reporter expression. Ectopic expression of wild-type and mutant E1A proteins was monitored by Western blotting of lysed cell protein (~7.5 μ g/lane) derived from a pool of three replicate transfectants. Bands detected in each lane were quantified by densitometry using NIH Image 1.44 software. A correction factor for each mutant E1A was then calculated by dividing mutant band density by the density corresponding to wild-type E1A resolved on the same blot. For this experiment, the correction factors used for (a) and (b), respectively were: E1Awt, 1.00 and 1.00; d2-36, 0.68 and 0.64; d37-60, 0.69 and 0.74; d60-80, 0.77 and 0.97; dCR2, 1.41 and 1.69; d129-139, 1.31 and 1.31; RG2, 0.75 and 0.46; CG124, 1.51 and 1.70; YH47/CG124, 1.59 and 1.80; d2-36/CG124, 0.95 and 0.82. For each E1A transfectant, the CAT enzyme value measured by ELISA was normalized for both total cellular protein and E1A expression. Data sets are represented as a percentage of normalized CAT enzyme measured in control cells (defined as 100%). Error bars reflect the range of three plates from a representative experiment

treatment. As shown in Figure 4, wild-type E1A effectively repressed c-Fos-stimulated promoter activity, while the RB-deficient mutants were considerably less effective. These included the dCR2 deletion mutant and the point mutants CG124 and YH47/CG124. These results reinforce the experiments shown in Figure 3, and further suggest a requirement for one or more RB family proteins in c-Fos-dependent transcription.

Figure 4 Effect of wild-type and mutant E1A proteins on tissue factor promoter activity in cells overexpressing c-Fos. AKR-2B cells were cotransfected with 5 μ g API-TF60CAT, 3 μ g RSV-JunD, 5 μ g RSV-c-Fos, and 0.5 μ g of the indicated wild-type or mutant E1A expression vector. Control cells received 0.5 μ g empty pCI expression vector in place of E1A expression vector. After transfection, cells were serum-starved for 48 h and quiescent cell lysates were prepared. Lysates were assayed for total protein and CAT enzyme. CAT values were normalized to correct for E1A protein levels as described in the legend to Figure 3 and expressed as a percentage of control. Error bars reflect the range of three plates from a representative experiment

Expression of pRB is necessary but not sufficient to restore E1A-inhibited promoter activity

To verify a requirement for pRB in the activation of TF promoter activity, we attempted to rescue promoter activity from E1A inhibition by coexpressing pRB in the same cells. In repeated experiments, we were unable to rescue promoter activity from wild-type E1A inhibition using this approach. Thus, it appeared that either a protein unrelated to pRB bound to the same E1A domains as members of the pRB family, or alternatively, pRB could be necessary but not sufficient for full activation of promoter activity. Because the double mutant d2-36/CG124 was the least effective in inhibiting promoter activity in response to serum plus TGF- β 1 (Figure 3a), we reasoned that an additional protein interacting with E1A N-terminus, perhaps CBP/p300, may potentiate the ability of pRB to rescue promoter activity. To test this hypothesis, we attempted to rescue promoter activity by pRB overexpression in cells cotransfected with either wild-type E1A, or the deletion mutant d2-36, or the point mutant RG2. All effectively repress TF promoter activity (Figure 3), but the latter two are specifically defective in CBP/p300 binding. As shown in Figure 5, coexpression of pRB effectively rescued promoter activity from inhibition by the d2-36 mutant, but had no effect on inhibition mediated by wild-type E1A or the RG2 point mutant. This rather striking result suggested that a protein other than CBP/p300 is capable of interacting with the N-terminal portion of the CBP/p300 binding domain, but is insensitive to the single amino acid change at position two. The requirement for deletion of the E1A N-terminus to permit rescue by pRB suggests that this factor is required in combination with pRB for full activation of TF promoter activity. This view was reinforced by a failure to rescue wild-type E1A-inhibition of promoter activity by overexpressing a combination of CBP/p300 and pRB (data not shown).

Figure 5 Rescue of E1A-inhibited TF promoter activity by coexpression of pRB. AKR-2B cells were cotransfected with 5 μ g AP1-TF60CAT, 0.5 μ g pCMV β , 0.5 μ g of either E1Aw1, d2-36, or RG2 expression vector, and varying amounts (0, 3, 6, or 9 μ g) of pSG5RB. Empty pCI vector was added as needed to maintain a total of 15 μ g DNA per transfection. Control cells were cotransfected with 5 μ g AP1-TF60CAT, 0.5 μ g pCMV β , and 9.5 μ g pCI vector. After being rendered quiescent, transfectants were stimulated for 5 h with serum plus TGF- β 1. Cell lysates were assayed for total protein and for CAT and β -galactosidase reporter expression. CAT protein levels were expressed as a percentage of control. Error bars reflect the range of three plates from a representative experiment

The identity of this factor is currently unknown, but a number of proteins other than CBP/p300 have been described that can bind sequences within the E1A N-terminus. These include the TATA-box binding protein TBP (Song *et al.*, 1995), Dr1, a transcriptional repressor (Kraus *et al.*, 1994), YY1, a multifunctional transcription factor (Lewis *et al.*, 1995), the bHLH protein myogenin (Taylor *et al.*, 1993), and a factor necessary for cardiac myocyte-specific gene expression (Bishopric *et al.*, 1997).

