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Dissociation of Retinoblastoma Gene Protein Hyperphosphorylation and Commitment To Enter S Phase

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Mitogenic activities of simian virus 40 large T and small t antigens were studied in serum-deprived human diploid fibroblasts. Wild-type large T and small t cooperated in stimulating DNA synthesis and in inducing hyperphosphorylation of the *Rb* gene product (pRb). In contrast, a T antigen mutant defective for pRb binding (Rb^- T) possessed no detectable mitogenic activity alone and failed to complement small t in stimulating DNA synthesis. Surprisingly, Rb^- T and small t cooperated as strongly as wild-type T and small t with respect to pRb hyperphosphorylation. As a consequence, in two closely related conditions (i.e., stimulation by small t plus wild-type T versus small t plus Rb^- T), the fraction of pRb in hyperphosphorylated forms dissociated from the fraction of cells in the S phase. These results indicate that pRb hyperphosphorylation is not always tightly coupled with a commitment to initiate DNA replication.

The retinoblastoma gene product (pRb) is believed to have a pivotal role in control of the mammalian cell cycle. Support for this role derives from observations that the *Rb* gene is inactivated in a number of malignant states (7, 23, 38, 39), as well as from extensive evidence that pRb directly interacts with the transforming proteins encoded by several DNA tumor viruses (12, 17, 41, 48, 67). Involvement of pRb in control of cell proliferation is further suggested by its interactions with cyclins (15, 18, 36), cdc2/cdk-kinase family members (32, 68), and cellular transcription factors (2, 3, 9, 28, 30, 50, 58). Moreover, it has been demonstrated that introduction of a pRb expression vector or microinjection of pRb can result in G_1 arrest (26, 31) or suppression of tumorigenicity (5, 62).

Because phosphorylation-dephosphorylation reactions are crucial to the control of nuclear protein functions, considerable attention has been focused on variations in pRb phosphorylation with the cell cycle (6, 13, 45, 65). The level of pRb phosphorylation increases during the mid-G_1 phase or concomitant with onset of the S phase (11, 13, 41, 43) and remains elevated at least through the metaphase stage of mitosis (42). There is good evidence that cell cycle-dependent shifts in cdc2/cdk-kinase and phosphatase activities play important roles in the modulation of pRb phosphorylation (1, 16). Less clear, however, is precisely what functional consequences ensue from this cyclic modification of pRb. Over the past several years, emphasis has been placed on the strong correlation between the capacity of pRb to arrest cells in the mid- to late-G_1 phase and its hypophosphorylated state during this phase of the cell cycle (6, 11, 13, 41, 43, 45). On the basis of this correlation, it has been hypothesized that the interaction of pRb with critical target proteins, and therefore the capacity of pRb to inhibit cell cycle progression, is regulated by phosphorylation-dephosphorylation reactions acting on pRb (15, 18, 34, 41, 43, 51). While the available evidence is consistent with this idea, there is a need for further experimental observations to determine whether the interrelationship(s) between pRb phosphorylation and cell cycle control is more complex than is currently appreciated.

One highly informative approach to the investigation of the functions of pRb, p53, and other antiproliferative proteins has been to study the interactions of these proteins with polypeptides encoded by viral transforming genes. Functional interactions are potentially of two types: direct binding, exemplified by pRb–simian virus 40 (SV40) T-antigen complex formation, and indirect, e.g., via virus protein-induced changes in the phosphorylation states of target cellular proteins. A precedent for the latter mechanism has been described for SV40 small t antigen in which increased in vitro p53 phosphorylation can result from inhibition of protein phosphatase 2A (PP2A) by small t (56). Interestingly, it has been reported that neither the level nor the cell cycle pattern of pRb hyperphosphorylation in SV40-transformed cells differs from that observed in nontransformed cells (41, 43). One explanation for this finding is that PP2A does not contribute significantly to maintenance of the hypophosphorylated pRb state during G_1; if this is so, then inhibition of PP2A by small t should not directly influence the phosphorylation state of pRb. An alternative possibility is that the effect(s) on pRb phosphorylation mediated by the large T or small t antigen can be efficiently counteracted by cellular homeostatic mechanisms. If this is the case, and such homeostatic mechanisms are activated in SV40-transformed cell lines, effects of these viral oncogenic proteins on pRb phosphorylation might have been masked in earlier studies.

To gain further insight into these issues, we decided to investigate levels of pRb phosphorylation and DNA synthesis in human diploid fibroblasts (HDF) transiently transfected with expression plasmids encoding SV40 T and/or t antigens. The domain in T antigen required for pRb binding has been mapped to amino acid residues 105 to 114; point mutations within this region can completely abrogate in vitro formation of complexes between pRb (or other pRb-like proteins) and T antigen (12). Since the same mutations dramatically impair T-antigen-dependent cellular transformation (10, 35), it is

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generally accepted that these mutations in the T-antigen pRb-binding domain also reduce or abolish T-pRb interactions in vivo. By expressing large T antigens that are either competent or defective for pRb binding and simultaneously monitoring the fraction of transfected cells in the S phase, we were able to demonstrate that the level of pRb phosphorylation does not always correlate closely with a commitment to enter replicative DNA synthesis in HDF.

MATERIALS AND METHODS

Cell culture and transfections. WI-38 human lung embryo fibroblasts were obtained from the American Type Culture Collection and propagated as previously described (27). Electroporation was performed as previously described (25), except that in all experiments transfected cells were maintained in 0.5 or 0.3% fetal bovine serum (FBS) and 5 mM sodium butyrate for 48 h prior to harvesting.

