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E1A Blocks Hyperphosphorylation of p130 and p107 without Affecting the Phosphorylation Status of the Retinoblastoma Protein

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The phosphorylation status of the pRB family of growth suppressor proteins is regulated in a cell cycle entry-, progression-, and exit-dependent manner in normal cells. We have shown previously that p130, a member of this family, exhibits patterns of phosphorylated forms associated with various cell growth and differentiation stages. However, human 293 cells, which are transformed cells that express the adenoviral oncoproteins E1A and E1B, exhibit an abnormal pattern of p130 phosphorylated forms. Here we report that, unlike pRB, the phosphorylation status of both p130 and p107 is not modulated during the cell cycle in 293 cells as it is in other cells. Conditional overexpression of individual G_1/S cyclins in 293 cells does not alter the phosphorylation status of p130, suggesting that the expression of E1A and/or E1B blocks hyperphosphorylation of p130. In agreement with these observations, transient cotransfection of vectors expressing E1A 12S, but not E1B, in combination with pocket proteins into U-2 OS cells blocks hyperphosphorylation of both p130 and p107. However, the phosphorylation status of pRB is not altered by cotransfection of E1A 12S vectors. Moreover, MC3T3-E1 preosteoblasts stably expressing E1A 12S also exhibit a block in hyperphosphorylation of endogenous p130 and p107. Direct binding of E1A to p130 and p107 is not required for the phosphorylation block since E1A 12S mutants defective in binding to the pRB family also block hyperphosphorylation of p130 and p107. Our data reported here identify a novel function of E1A, which affects p130 and p107 but does not affect pRB. Since E1A does not bind the hyperphosphorylated forms of p130, this function of E1A might prevent the existence of "free" hyperphosphorylated p130, which could act as a CDK inhibitor.

The retinoblastoma family of proteins (also designated as pocket proteins) comprises the product of the retinoblastoma tumor suppressor gene and the structurally and functionally related proteins p107 and p130 (for a review, see references 15, 20, and 21). The phosphorylation status of the three pocket proteins is regulated in a cell cycle-dependent manner. In normal quiescent cells (cells in G_0), pRB and p107, when detectable, are found hypophosphorylated, whereas p130 is resolved as two bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) corresponding to differently phosphorylated forms (named p130 forms 1 and 2) (18). As the cells are restimulated to enter the cell cycle and progress through mid-G_1, the three pocket proteins are hyperphosphorylated abruptly, most likely by G_1 cyclin-CDK complexes (15, 29). Hyperphosphorylation of p130 results in a band shift to a more slowly migrating band, which we named p130 form 3. This form of p130 is dramatically downregulated as cells progress through S phase and mitosis (18, 19). As cells exit mitosis to enter the next G_1 phase, the three pocket proteins become hypophosphorylated. It has been shown that, at least in the case of pRB, this later shift occurs by the action of specific protein phosphatases (17).

Among the pocket proteins, p130 is the one that exhibits the most conserved pattern of phosphorylated forms in normal cells. While a variety of differently phosphorylated pRB forms are detected when different cell types are compared under similar physiological conditions, the patterns of p130 forms detected by SDS-PAGE followed by Western blot analysis are precisely coupled to cell cycle phases, as well as to the quiescent stage. In addition, unlike pRB and p107, the levels of p130 seem to be linked also to the phosphorylation status of p130 and, thus, to the cell cycle stage (9, 18, 19). While these patterns are highly conserved in normal mammalian cells of different origin, forms with aberrant mobility have been detected in transformed cells such as human 293 cells (18) and HeLa cells (unpublished data). In asynchronously growing 293 cells, p130 is detected by Western blot analysis primarily as a single form with a faster mobility than p130 form 3; we have named this faster-migrating form form 2b (18). p130 form 3 is typically seen as the primary form in rapidly growing nontransformed cell lines in which most cells are at stages of the cell cycle other than early G_1 (19). Since 293 cells are transformed cells that express the products of two adenovirus oncogenes, E1A and E1B, it is conceivable that one of the expressed gene products is responsible for the abnormal phosphorylation of the p130 protein. While p130 form 2b physically associates with E1A, p130 form 3 does not (18), suggesting that the block to hyperphosphorylated form 3 observed in 293 cells might function to ensure that all p130 forms are compromised by E1A. The abnormal phosphorylation pattern of p130 observed in 293 cells is specific since normal pRB hyperphosphorylated forms