The RB-family member, p107, can substitute for pRB in promoter activation

The functional relationships between members of the RB family are still poorly understood (reviewed by Mulligan and Jacks, 1998). Thus, it was of interest to determine whether p107 and p130 could functionally substitute for pRB in stimulating TF promoter activity. To address this question, we tested the ability of pRB, p107 and p130 to rescue TF promoter activity in cells cotransfected with the E1A d2-36 mutant. As shown in Figure 6, both pRB and p107 effectively rescued promoter activity from E1A inhibition but p130 lacked any detectable effect. This was somewhat surprising given that p107 and p130 are more closely related to each other than to pRB (Mulligan and Jacks, 1998). Although differential effects of p107 and p130 on transcription have been previously noted (Wang *et al.*, 1994; Tevosian *et al.*, 1997), we could not discount the possibility that our results could simply reflect differences in the levels of ectopically expressed pRB, p107, and p130 in AKR-2B fibroblasts. To address this issue, immunoblot analysis was used to compare the levels of each endogenous pocket protein with the corresponding over-expressed pocket protein in lysates from transfected cells. While pRB and p107 over-expression was detectable, we were unable to reproducibly distinguish any difference in the levels of p130 in lysates from cells transfected with expression vector

Figure 6 Effect of pRB, p107, and p130 expression on E1A-inhibition of TF promoter activity. (a) AKR-2B cells were cotransfected with 5 μ g AP1-TF60CAT and 0.5 μ g pCMV β , along with 0.5 μ g of the d2-36 E1A deletion mutant expression vector alone, or including 6 μ g of pSG5RB, pcDEF3-p130, or CMV-p107. Empty pCI vector was added to maintain a total of 15 μ g DNA per transfection. Control cells were cotransfected with 5 μ g AP1-TF60CAT, 0.5 μ g pCMV β , and 9.5 μ g pCI vector. The treatment is the same as that described in Figure 5. Cell lysates were assayed for total protein and for CAT and β -galactosidase reporter expression. CAT protein levels were expressed as a percentage of control. Error bars reflect the range of three plates from a representative experiment. (b) The relative level of ectopic pRB, p107 or p130 expression was assessed by immunoblotting of hypertonic cell lysates from AKR-2B fibroblasts transiently transfected with 15 μ g of the indicated expression plasmids. Lysed cell protein (18 μ g per lane) was resolved on a 6% SDS-polyacrylamide gel and the resultant blot was sequentially probed with anti-p107 (SD9), anti-p130 (C-20) and then anti-pRB (IF8) antibodies. Graph on the right shows the relative band densities as measured by densitometry

compared to control cells (Figure 6b). As a consequence, we cannot exclude the possibility that the apparent failure of p130 to rescue is simply due to a deficiency of p130 over-expression relative to pRB and p107. Notwithstanding these results, the TF promoter was consistently rescued from inhibition by the E1A d2-36 mutant in cells transfected with either pRB or p107 expression vectors (Figure 6a). This suggests that the nominal level of ectopic expression observed for pRB and p107 was sufficient to elicit an effect on transcription.

E1A inhibition of TF promoter activity is cell synchronization dependent

To further test whether E1A-mediated inhibition of AP1-dependent TF promoter activity was due to

physical interaction between E1A and pRB, we analysed the need of cell synchrony on this effect. E1A specifically interacts with hypophosphorylated pRB, a form that predominates only in cells growth arrested in G₀ or in the early G₁ phase of the cell cycle (reviewed by Nevins, 1992). In AKR-2B fibroblasts, asynchronous rapidly-growing cells appear to express predominately hyperphosphorylated forms of pRB which migrate more slowly in SDS-polyacrylamide gels (Figure 7c, ppRB). Thus, if E1A-mediated inhibition of TF promoter activity is due to the direct binding of pRB, we would expect that inhibition would be similarly restricted to G₀ or to early G₁. To test this prediction, we compared the ability of E1A to inhibit TF promoter activity in cells undergoing the G₀ to G₁ transition with its ability to inhibit in asynchronous cells. As shown in Figure 7a, E1A strongly repressed TF reporter activity in quiescent cells stimulated for 5 h with serum and TGF- β 1 (FBS+1). In contrast, E1A failed to inhibit promoter activity in asynchronous, rapidly growing (RG) cells and even appeared slightly stimulatory at lower doses. The inability of E1A to repress the TF promoter in rapidly growing cells was not due to low levels of ectopic expression. In fact, E1A expression appeared to be slightly more robust in asynchronous, rapidly growing cells compared to synchronized cells stimulated with serum and TGF- β 1 even after normalizing for differences in protein load (Figure 7b). Importantly, Western blot analysis of lysed whole cell protein from parallel cultures confirmed that the hypophosphorylated form of pRB still predominated in 5 h serum and TGF- β 1 stimulated cells, while asynchronous RG cells exhibited proportionately more hyperphosphorylated pRB (Figure 7c, compare lanes 1 and 3). These results, taken together with those described above, lead us to conclude that E1A likely inhibits c-Fos-dependent activation of TF transcription through direct physical interaction with hypophosphorylated pRB. This conclusion is substantiated by data indicating that only the hypophosphorylated form of pRB coimmunoprecipitates with E1A from extracts of synchronized cells stimulated with serum and TGF- β 1 (Figure 7d, compare lanes 2 and 4).