Plasmids. Expression plasmids pRSV-TAg and pRSV-TAg(Rb$^-$) were described earlier (55); the latter is identical to pRSV-TAg-K1 (55). pRSV-TAg(p53$^-$) was constructed by ligating a *Pflm*I-*Nsi*I fragment from p402DN (40) to a *Pflm*I-*Nsi*I fragment from pRSV-TAg. CMV.3 was derived by combining an *Nru*I-*Xba*I fragment from CMV-IL2R (24) with an *Nru*I-*Xba*I cytomegalovirus (CMV) promoter-containing fragment from pcDNA I (InVitrogen). pCMV-TAg was obtained by ligating a *Hin*dIII-*Bam*HI fragment from CMV.3 with *Bam*HI-*Taq*I and *Taq*I-*Hin*dIII fragments from pRSV-TAg; pCMV-TAg(Rb$^-$) was constructed from corresponding fragments from CMV.3 and pRSV-TAg-K1. Two versions of plasmid CMV-t were generated by ligation of the larger *Hin*dIII-*Xba*I fragment from CMV-IL2R with fragments containing the small t coding region. The first small t coding sequence was obtained from plasmid pW2-t (8); this sequence was amplified by PCR with primers which generated 5′ *Hin*dIII and 3′ *Xba*I ends. The second small t coding sequence fragment, derived from pRSV-TAg, was likewise amplified by PCR with primers which generated 5′ *Hin*dIII and 3′ *Xba*I ends. DNA sequence analysis demonstrated that the small t coding sequence obtained from pRSV-TAg was identical to that published for SV40 strain 776 (20, 52). Parallel analysis of the small t coding sequence from plasmid pW2-t revealed differences leading to amino acid changes at three positions: Met to Ile at position 95 (ATG→ATA), Ala to Val at position 104 (GCA→GTA), and Ala to Thr at position 109 (GCT→ACT). Both small t variants possessed equivalent mitogenic and transcriptional activation activities (unpublished data). CMV-cTAg was constructed by site-directed deletion of the small t intron from a subcloned *Hin*dIII fragment, followed by excision of the mutagenized region with *Pflm*I and *Hin*dIII and ligation in a three-fragment reaction with *Hin*dIII-*Bam*HI and *Bam*HI-*Pflm*I fragments from CMV-TAg. CMV-cTAg(Rb$^-$) was obtained by rejoining the small t intron deletion fragment with corresponding fragments from CMV-TAg(Rb$^-$).

Affinity cell sorting and flow cytometry. Magnetic affinity cell sorting was performed as previously described (54); investigators interested in implementing this technique should contact the corresponding author for a detailed protocol. For flow cytometry, nuclei from sorted cells were either stained directly with propidium iodide or fixed with ethanol, denatured with 2 N HCl, and stained with anti-bromodeoxyuridine (Becton-Dickenson) and propidium iodide. Analysis was performed by using LysisII software on a FACscan flow cytometer equipped with a doublet discrimination module (Becton-Dickinson).

Western immunoblot analysis. Western analysis was performed as previously described (65). Antibodies obtained from PharMingen included anti-large T (Pab101), anti-small t (Pab108), and anti-pRb (G3-245). Quantification of pRb hyperphosphorylated forms was performed by laser densitometry using ImageQuant software (Molecular Dynamics).

RESULTS

Since we wished to gain information concerning the role of pRb, in particular pRb hyperphosphorylation, in cell cycle regulation, it was essential to employ conditions under which entry into the S phase was demonstrably dependent on neutralization of *Rb* family proteins. Such conditions were found previously in a study which we performed to determine whether functional inactivation of pRb (and/or other *Rb* family proteins) is required for stimulation of DNA synthesis by SV40 large T and small t antigens in serum-deprived WI-38 HDF (55). In that study, we compared the mitogenic activities of wild-type T antigen and a pRb binding-defective T-antigen mutant in the presence of small t antigen. The initial expectation was that pRb binding by T antigen would be required for efficient stimulation of G_1→S transit and that the degree of pRb hyperphosphorylation would closely correlate with the extent of mitogenic stimulation. Surprisingly, in WI-38 HDF subjected to serum deprivation for 48 h, the mitogenic activity of T exhibited only a weak, poorly reproducible dependence on its pRb-binding domain. This was unsatisfactory, since under conditions in which mitogenic activity did not require T-mediated pRb inactivation, there would be no reason to believe that functional inactivation of pRb by an alternate pathway, e.g., hyperphosphorylation, should regulate entry into the S phase. Fortuitously, it was found that serum deprivation of WI-38 cells in the presence of the differentiating agent sodium butyrate dramatically enhances the role of pRb-*Rb* family proteins in WI-38 cell cycle control (55). Under the latter conditions, as in cellular senescence, T antigen is strongly dependent on its pRb-binding domain for stimulation of DNA synthesis.

Comparison of wild-type and pRb binding-defective T antigens. To confirm the validity of serum deprivation in the presence of sodium butyrate as a model for studying control of G_1→S transit, two background experiments were performed. The first ensured that growth arrest would occur in the G_1 phase of the cell cycle when WI-38 cultures were serum starved under these conditions. Cultures were maintained in 0.3% FBS for 48 h with or without 5 mM sodium butyrate. As shown in Fig. 1c and d, butyrate treatment potentiated, but did not otherwise alter, the G_1 block caused by serum starvation. Control cultures maintained in 10% FBS with or without butyrate revealed that butyrate also induced G_1 arrest in WI-38 cells under high-serum conditions (Fig. 1a and b). In the second experiment, we demonstrated the feasibility of a new assay designed to provide more detailed information on S-phase entry and progression in cells transiently expressing SV40 T and t antigens. In this experiment, bromodeoxyuridine (BrdUrd)-labeled nuclei were prepared from surface-tagged cells which had been transfected with pRSV-IL2R (a plasmid encoding Tac antigen [21]) with or without pRSV-TAg, a plasmid encoding both large T and small t gene products. Nuclei were subjected to flow cytometric analysis by double staining with a fluorescein-tagged anti-BrdUrd monoclonal antibody and propidium iodide. In the transfected control cells, serum deprivation in the presence of sodium butyrate resulted in almost complete cessation of DNA synthesis (Fig. 2A). Conversely, when parallel HDF cultures were cotransfected with the SV40 T-t antigen expression vector, a large fraction of serum-deprived cells overcame the G_1 block and