Although sequestration of hypophosphorylated pRB by E1A is consistent with our experimental data, other mechanisms of E1A inhibition are equally plausible. For example, we cannot exclude the possibility that E1A does not inhibit AP1-TF promoter activity by direct physical association with hypophosphorylated pRB, but rather indirectly, perhaps by induction of pRB hyperphosphorylation as has been observed in E1A 12S virus-infected baby rat kidney cells (Wang *et al.*, 1991). One could argue that if hypophosphorylated pRB is solely responsible for AP1-TF promoter co-activation, then its hyperphosphorylation could manifest the same inhibitory effect as E1A-binding. Although we find no compelling evidence for E1A-induced hyperphosphorylation by immunoblot analysis of total cell extracts (data not shown), limitations imposed by the relative transfection efficiency of AKR-2B fibroblasts prevent us from accurately assessing pRB phosphorylation status in cells actually transfected with E1A versus non-transfected cells in the same culture. Thus, we cannot discount E1A-induced changes in pRB phosphorylation as a contributing

Figure 7 Influence of cell synchrony on E1A repression of TF promoter activity. (a) AKR-2B fibroblasts were cotransfected with 5 μ g AP1-TF60CAT along with the indicated amounts of pCIE1Awt expression vector. Empty pCI vector was added as needed to maintain a total of 10 μ g DNA transfected. Control cells were cotransfected with 5 μ g AP1-TF60CAT and 5 μ g pCI vector. After transfection, cells were fed with fresh media containing 5% FBS for 24 h (rapidly growing, RG) or rendered quiescent for 48 h, then restimulated for 5 h with serum plus TGF β 1 (FBS+T). Cell lysates were prepared and assayed for total protein and CAT enzyme. CAT protein levels were expressed as a percentage of control. Error bars reflect the range of three plates from a representative experiment. (b) Ectopic expression of E1A protein in asynchronous, rapidly growing (RG) cells versus synchronized cells stimulated with serum+TGF- β 1 (FBS+T) was analysed by Western blotting of lysed cell protein (10 μ g per lane) resolved on a 10% SDS-polyacrylamide gel. Blots were probed with anti-E1A M73. The major E1A band migrates as a $\sim$ 45 kDa protein in this gel system. (c) Expression of pRB in non-transfected AKR-2B fibroblasts was monitored by Western blotting of lysed cell protein (7.6 μ g per lane) prepared from rapidly growing (RG), quiescent (Q), and serum+TGF- β 1 (FBS+T)-stimulated cells and resolved on a 6% SDS-polyacrylamide gel. The blot was probed with a pRB antibody (G3-245, Pharmingen) that reacts with both hyper- and hypophosphorylated pRB (compare ppRB to pRB). The more rapidly migrating hypophosphorylated pRB band ($\sim$ 105 kDa) is proportionately greater in Q and FBS+T-stimulated cells while hyperphosphorylated pRB (ppRB) is more abundant in RG cells. (d) Hypophosphorylated pRB preferentially co-immunoprecipitates with E1A from extracts of FBS+T stimulated transfectants. Blot of a 6% SDS-polyacrylamide gel was probed with anti-pRB G3-245. Total starting lysed cell protein (1.4% of IP input) was run as a control. Abbreviations used are WB, Western blot; IP, immunoprecipitation; WCE, whole cell extract

factor to inhibition of TF promoter activity particularly in synchronized cells expressing a higher proportion of hypophosphorylated pRB (Figure 7c).

The role of p107, if any, as it relates to the data shown in Figure 7, is somewhat more ambiguous given that E1A is capable of interacting with both hypo- and hyperphosphorylated forms of p107 (Dyson *et al.*,

1989). Specifically, in rapidly-growing asynchronous cells, where p107 would be expected to be predominantly hyperphosphorylated, E1A should, in theory, have retained the ability to inactivate p107 (but not pRB). However, the absence of any discernable repressive effect of E1A on TF reporter activity in rapidly growing cells (Figure 7a) suggests that the importance of p107 in modulating TF transcription is secondary to that of pRB. This contention is also supported by failure of the E1A d2-36/CG124 mutant to repress TF promoter activity even though this mutant retains the ability to bind p107 (Figure 2, lane 10).

TF reporter activity in $Rb^{+/+}$, $Rb^{+/-}$, and $Rb^{-/-}$ mouse embryo fibroblasts

In an attempt to obtain more direct evidence for the involvement of pRB in the control of TF gene transcription in fibroblasts, we analysed the activity of the minimal AP1-TF60 reporter in transiently transfected $Rb^{+/+}$, $Rb^{+/-}$, and $Rb^{-/-}$ mouse embryo fibroblasts (MEFs). For consistency with experiments conducted with AKR-2B fibroblasts, transfected MEFs were serum-starved for 48 h then treated with 20% serum supplemented with 5 ng/ml TGF- β 1 for an additional 5 h. To account for differences in cell density and growth rate following serum-deprivation and growth stimulation, CAT enzyme values were normalized to total cell protein measured in transfected MEF lysates. As shown in Figure 8a, the expression of the CAT reporter gene in $Rb^{+/-}$ cells was ~twofold less than that of $Rb^{+/+}$ cells, while CAT expression in $Rb^{-/-}$ cells was ~sixfold less than that of wild-type MEFs. Residual AP1-TF60 promoter activity in $Rb^{-/-}$ cells may be due to compensatory effects mediated by endogenous p107. To ensure that the diminished activity of AP1-TF60 in Rb null cells was due, in fact, to the absence of pRB protein, $Rb^{-/-}$ cells were transiently co-transfected with the AP1-TF60 reporter and two different pRB expression vectors. As shown in Figure 8b, ectopic expression of pRB driven by either an SV40 promoter (pSG5RB) or CMV promoter (pCI-RB) markedly enhanced the activity of the TF reporter (7–8-fold) when using 1 μ g of expression plasmid. Curiously, when 5 μ g of expression plasmid was used, pSG5RB transfected cells retained high TF promoter activity, while pCI-RB transfected cells were indistinguishable from control cells. This latter result was reproducible and is likely due to a squelching effect imposed by over-expression of pRB by the stronger CMV promoter, or perhaps, to inhibition of endogenous *c-fos* gene expression (Robbins *et al.*, 1990). Furthermore, even in an Rb null background, ectopic expression of p107 augmented the activity of the AP1-TF reporter reinforcing the notion that pRB and p107 may be redundant in terms of their ability to function as coactivators of the TF promoter in fibroblasts.

Discussion

Several lines of evidence clearly implicate pRB and/or p107 as a required cofactor for c-Fos-dependent transcription of the TF gene in fibroblasts. First, E1A mutants deficient in their ability to bind pRB and

Figure 8 Tissue factor promoter activity in $Rb^{+/+}$, $Rb^{+/-}$ and $Rb^{-/-}$ mouse embryo fibroblasts. (a) Primary $Rb^{+/+}$, $Rb^{+/-}$ and $Rb^{-/-}$ mouse embryo fibroblasts were transfected with 10 μ g AP1-TF60CAT reporter and 0.5 μ g pCMV β using LipofectamineTM reagent. Cells were then treated as described in Figure 3a. Cell lysates were assayed for total protein and for CAT and β -galactosidase reporter expression. CAT levels were corrected for protein content and expressed as a percentage of the mean $Rb^{+/+}$ value (defined as 100%). Error bars represent the range of values in duplicate plates from a representative experiment. (b) $Rb^{-/-}$ fibroblasts were cotransfected with 5 μ g AP1-TF60CAT, 0.5 μ g pCMV β , and the indicated amounts of pRB and p107 expression vectors using LipofectamineTM reagent as above. Empty pCI vector was used to maintain a total of 10.5 μ g DNA per transfection. Control cells were cotransfected with 5 μ g AP1-TF60CAT, 0.5 μ g pCMV β , and 5 μ g pCI vector. Cell lysates were assayed for total protein and for CAT reporter expression. CAT enzyme levels were corrected for protein content. Error bars represent the range of values in duplicate plates from a representative experiment

p107 are impaired in their ability to repress TF promoter activity relative to wild-type E1A. Secondly, overexpression of pRB or p107 relieves repression mediated by the E1A d2-36 mutant, and thirdly, E1A repression of TF promoter activity is restricted to the G_0 and early G_1 phases of the cell cycle, a time in which the hypophosphorylated, E1A-binding form of pRB predominates. Because the activation of TF transcription in serum- or serum plus TGF- β 1-stimulated cells is dependent upon the rapid recruitment of c-Fos into heterodimeric AP-1 DNA-binding complexes (Felts *et al.*, 1995, 1997), these results point to a requirement for pRB and/or p107 in c-Fos-dependent transcription of the TF gene. More direct evidence in support of this conclusion derives from the fact that E1A inhibits the stimulation of TF transcription which results from c-Fos overexpression in the absence of growth factors, and that all tested mutations which abrogate pocket protein-binding (dCR2, CG124, YH47/CG124) relieve this inhibitory effect. Moreover, $Rb^{-/-}$ mouse embryo fibroblasts are deficient (relative to $Rb^{+/+}$ cells) in their ability to drive

high level AP1-TF promoter activity. Importantly, this deficiency is ameliorated by ectopic expression of either pRB or p107. Thus, pRB and/or p107 appear to function as required cofactors for c-Fos-dependent transcription of the TF gene in mouse fibroblasts.

How might c-Fos and pRB cooperate in stimulating TF transcription? Because pRB has been reported to directly interact with other transcription factors including members of the E2F family (reviewed by Nevins *et al.*, 1997), MyoD (Gu *et al.*, 1993), members of the CCAAT/enhancer-binding protein (C/EBP) family (Chen *et al.*, 1996a,b), and, in particular, c-Jun and c-Fos (Nead *et al.*, 1998; Nishitani *et al.*, 1999), we considered the possibility that pRB may similarly interact with c-Fos in AKR-2B fibroblasts. Although *in vitro* transcribed and translated c-Fos has been shown to interact with the B pocket region of pRB fused to glutathione-S-transferase via 'pull down' assay (Nead *et al.*, 1998), we were unable to detect such an interaction between c-Fos and full-length pRB by co-immunoprecipitation experiments conducted with AKR-2B fibroblast extracts and a variety of antibodies recognizing different c-Fos and pRB epitopes (data not shown). Thus, our data do not support a model in which c-Fos and pRB cooperate through direct physical interaction at least in AKR-2B extracts.

An alternative model to explain the cooperative effects of c-Fos and pRB on TF transcription is that both proteins may interact with one or more components of the TF basal transcription complex. For example, c-Fos has been shown to directly interact with the TATA-binding protein (TBP) component of the TFIID complex (Metz *et al.*, 1994) while pRB has been shown to bind directly to the TBP-associated factor TAF_{II}250 (Shao *et al.*, 1995). It is not difficult to imagine that such a dual interaction with the TFIID complex would have a cooperative effect on promoter activity. This model is also consistent with the specific requirement for c-Fos in TF transcription since other members of the Fos family lack the ability to bind TBP (Metz *et al.*, 1994).