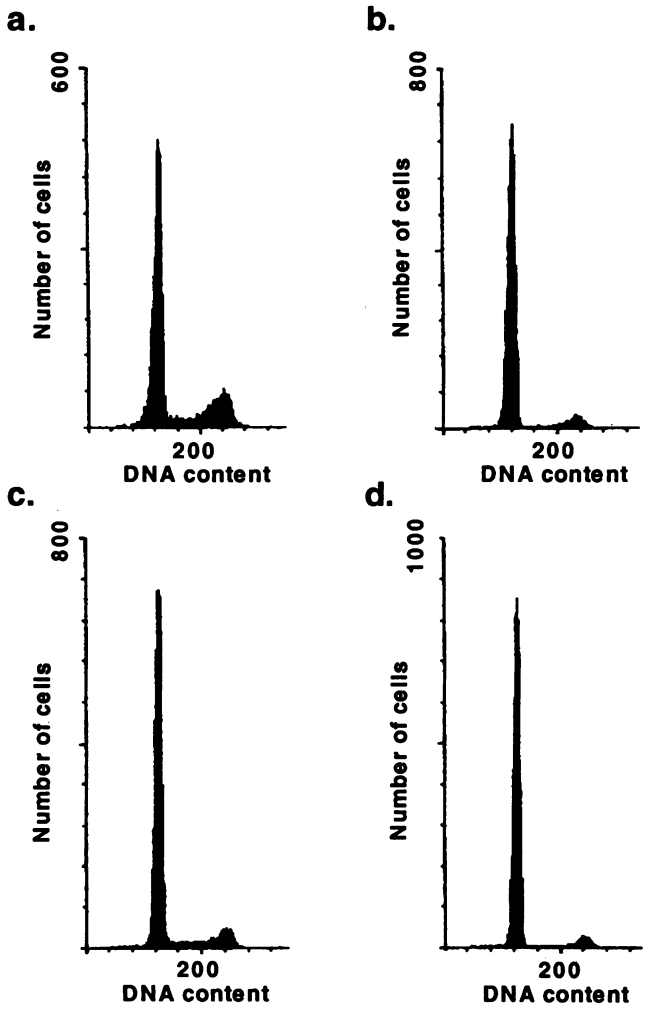


FIG. 1. Effects of sodium butyrate treatment on HDF cell cycle. Subconfluent WI-38 HDF were maintained for 48 h in medium containing 10% FBS (a), 10% FBS with 5 mM sodium butyrate (b), 0.3% FBS (c), or 0.3% FBS with 5 mM sodium butyrate (d). Nuclei prepared from harvested cells were stained with propidium iodide to determine DNA content.

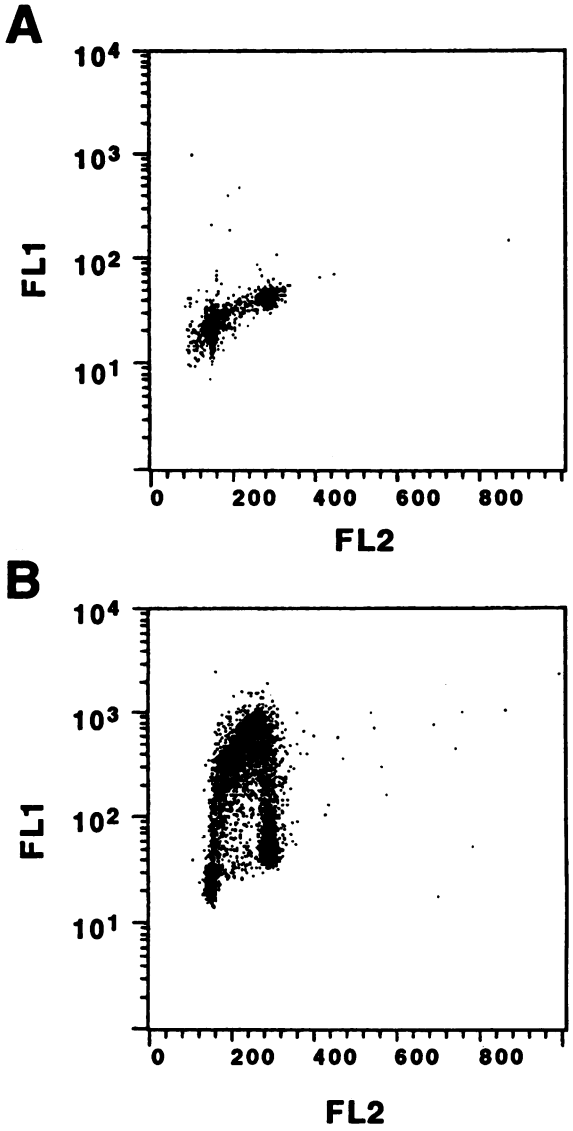


FIG. 2. Cell cycle analysis of transiently transfected HDF. (A) Subconfluent replicating WI-38 HDF were transfected with pRSV-IL2R (5 μg) and maintained in medium containing 0.5% FBS with 5 mM sodium butyrate for 48 h. Cultures were incubated in 10 μM BrdUrd for 30 min to label cells in the S phase and then subjected to affinity sorting to isolate Tac-antigen-positive cells. Nuclei prepared from sorted cells were stained with fluorescein-coupled anti-BrdUrd (FL1) and propidium iodide (FL2). Abscissa and ordinate values represent fluorescence intensity (arbitrary units). (B) Transfection with pRSV-IL2R (5 μg) plus pRSV-TAg (4 μg).

incorporated BrdUrd in a pattern consistent with the ability to complete at least one round of replicative DNA synthesis (Fig. 2B).