Does pRB and/or p107 correspond to the factor previously designated CAF for coactivator of Fos (Felts *et al.*, 1997)? CAF was initially defined as a c-Fos- and E1A-interacting protein that mediated synergistic activation of TF transcription by serum and TGF- β 1. Specifically, we postulated that while the recruitment of c-Fos into specific AP-1 DNA-binding complexes was stimulated by serum-dependent signaling pathways (Felts *et al.*, 1995), the functional activity of CAF was modulated by TGF- β 1 (Felts *et al.*, 1997). Although, as noted above, our data do not support a direct interaction between pRB and c-Fos, pRB has been implicated in TGF- β 1 signaling pathways controlling both *c-myc* transcription in keratinocytes (Pietenpol *et al.*, 1990, 1991) and *jumB* transcription in epithelial cells (Giannini *et al.*, 1997). It is therefore possible that pRB plays a similar role in TGF- β 1-dependent transcriptional synergism in fibroblasts. However, the requirement for pRB is clearly not restricted to cells costimulated with TGF- β 1 because E1A mutants defective in pRB binding are also ineffective in inhibiting TF transcription in cells stimulated with serum alone (Figure 3b) or in cells stimulated by c-Fos overexpression (Figure 4). It will be of interest to determine whether TGF- β 1 affects the

phosphorylation status of pRB in fibroblasts as it does in mink lung epithelial cells (Giannini *et al.*, 1997).

Smads comprise a recently identified family of intracellular proteins that function as mediators of transducing signals originating from 'activated' type I receptors for the TGF- β superfamily of related growth factors (reviewed by Massagué *et al.*, 1997). Because Smad3 and Smad4 have been shown to directly interact with Jun family components of AP-1 complexes (Liberati *et al.*, 1999), and because Smads 1, 2, and 3 have been reported to interact with E1A (Nishihara *et al.*, 1999), we considered the possibility that Smads may play a role in TGF- β 1-dependent synergy of TF transcription in serum-stimulated fibroblasts. However, coexpression of Smad4 with either Smad2 or Smad3 did not potentiate serum-stimulated transcription of the AP1-TF60 reporter, nor did expression of a Smad3 dominant negative mutant inhibit synergy (data not shown). Interestingly, however, coexpression of Smad4 with either Smad2 or Smad3 specifically squelched TGF- β 1-dependent synergy, suggesting that at least one Smad-interacting protein may be integral to this effect in fibroblasts. This is unlikely to be c-Fos, however, because unlike the Jun proteins, Fos family components of AP-1 exhibit no detectable interaction with Smad proteins (Liberati *et al.*, 1999). Further study will be required to clarify the role, if any, of Smad proteins in this system.

Although our data strongly support a role for pRB and p107 as c-Fos cooperating factors, the failure of pRB to rescue TF promoter activity in cells expressing wild-type E1A (Figure 5) strongly suggests that these are not the only proteins functioning to augment c-Fos dependent transcription in fibroblasts. Because deletion of the E1A N-terminus is required for rescue of TF promoter activity by pRB or p107, it is likely that another protein with affinity for the E1A N-terminus may potentiate the coactivating functions of pRB and p107. That this protein is not CBP/p300 is evidenced by the fact that pRB expression does not relieve repression by the point mutant RG2 which, like the d2-36 mutant, is defective in CBP/p300 binding (Wang *et al.*, 1993 and Figure 2), nor does coexpression of pRB with CBP/p300 relieve repression by wild-type E1A. Thus, CAF, as previously defined (Felts *et al.*, 1997), may not represent a single entity, but a combination of factors which include pRB, and/or p107, and another, as yet unidentified E1A-binding protein. Functional cooperation between multiple coactivators and their targets has been observed in steroid receptor- and NF- κ B-dependent transcription (Smith *et al.*, 1996, Shepard *et al.*, 1999) and indeed, appears to be an emerging theme in eukaryotic gene regulation in general (reviewed by Kingston, 1999).

Finally, it is worth briefly commenting on the ability of p107, but not p130, to substitute for pRB in coactivating c-Fos-dependent transcription. While studies of knockout mice have suggested considerable functional overlap between p107 and p130, distinct biochemical properties and regulatory mechanisms have been observed (reviewed by Mulligan and Jacks, 1998; Kiess *et al.*, 1995; Garriga *et al.*, 1998; Smith *et al.*, 1998). These differences include protein-binding properties that affect transcriptional regulation, as well as distinct patterns of accumulation during cell proliferation. Differences in the spectrum of p107 and

p130 binding proteins may well account for their differential ability to coactivate c-Fos dependent transcription of the TF gene in fibroblasts. Delineation of amino acid sequences in pRB and p107 which are required for coactivation in this system seems likely to provide additional insight into the molecular mechanisms involved.