To compare relative mitogenic activities of wild-type large T antigen and a T antigen defective for binding to *Rb* family proteins by using this assay, we next transfected subconfluent HDF with pRSV-IL2R alone or together with either pRSV-TAg or pRSV-TAg(Rb^-); the latter plasmid encodes a pRb binding-defective T antigen (Rb^- T) described by Kalderon and Smith (mutant K1; Glu-107→Lys) (35). Equal amounts of wild-type and Rb^- T antigens are produced by these plasmids (55). After HDF had been maintained for 48 h in medium containing 0.5% FBS and 5 mM sodium butyrate, they were labeled for 30 min with BrdUrd and nuclei from the transfected subpopulations were isolated. As determined by flow cytometry of anti-BrdUrd-propidium iodide-stained singlet nuclei, the percentage of control transfected cells in the S phase was 0.4%, whereas cells transfected with wild-type T or Rb^- T exhibited labeling indices of 55 and 6%, respectively (Fig. 3).

Sodium butyrate induces pRb hypophosphorylation. The strong dependence of T mitogenic activity on the preservation of an intact T pRb-binding domain in the presence of sodium butyrate led us to investigate whether this differentiation-inducing and growth-inhibitory agent (22, 37, 49, 63) might functionally activate pRb, either by increasing the level of pRb or by altering its phosphorylation state. HDF were maintained in various concentrations of butyrate for 16 or 40 h and then harvested for quantification of pRb by Western analysis. As shown in Fig. 4, these treatments did not alter pRb protein levels but rather caused a time- and dose-dependent decrease

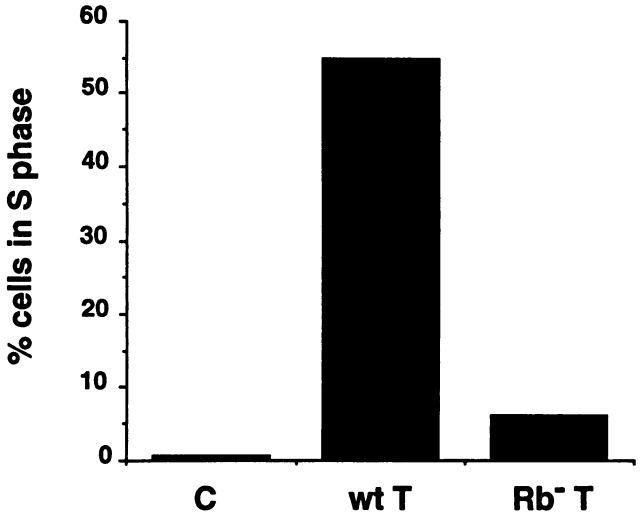


FIG. 3. Relative mitogenic activities of wild-type and pRb binding-defective T antigens. Subconfluent WI-38 cells were transfected, maintained in medium containing 0.5% FBS–5 mM sodium butyrate for 48 h, labeled with BrdUrd, and analyzed as described in the legend to Fig. 2. C, pRSV-IL2R (5 μg) alone; wt T, pRSV-IL2R (5 μg) plus pRSV-TAg (4 μg); Rb⁻ T, pRSV-IL2R (5 μg) plus pRSV-TAg(Rb⁻) (4 μg).

in pRb phosphorylation. Butyrate-induced pRb hypophosphorylation was also observed for cells cultured in either high or low serum concentrations (Fig. 4 and unpublished results). Thus, to the extent that butyrate enhances the effective activity of *Rb* family proteins in HDF cell cycle regulation, this change correlates with pRb hypophosphorylation rather than altered pRb synthesis or degradation. Although it is tempting to speculate that pRb is a primary target in butyrate-mediated growth inhibition, a much more likely possibility is that this agent has pleiotropic effects on cell cycle functions. Consistent with this idea, Saos-2 osteosarcoma cells, which are defective for both *p53* and *Rb* gene functions (5, 33), are subject to growth suppression by sodium butyrate; furthermore, transfection of these cells with a pRb expression vector did not substantially increase their susceptibility to the inhibitory properties of this agent (unpublished results).

pRb hyperphosphorylation in cells transfected with T and t. Given the low background pRb phosphorylation level in butyrate-treated, serum-deprived HDF and the reproducibly

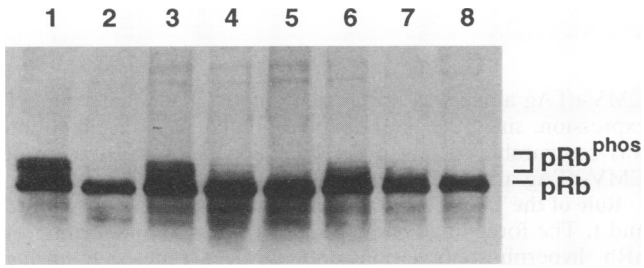


FIG. 4. Effect of sodium butyrate on pRb phosphorylation. Western blot of pRb extracted from WI-38 HDF maintained in complete medium with 10% FBS. Lanes: 1, subconfluent cells in the absence of butyrate; 2, confluent cells in the absence of butyrate; 3 to 5, subconfluent cells treated for 16 h with 1, 3, or 5 mM butyrate; 6 to 8, subconfluent cells treated for 40 h with 1, 3, or 5 mM butyrate.

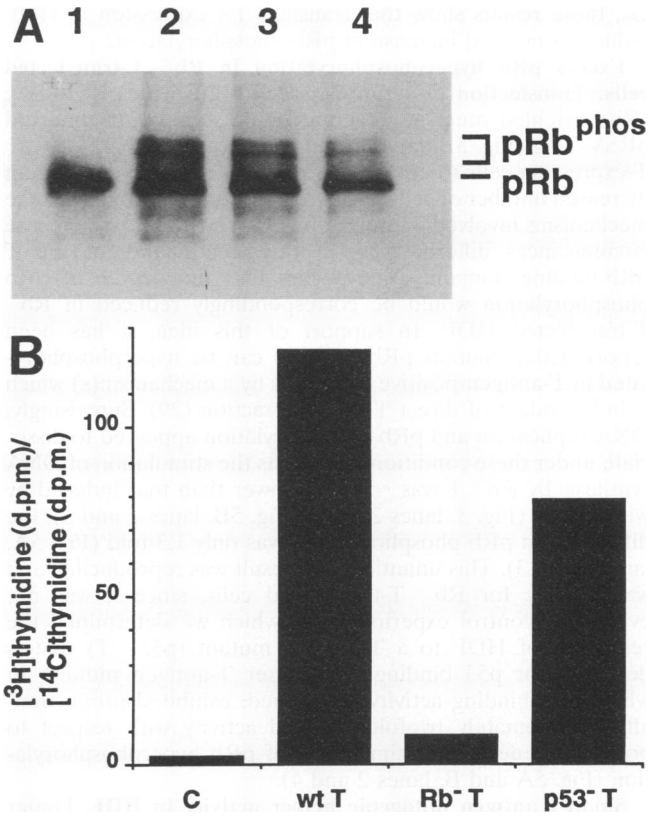


FIG. 5. Excess pRb hyperphosphorylation in cells expressing pRb-binding-defective T antigen. WI-38 cells were electroporated, maintained for 48 h in medium containing 0.5% FBS–5 mM butyrate, and harvested by affinity sorting. (A) Western blot of pRb extracted from cells transfected with the following: lane 1, pRSV-IL2R (5 μg) alone; lane 2, pRSV-IL2R (5 μg) plus pRSV-TAg (5 μg); lane 3, pRSV-IL2R (5 μg) plus pRSV-TAg(Rb⁻) (5 μg); lane 4, pRSV-IL2R (5 μg) plus pRSV-TAg(p53⁻) (5 μg). (B) Determination of DNA synthesis in cells transfected and maintained for 48 h in medium containing 0.5% FBS–5 mM butyrate as for panel A. Prior to electroporation, cells were labeled for 4 h in 0.05 μCi of [^{14}C]thymidine per ml (this facilitated normalization for small variations in transfection efficiency and/or recovery in cell sorting). Cells were then labeled with 1 μCi of [^{3}H]thymidine per ml for 2 h immediately before harvesting to determine mitogenic effects of transfection with the indicated plasmids.

strong mitogenic activity of pRSV-TAg under these conditions (Fig. 3; reference 55), we expected dramatic stimulation of pRb hyperphosphorylation in cells transiently transfected with this plasmid. To determine whether this would occur, HDF were cotransfected with pRSV-IL2R and pRSV-TAg, serum deprived in the presence of butyrate for 48 h, harvested by affinity sorting, and processed for Western blot analysis. As shown in Fig. 5A (lanes 1 and 2), in cells transiently expressing wild-type large T and small t antigens, approximately 50% of pRb migrated within a smear of hyperphosphorylated forms. Parallel experiments in which an equivalent amount of pRSV-TAg was transfected revealed that this level of pRb hyperphosphorylation correlated with ≈50% of cells being in the S phase or ≈75% of cells being in S plus G_2-M (Fig. 3). Although it is uncertain whether normal cell cycle-dependent kinase activation under these conditions should yield a percentage of hyperphosphorylated pRb comparable to the percentage of cells in the S phase or closer to the percentage of cells in S plus

G_2, these results show that transient T-t expression in HDF induces a marked increase in pRb phosphorylation.

Excess pRb hyperphosphorylation in Rb$^-$ T-transfected cells. Transfection of serum-deprived HDF with pRSV-TAg (Rb$^-$) yielded much weaker mitogenic stimulation than did pRSV-TAg (Fig. 5B, lanes 2 and 3). Since the failure of Rb$^-$ T-expressing cells to enter the S phase was associated with an increased number of cells in G_1 (data not shown), and since the mechanisms involved in promoting DNA synthesis under these circumstances differed only in the functionality of the T pRb-binding domain, we expected that the degree of pRb phosphorylation would be correspondingly reduced in Rb$^-$ T-transfected HDF. In support of this idea, it has been reported that mutant pRb proteins can be hyperphosphorylated in T-antigen-positive COS cells by a mechanism(s) which is independent of direct T-pRb interaction (29). Surprisingly, DNA replication and pRb phosphorylation appeared to dissociate under these conditions. Whereas the stimulation of DNA synthesis by Rb$^-$ T was ≈10-fold lower than that induced by wild-type T (Fig. 3, lanes 2 and 3; Fig. 5B, lanes 2 and 3), the difference in pRb phosphorylation was only 1.3-fold (Fig. 5A, lanes 2 and 3). This unanticipated result was reproducible and was specific for Rb$^-$ T-transfected cells, since it was not evident in control experiments in which we determined the responses of HDF to a T-antigen mutant (p53$^-$ T) that is defective for p53 binding. The latter T-antigen mutant, in which pRb-binding activity is retained, exhibited proportionally approximately twofold reduced activity with respect to both mitogenesis and stimulation of pRb hyperphosphorylation (Fig. 5A and B, lanes 2 and 4).

Small t antigen mitogenic helper activity in HDF. Under restrictive growth conditions, e.g., serum deprivation or confluence, a role for small t antigen in SV40-mediated transformation of rodent cells can be demonstrated (4, 44, 59, 61). A role has also been reported for small t in SV40-induced dense focus formation in HDF (14). Since small t antigen was expressed in the above-described experiments, one obvious question was whether the relatively weak stimulation of DNA synthesis observed following transfection with the pRSV-TAg(Rb$^-$) plasmid was due to Rb$^-$ T alone or required small t. A related, and for us particularly interesting, question was whether the PP2A-inhibitory activity of small t (47, 53, 56, 69) contributed to the anomalously high pRb phosphorylation in pRSV-TAg(Rb$^-$)-transfected cells.

Initially, the mitogenic activity of small t alone was tested in experiments in which HDF were transfected with a small t expression vector, CMV-t. When DNA input was adjusted such that the level of small t expression achieved was comparable to that obtained with the genomic vector pRSV-TAg, only a low level of small t-induced DNA synthesis was observed and the level of pRb phosphorylation was minimally increased over that in control cells (data not shown). This contrasted with experiments in which small t was overexpressed and small t mitogenic activity could be readily detected (see below).