Materials and methods

Construction of E1A mutants

Full-length adenovirus E1A 12S cDNA was amplified from pCMV12SE1A (a gift of J Nevins) with primers '12SS1a', 5'-GGA TCCACGCGTGAGT GCCAGCGAGTA GAGTTTCTCCT-3', and '12SAS1a', 5'-GGATCCGGTACCCACACGCAATCACAGTTTACACCTTA-3', then subcloned into pCI (Promega) expression vector at *Mlu*I and *Kpn*I sites to obtain pCIE1Awt. The deletion mutant d2-36 was also generated using the same primers but with pCMVE1Ad2-36 as template (also a gift of J Nevins). The RG2 mutant was then directly amplified from pCIE1Awt with primers (altered base underlined) 5'-GGATCCACGCGTGGACTGAAAATGGGACATATTATCTGC-3', and 12SAS1a (above). Deletion mutants d37-60, d60-80, dCR2, and d129-139, and point mutant CG124 were constructed in pCIE1Awt by overlap extension using PCR amplification (Horton *et al.*, 1989). Fragments AB were generated using 12SS1a as primer A and the following B primers: d37-60, 5'-CTCGGGAATAATCTGCGAAACC GCGCTAGGAGGTGG AAGATTATCAGC-3'; d60-80, 5'-CGGGCGCCGGCGGAAAAGTGAACCTCGTTGGGATCTTCGGG-3'; dCR2, 5'-GCTCAGGCTCAGGTT CAGACCA CCACTCCGG CACA AGGT TTGGC-3'; d129-139, 5'-CTCAGGTTTCAGACACAGGGCCAGCCTCGTGGCAGGT-3'; and CG124 (altered base underlined) 5'-TGG AAAGCCAGCCTCG TGGCCGGTAAGA TCGATCACCTCCGG-3'. Fragments CD were generated using 12SAS1a as primer D and the following C primers: d37-60, 5'-GCTGATAATCTTCCACCTCCTAGC GCGGTTTCGCAGATTTTCCCGAG-3'; d60-80, 5'-CCCGAAGATCCCAACGAGTTCACTTTTCCGCCGGCGCCCC-3'; dCR2, 5'-GCCAAACCTTGTGCCGGAGGTGGTGTCTGAACCTGAGCCTGAGC-3'; d129-139, 5'-ACCTGCCACGAGGCTGGCCCTGTGTCTGAACCTGAG-3'; and CG124 (altered base underlined) 5'-ATCGATCTTACCGCCACGAGGCT-3'. The corresponding AB and CD fragments were 'SOEn' together by PCR amplification with 12SS1a and 12SAS1a primers. The final products were gel purified with spin-X columns (Costar), digested with *Mlu*I and *Kpn*I, and ligated into the corresponding sites in the pCI vector.

The YH47/CG124 double-point mutant was generated using pCIE1ACG124 as the template and the following primers (altered base underlined) for overlap extension: primer A, 12SS1a; primer B, 5'-CGTCACGTCTAAATCATG CAGTTCGTGAAGGGTAGG-3'; primer C, 5'-CCTACCTTCACGAAGTGCATGATTTAGACGTGACG-3'; and primer D, 12SAS1a. The d2-36/CG124 mutant was generated by using pCIE1Ad2-36 mutant as template and the primers for overlap extension as described above for the CG124 mutant. The fidelity of all constructs was confirmed by DNA sequencing and plasmids used for transfection were purified by double cesium chloride gradient centrifugation.

DNA constructs

AP1-TF60CAT reporter was constructed as described (Felts *et al.*, 1995). The expression vectors RSV-c-Fos, and RSV-JunD were obtained from H Iba. Retinoblastoma tumor suppressor protein (pRB) expression vector was a gift of WG Kaelin, Jr, while p107 and p130 expression vectors were gifts of B Dynlacht.

Cell culture, transient transfection and CAT ELISA assay

Mouse embryo-derived AKR-2B cells were maintained and transiently transfected using DEAE-Dextran and a total of 10 or 15 μ g plasmid DNA per 8×10^5 cells as described previously (Stoflet *et al.*, 1992; Felts *et al.*, 1995). Typically, cells were cotransfected with 5 μ g of a TF reporter plasmid, AP1-TF60CAT, and varying amounts (0.5 to 9 μ g) of expression plasmid. Empty pCI vector was supplemented as required to maintain a total of 10 or 15 μ g DNA per transfection. In some experiments, 0.5 μ g of a control plasmid, pCMV β (Clontech, β -galactosidase reporter), was included. After transfection, cells were allowed to recover in McCoy's 5A medium (Life Technologies Inc.) containing 10% fetal bovine serum (FBS) (Hyclone) for 18–24 h. Cells were then washed and rendered quiescent in serum-free MCDB 402 medium (JRH Biosciences) for 36–48 h. Quiescent cells were restimulated for 5 h with MCDB 402 containing 20% (v/v) serum alone or plus 5 ng/ml TGF- β 1 (Austral Biologicals). To obtain cycling cells, after transfection and recovery, cells were switched to McCoy's 5A medium supplemented with 5% FBS and grown for an additional 24 h. *Rb*^{+/+}, *Rb*^{+/-} and *Rb*^{-/-} mouse embryo fibroblasts (MEFs), obtained from T Jacks) were maintained in DME medium (Hyclone) supplemented with 10% FBS and transfected at passage 6 or 7. MEFs were transiently transfected using a ratio of 2 μ l LipofectamineTM reagent (Life Technologies, Inc.) per μ g plasmid DNA in DME as recommended by the manufacturer. After an overnight (18–24 h) recovery period in DME containing 20% FBS, transfected MEFs were washed, serum-starved in DME for 48 h, then restimulated with DME containing 20% (v/v) FBS plus 5 ng/ml TGF- β 1 for 5 h.

At the time of harvesting, cells were washed three times with phosphate-buffered saline, then lysed in 1 $\times$ hypotonic lysis buffer provided with the CAT-ELISA kit (Roche Molecular Biochemicals) and supplemented with a protease inhibitor cocktail (completeTM, Roche Molecular Biochemicals). Whole cell lysate was collected after centrifugation at 4°C, 14 000 r.p.m. for 10 min. Chloramphenicol acetyltransferase (CAT) and β -galactosidase reporter proteins were measured using commercial ELISA kits (Roche Molecular Biochemicals) as directed by the manufacturer. β -galactosidase served as reference to monitor transfection efficiency between replicate transfectants in selected experiments. Total protein content of the cell lysates was determined by BCA dye-binding assay (Pierce) using bovine serum albumin as standard.