To determine whether small t complemented large T in this transfection assay, we evaluated the extent to which large T alone could increase the fraction of S-phase cells and/or the level of pRb hyperphosphorylation. This was accomplished by transfecting HDF with CMV-cTAg, a cDNA expression plasmid that encodes large T only, with or without CMV-t. In the absence of small t antigen, large T induced only a low level of pRb hyperphosphorylation (Fig. 6A) and exhibited weak mitogenic activity (Fig. 6C). By comparison, strong pRb hyperphosphorylation and DNA synthesis induction were evident following cotransfection of plasmids CMV-cTAg and CMV-t. The reduced mitogenic response following transfection with

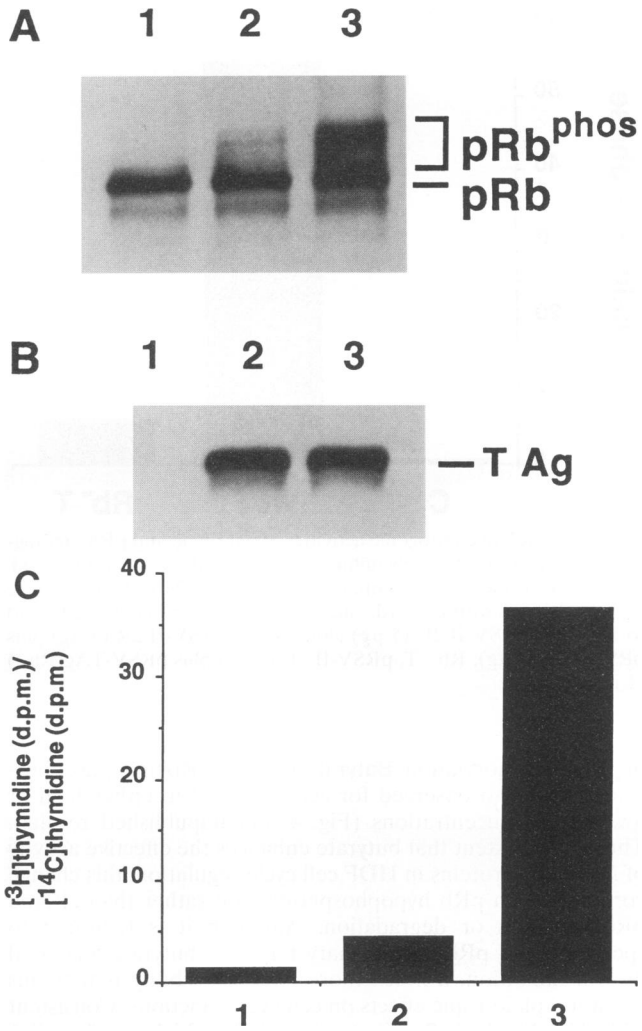
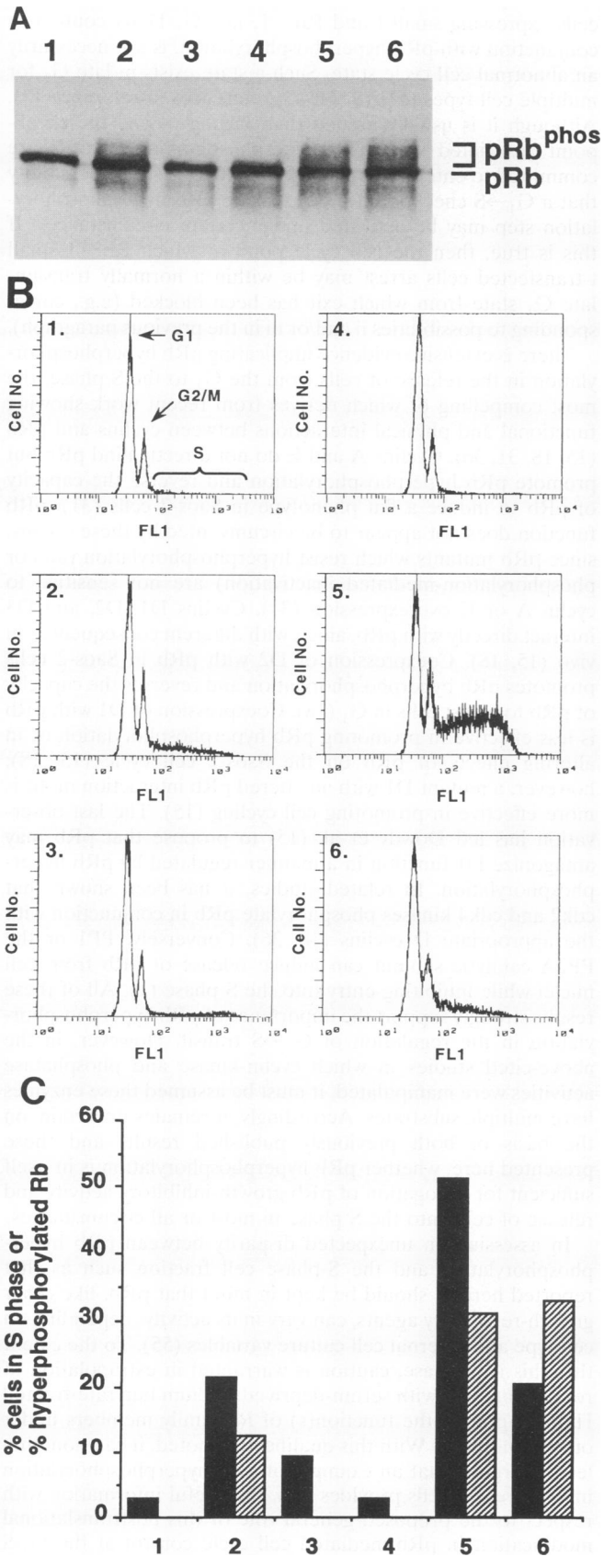