Immunoprecipitation and Western blot analysis

Approximately 8×10^5 AKR-2B cells per 100 mm dish were transiently transfected with 10 μ g of the appropriate expression vectors (wild-type E1A and various E1A mutant cDNAs in pCI). After treatments as described above, cells were lysed and collected in 1 ml ice cold 'E1A buffer' (50 mM HEPES, pH 7.0, 250 mM NaCl, 0.1% NP-40) containing protease inhibitor cocktail. After spinning down the cell debris, lysates were precleared with protein A/G agarose (Pierce), then incubated with 1 μ g anti-E1A monoclonal antibody (M73, Santa Cruz) at 4°C overnight. The immunocomplexes were pulled down by protein A/G gel followed by four washes with lysis buffer. The co-precipitated proteins were resuspended in 1 $\times$ SDS-PAGE sample preparation buffer, boiled for 3 min, and resolved on 6% SDS-polyacrylamide gel followed by transfer onto Immobilon-P PVDF membrane (Millipore). Blots were blocked in 5% nonfat dry milk in TBST (20 mM Tris-HCL, pH 7.6, 137 mM NaCl, 0.1% Tween-20), then probed with primary antibodies diluted 1–2 μ g/ml in blocking buffer for 1–3 h. Commercially obtained antibodies used in this studies included anti-p300 (C-20, Santa Cruz), anti-CBP (A-22, Santa Cruz), anti-

p130 (C-20, Santa Cruz), anti-p107 (SD9, Santa Cruz) anti-pRB (G3-245, Pharmingen). Following incubation with primary antibody, blots were washed four times with TBST then horseradish peroxidase-conjugated secondary antibodies (Santa Cruz) diluted 1:2000 in blocking buffer were applied for 1 h. Following four TBST washes, immune complexes were detected with ECL reagents (Amersham) and visualized on BioMax MR film (Kodak).

In order to confirm E1A expression in transfected cells, Western blot analysis of total lysed cell protein was performed using a mouse monoclonal anti-E1A antibody (M73, Santa Cruz) at 1 µg/ml. To assess ectopic pRB, p107 and p130 expression, transfectants were lysed with hypertonic lysis buffer consisting of 20 mM HEPES pH 8, 440 mM NaCl, 1 mM EDTA, 25% v/v glycerol, 1% v/v Triton X-100, 7 mM β-mercaptoethanol plus protease inhibitors. Lysed cell protein was analysed by Western blotting using pocket

protein antibodies specific for pRB (IF8, Santa Cruz), p107 (SD9, Santa Cruz), and p130 (C-20, Santa Cruz).

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References

- Arany Z, Sellers WR, Livingston DM and Eckner R. (1994). *Cell*, **77**, 799–800.
- Arias J, Alberts AS, Brindle P, Claret FX, Smeal T, Karin M, Feramisco J and Montminy M. (1994). *Nature*, **370**, 226–229.
- Bannister AJ and Kouzarides T. (1995). *EMBO J.*, **14**, 4758–4762.
- Barbeau D, Charbonneau R, Whalen SG, Bayley ST and Branton PE. (1994). *Oncogene*, **9**, 359–373.
- Bisphopric NH, Zeng G-Q, Sato B and Webster KA. (1997). *J. Biol. Chem.*, **272**, 20584–20594.
- Bromberg ME, Konigsberg WH, Madison JF, Pawashe A and Garen A. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 8205–8209.
- Bugge TH, Xiao Q, Kombrinck KW, Flick MJ, Holmbäck K, Danton MJS, Colbert MC, Witte DP, Fujikawa K, Davie EW and Degen JL. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 6258–6263.
- Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, Van Vlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Müller M, Risau W, Edgington T and Collen D. (1996). *Nature*, **383**, 73–75.
- Chen P-L, Riley DJ, Chen Y and Lee W-H. (1996a). *Genes Dev.*, **10**, 2794–2804.
- Chen P-L, Riley DJ, Chen-Kiang S and Lee W-H. (1996b). *Proc. Natl. Acad. Sci. USA*, **93**, 465–469.
- Contrino J, Hair G, Kreutzer DL and Rickles FR. (1996). *Nature Med.*, **2**, 209–214.
- Dyson N, Buchkovich K, Whyte P and Harlow E. (1989). *Cell*, **58**, 249–255.
- Edgington TS, Mackman N, Brand K and Ruf W. (1991). *Thromb. Haemost.*, **66**, 67–79.
- Erlich J, Parry GCN, Fearn C, Muller M, Carmeliet P, Luther T and Mackman N. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 8138–8143.
- Felts SJ, Stang MT and Getz MJ. (1997). *Oncogene*, **14**, 1679–1685.
- Felts SJ, Stofflet ES, Eggers CT and Getz MJ. (1995). *Biochemistry*, **34**, 12355–12362.
- Garriga J, Limon A, Mayol X, Rane SG, Albrecht JH, Reddy EP, Andres V and Grana X. (1998). *Biochem. J.*, **333**, 645–654.
- Giannini G, Marcotullio LD, Zazzeroni F, Alesse E, Zani M, T'Ang A, Sorrentino V, Screpanti I, Frati L and Gulino A. (1997). *J. Biol. Chem.*, **272**, 5313–5319.
- Gu W, Schneider JW, Condorelli G, Kaushal S, Mahdavi V and Nadal-Ginard B. (1993). *Cell*, **72**, 309–324.
- Harlow E, Franza Jr. BR and Schley C. (1985). *J. Virol.*, **55**, 533–546.
- Horton RM, Hunt HD, Ho SN, Pullen JK and Pease LR. (1989). *Gene*, **77**, 61–68.
- Kiess M, Gill RM and Hamel PA. (1995). *Cell Growth and Differ.*, **6**, 1287–1298.
- Kingston RE. (1999). *Nature*, **399**, 199–200.
- Kraus VB, Inostroza JA, Yeung K, Reinberg D and Nevins J. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 6279–6282.
- Kwok RPS, Lundblad JR, Chrivia JC, Richards JP, Bächinger HP, Brennan RG, Roberts SGE, Green MR and Goodman RH. (1994). *Nature*, **370**, 223–226.
- Lewis BA, Tullis G, Seto E, Horikoshi N, Weinmann R and Shenk T. (1995). *J. Virol.*, **69**, 1628–1636.
- Liberati NT, Datto MB, Frederick JP, Shen X, Wong C, Rougier-Chapman EM and Wang X-F. (1999). *Proc. Natl. Acad. Sci. USA*, **96**, 4844–4849.
- Lundblad JR, Kwok RPS, Lurance ME, Harter ML and Goodman RH. (1995). *Nature*, **374**, 85–88.
- Massagué J, Hata A and Liu F. (1997). *Trends in Cell Biol.*, **7**, 187–192.
- Metz R, Bannister AJ, Sutherland JA, Hagemer C, O'Rourke EC, Cook A, Bravo R and Kouzarides T. (1994). *Mol. Cell. Biol.*, **14**, 6021–6029.
- Mueller BM, Reisfeld RA, Edgington TS and Ruf W. (1992). *Proc. Natl. Acad. Sci. USA*, **89**, 11832–11836.
- Mulligan G and Jacks T. (1998). *Trends in Genet.*, **14**, 223–229.
- Nead MA, Baglia LA, Antinore MJ, Ludlow JW and McCance DJ. (1998). *EMBO J.*, **17**, 2342–2352.
- Nevins JR. (1992). *Science*, **258**, 424–429.
- Nevins JR, Gustavo L, DeGregori J and Jakoi L. (1997). *J. Cell. Physiol.*, **173**, 233–236.
- Nishihara A, Hanai J, Imamura T, Miyazono K and Kawabata M. (1999). *J. Biol. Chem.*, **274**, 28716–28723.
- Nishitani J, Nishinaka T, Cheng C-H, Rong W, Yokoyama KK and Chiu R. (1999). *J. Biol. Chem.*, **274**, 5454–5461.
- Pietenpol JA, Mürger K, Howley PM, Stein RW and Moses HL. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 10227–10231.
- Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Mürger K, Howley PM and Moses HL. (1990). *Cell*, **61**, 777–785.
- Prestridge DS. (1991). *Comput. Appl. Biosci.*, **7**, 203–206.
- Reddy JC, Hosono S and Licht JD. (1995). *J. Biol. Chem.*, **270**, 29976–29982.
- Robbins PD, Horowitz JM and Milligan RC. (1990). *Nature*, **346**, 668–671.
- Shao Z, Ruppert S and Robbins PD. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 3115–3119.
- Shen X, Hu PP, Liberati NT, Datto MB, Frederick JP and Wang X-F. (1998). *Mol. Biol. Cell*, **9**, 3309–3319.
- Sheppard K-A, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK and Collins T. (1999). *Mol. Cell. Biol.*, **19**, 6367–6378.
- Singh P, Coe J and Hong W. (1995). *Nature*, **374**, 562–565.

- Smith CL, Onate SA, Tsai M-J and O'Malley BW. (1996). *Proc. Natl. Acad. Sci. USA*, **93**, 8884–8888.
- Smith EJ, Leone G and Nevins JR. (1998). *Cell Growth Differ*, **9**, 297–303.
- Song C-Z, Loewenstein PM, Toth K and Green M. (1995). *Proc. Natl. Acad. Sci. USA*, **92**, 10330–10333.
- Stoflet ES, Schmidt LJ, Elder PK, Korf GM, Foster DN, Strauch AR and Getz MJ. (1992). *Mol. Biol. Cell*, **3**, 1073–1083.
- Taylor DA, Kraus VB, Schwartz JJ, Olson EN and Kraus WE. (1993). *Mol. Cell. Biol.*, **13**, 4714–4727.
- Tevosian SG, Shin HH, Mendelson KG, Sheppard K-A, Paulson KE and Yee AS. (1997). *Genes Dev.*, **11**, 383–396.
- Topper JN, DiChiara MR, Brown JD, Williams AJ, Falb D, Collins T and Gimbrone MA Jr. (1998). *Proc. Natl. Acad. Sci. USA*, **95**, 9506–9511.
- Vrana JA, Stang MT, Grande JP and Getz MJ. (1996). *Cancer Res.*, **56**, 5063–5070.
- Wang HGH, Draetta G and Moran E. (1991). *Mol. Cell. Biol.*, **11**, 4253–4265.
- Wang HGH, Rikitake Y, Corrigan Carter M, Yaciuk P, Abraham SE, Zerler B and Moran E. (1993). *J. Virol.*, **67**, 476–488.
- Wang JK, Knudsen ES and Welch PJ. (1994). *Adv. Cancer Res.*, **64**, 25–85.