FIG. 6. Cooperation of large T and small t antigens. (A and B) Western blots of pRb and T antigen, respectively, extracted from WI-38 cells transfected, maintained for 48 h in medium containing 0.3% FBS–5 mM butyrate, and harvested by affinity sorting. Transfections included pCMV-IL2R (2 μg) and the following: lane 1, pCMV.3 control (25 μg); lane 2, pCMV-cTAg (20 μg); lane 3, pCMV-cTAg (20 μg) plus pCMV-t (5 μg). (C) Determination of DNA synthesis in cells transfected and maintained in medium containing 0.3% FBS–5 mM butyrate as in panels A and B. Cultures were sequentially labeled with [^{14}C]thymidine and [^{3}H]thymidine as described in the legend to Fig. 5B. Lanes: 1, pCMV.3 control (20 μg); 2, pCMV-cTAg (15 μg) plus CMV.3 (5 μg); 3, pCMV-cTAg (15 μg) plus pCMV-t (5 μg).

CMV-cTAg alone was not attributable to inadequate large T expression, since the level of T accumulated in this situation was comparable to that observed following cotransfection of CMV-cTAg and CMV-t (Fig. 6B).

Role of the T pRb-binding domain in cooperation between T and t. The foregoing results [in particular, the dissociation of pRb hyperphosphorylation and DNA synthesis following pRSV-TAg(Rb$^-$) transfection] predicted that introduction of cDNA plasmid CMV-cTAg(Rb$^-$) together with CMV-t might induce pRb hyperphosphorylation without concomitant stimulation of DNA synthesis. Results of an experiment in which these plasmids were tested alone and in combination are shown in Fig. 7. As an internal control in this experiment, we



retested cooperation between CMV-t and CMV-cTAg, albeit with an adjusted CMV-t input. Since we wanted to ensure that a potentially weak capacity of small t to complement Rb^- T would not be missed, the amount of transfected CMV-t was increased. At this level, the ability of small t to induce DNA synthesis was easily detected (Fig. 7B, histograms 1 and 2) and in fact was greater than the mitogenic activity of large T alone (histograms 2 and 3). The cooperation between large T and small t to induce S-phase entry, while less dramatic, was still readily seen (histogram 5); again, mitogenic cooperation was not due to a change in the expression of large T, since T levels were independent of small t expression (data not shown). Comparison of pRb phosphorylation levels in cells transfected with small t alone or large T plus small t revealed that hyperphosphorylated forms increased roughly in parallel with DNA synthesis, although the maximum percentage of pRb in hyperphosphorylated forms remained less than the percentage of cells in the S phase (Fig. 7A and B, lanes and histograms 2 and 5; quantified in Fig. 7C). The reason for this is not known but might be related, paradoxically, to a relatively high level of small t expression in this experiment, e.g., if small t-induced mitogen-activated protein (MAP) kinase activation (60) promotes S-phase entry in the absence of normal pRb phosphorylation.

Quite different results were observed following transfection with CMV-cTAg(Rb^-), alone or together with CMV-t. (i) Rb^- T expression in the absence of small t had little or no effect on either pRb phosphorylation or DNA synthesis (Fig. 7A, lane, 4 and 7B, histogram 4). (ii) Although an increased fraction of S-phase cells was seen following coexpression of Rb^- T and small t, mitogenic activity was no greater than that obtained with small t alone (Fig. 7B, histograms 2 and 6). (iii) Despite the failure of Rb^- T and small t to cooperate in stimulating DNA synthesis, these proteins together increased pRb phosphorylation 2.5-fold, to a level equivalent to that associated with coexpression of small t and wild-type T (Fig. 7A, lanes 5 and 6). We conclude that large T has the potential to act in conjunction with small t to stimulate pRb hyperphosphorylation and that this activity is at least partly independent of a commitment to enter the S phase.

DISCUSSION

In this study, we used a novel transfection-affinity cell sorting approach to monitor simultaneously the effects of SV40 large T- and small t-antigen expression on both cell cycle distribution and pRb hyperphosphorylation. A number of the results obtained were expected on the basis of previous reports. For

FIG. 7. Dissociation of pRb hyperphosphorylation and the fraction of cells in the S phase. WI-38 cells were electroporated, maintained for 48 h in medium containing 0.3% FBS–5 mM butyrate, and harvested by affinity sorting. (A) Western blot of pRb extracted from cells transfected with pCMV-IL2R (4 μg) and the following: lane 1, pCMV.3 control (25 μg); lane 2, pCMV-t (10 μg) plus pCMV.3 (15 μg); lane 3, pCMV-cTAg (15 μg) plus pCMV.3 (10 μg); lane 4, pCMV-cTAg(Rb) (15 μg) plus pCMV.3 (10 μg); lane 5, pCMV-t (10 μg) plus pCMV-TAg (15 μg); lane 6, pCMV-t (10 μg) plus pCMV-cTAg(Rb^-) (15 μg). (B) Histograms showing cell fractions in the S phase. Box numbers correspond to the lane numbers in panel A. FL1 (log scale) indicates fluorescence intensity due to staining with fluorescein-tagged anti-BrdUrd monoclonal antibody. (C) Comparison between the fraction of transfected cells in the S phase (solid columns) and the fraction of pRb in hyperphosphorylated forms (cross-hatched columns). The numbers below the columns correspond to the lane numbers in panel A.

example, it was confirmed that large T and small t antigens can cooperate strongly in HDF to stimulate DNA synthesis. This was anticipated on the basis of earlier evidence that coexpression of these SV40 early gene products is required for dense focus formation in HDF (14). It was further found that coexpression of wild-type T and small t efficiently induces pRb hyperphosphorylation in association with DNA synthesis. This was also expected, since appropriate levels of pRb phosphorylation had previously been observed in proliferating SV40-transformed cell lines (29, 41, 43).

A surprising result which emerged from our investigation is that under certain conditions, mitogenic cooperation between large T and small t could be shown to depend on an intact large T pRb-binding domain. This result was not predicted by the proposal that small t stimulates DNA synthesis by inducing pRb hyperphosphorylation when large T synthesis is limiting (for example, see reference 57). If this proposal were correct, small t-mediated pRb hyperphosphorylation should directly inactivate pRb and thereby complement Rb^- T at least as strongly as wild-type T. An alternative hypothesis which has been set forth is that small t-induced changes in T phosphorylation facilitate large T binding to pRb (19). Our results are consistent with the latter suggestion; however, there is no direct evidence that small t expression influences large T phosphorylation (70). A third possibility, that small t indirectly stimulates the MAP kinase pathway by interacting with PP2A (60), is also consistent with our data if it is postulated that the G_1 block induced by *Rb* family proteins under these conditions is epistatic to the mitogenic effect of MAP kinase activation unless pRb and its relatives are inactivated by direct interaction with T antigen.

When the activity of small t alone was compared with that of small t in combination with Rb^- T, it was found that these proteins failed to act together to stimulate DNA synthesis but did cooperation with respect to pRb phosphorylation. The resulting dissociation between pRb hyperphosphorylation and DNA synthesis, as measured by the fraction of cells in the S phase, is the first such example reported (although a disparity between pRb phosphorylation and induction of mitosis by adenovirus E1A mutants has been described previously [66]). Since under the conditions studied here DNA synthesis is strongly dependent on an intact pRb-binding domain in T antigen, it is reasonable to conclude that the limiting step(s) leading to S-phase entry involve neutralization of one or more *Rb* family members. This, in turn, suggests that a limited number of mechanisms can be considered likely to account for the failure to proceed into the S phase despite pRb hyperphosphorylation. The most straightforward possibilities are the following: (i) pRb hyperphosphorylation induced by small t-Rb^- T cooperation, although seemingly equivalent to that induced by small t–wild-type T cooperation, is nevertheless qualitatively deficient and thus is insufficient to inactivate pRb. (ii) pRb hyperphosphorylation is necessary but not sufficient for entry into the S phase—this could apply if pRb also controls S phase entry by a mechanism that is relatively independent of its phosphorylation state. (iii) S-phase entry is blocked by a second *Rb* family protein, such as p107 (71), which may not be subject to (or regulated by) small t-Rb^- T-mediated hyperphosphorylation. We cannot distinguish between these alternatives. As for the first possibility, however, we found that either small t–wild-type T- or small t-Rb^- T-induced hyperphosphorylated pRb forms could be washed out from nuclei by treatment with nonionic detergent in hypotonic buffer (46, 64), so by this criterion they are not distinguishable from putatively nonfunctional forms of pRb (unpublished results).

It is important to point out that the arrest state observed in cells expressing small t and Rb^- T, i.e., G_1 DNA content in conjunction with pRb hyperphosphorylation, is not necessarily an abnormal cell cycle state. Such a state exists in late G_1 for multiple cell types (11, 13, 41, 43; references in reference 15). Although it is usually argued that, having passed the checkpoint associated with pRb hyperphosphorylation, cells are committed to enter the S phase, our results raise the possibility that a $G_1 \rightarrow S$ checkpoint(s) beyond the pRb hyperphosphorylation step may be activated under certain circumstances. If this is true, then the cell cycle point at which Rb^- T-small t-transfected cells arrest may be within a normally transient late G_1 state from which exit has been blocked (e.g., corresponding to possibilities ii and/or iii in the previous paragraph).

There is extensive evidence implicating pRb hyperphosphorylation in the release of cells from the G_1 to the S phase, the most compelling of which derives from recent work showing functional and physical interactions between cyclins and pRb (15, 18, 31, 36). Cyclins A and E do not directly bind pRb but promote pRb hyperphosphorylation and reverse the capacity of pRb to induce a flat phenotype in Saos-2 cells (31). pRb function does not appear to be circumvented by these cyclins, since pRb mutants which resist hyperphosphorylation (and/or phosphorylation-mediated inactivation) are not sensitive to cyclin A or E overexpression (31). Cyclins D1, D2, and D3 interact directly with pRb, albeit with different consequences in vivo (15, 18). Coexpression of D2 with pRb in Saos-2 cells promotes pRb hyperphosphorylation and reverses the capacity of pRb to arrest cells in G_1 (18). Coexpression of D1 with pRb is less effective in promoting pRb hyperphosphorylation or in altering effects of pRb on the Saos-2 cell cycle (15, 18); however, a mutant D1 with an altered pRb interaction motif is more effective in promoting cell cycling (15). The last observation has led Dowdy et al. (15) to propose that pRb may antagonize D1 function in a manner regulated by pRb hyperphosphorylation. In related studies, it has been shown that cdk2 and cdk4 kinases phosphorylate pRb in conjunction with the appropriate D cyclins (18, 36). Conversely, PP1 or the PP2A catalytic subunit can induce release of pRb from cell nuclei while inhibiting entry into the S phase (1). All of these results strongly support the importance of pRb hyperphosphorylation in the regulation of $G_1 \rightarrow S$ transit. However, in the above-cited studies in which cyclin-kinase and phosphatase activities were manipulated, it must be assumed these enzymes have multiple substrates. Accordingly, it remains uncertain, on the basis of both previously published results and those presented here, whether pRb hyperphosphorylation is in itself sufficient for abrogation of pRb growth-inhibitory activity and release of cells into the S phase in most or all circumstances.

In assessing an unexpected disparity between pRb hyperphosphorylation and the S-phase cell fraction such as that reported here, it should be kept in mind that pRb, like other growth-regulatory agents, can vary in its activity, depending on cell type and external cell culture variables (55). To the extent that this is the case, caution is warranted in extrapolation of results obtained with serum-deprived, sodium butyrate-treated HDF to predict the function(s) of *Rb* family members under other conditions. With this qualification noted, it can nonetheless be argued that an example of pRb hyperphosphorylation in G_1-arrested cells provides new and useful information with respect to the proposed general role of this posttranslational modification in pRb-mediated cell cycle control at the G_1-S boundary.

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