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are detected in these cells. The presence of hyperphosphorylated forms of pRB, which do not associate with E1A, does not seem to be an obstacle for E1A- and E1B-mediated transformation of these cells, presumably because phosphorylation inactivates them. The suggestion that the presence of hyperphosphorylated p130 is not compatible with DNA tumor virus-induced transformation is supported by studies with simian virus 40 (SV40). SV40 large-T antigen (TAg), which also associates with and inactivates the pRB family of proteins, has been shown to induce p130 degradation (27, 28). The fact that two different tumor viruses have evolved to inactivate p130 through likely two different mechanisms suggests that p130 form 3 performs a cellular function whose inactivation may contribute to cellular transformation. This function of p130 is not shared by pRB, since hyperphosphorylated forms of pRB are not targeted by E1A and coexist in E1A-expressing cells.

In this report we have investigated the mechanism responsible for the abnormal phosphorylation of p130 in cells expressing adenoviral E1A. We have found that E1A modulates the phosphorylation status of both p130 and p107 without affecting the phosphorylation of pRB. In MC3T3-E1 cells, a preosteoblast normal cell line useful as a model of cell cycle regulation and differentiation (7, 8, 23), expression of E1A 12S produces the same p130 phenotype seen in 293 transformed cells. This supports the suggestion that blocking the appearance of p130 form 3 is a basic part of the mechanism by which E1A redirects growth and differentiation controls in normal mammalian cells. Our results suggest that the hyperphosphorylated forms of p130 and perhaps p107 perform a function whose inactivation is important for E1A-mediated transformation. In contrast, hyperphosphorylation seems to be sufficient to inactivate the tumor suppressor activities of pRB.

MATERIALS AND METHODS

Plasmids. The tetracycline system plasmids pUHD15-1neo and pUHD10-3 (13), as well as the TK-hygromycin and pUHD10-3 cyclin D3 (tet-cyclin D3) vectors, were provided by S. Reed (25). pUHD10-3 cyclin E (tet-cyclin E) and pUHD10-3 cyclin A (tet-cyclin A) plasmids were obtained by digesting Rc-cyclin E and Rc-cyclin A (provided by P. Hinds) (16) with *Spe*I-*Xba*I and inserting the full-length cDNA of each cyclin into the *Xba*I site of pUHD10-3. The Rc-cyclin D1 and Rc-cyclin D3 vectors were also provided by P. Hinds. The ptTAs vector is a pCMV vector that was generated and kindly provided by C. Lee and E. P. Reddy (unpublished data). pUHD10-3-HA-p130 was generated in two steps to transfer the hemagglutinin (HA)-tagged full-length p130 from pcDNA-HA-p130 (12) to the pUHD10-3 vector. ptTAs-HA-p130 was obtained by transferring a *Kpn*I-*Bam*HI cDNA fragment containing HA-tagged full-length p130 from pUHD10-3-HA-p130 to the vector ptTAs (full subcloning details can be obtained from those authors). pCMV-pRB was provided by W. Kaelin, and the pCMV-HA-p107 was provided by Liang Zhu (33). pcDNAI-E1A and pcDNAI-E1A mutant (mt) 928-961(mt1) were generated by replacing the E2F4 cDNA in pcDNA-E2F4 (12) with 700-bp *Bam*HI-*Eco*RI restriction fragments from the pGEX-2T-E1A (12S E1A) and pGEX-2T-E1A mt 928-961 (E1A 12S-expressing plasmids were donated by J. Nevins). The pCMV-E1B19K and pCMV-E1B55K plasmids were kindly donated by E. White (30).

Cell culture, cell treatments, and flow cytometric analysis. U-2 OS osteosarcoma, T98G human glioblastoma, HaCat keratinocyte, HeLa, and 293 cell lines were maintained in Dulbecco modified Eagle medium (DMEM; Cellgro) supplemented with 10% fetal bovine serum (FBS; Sigma). For the synchronization experiments, 293 cells were cultured in low serum (0.5% FBS) for 24 to 48 h and then were synchronized in G_1/S phase with 1.5 mM hydroxyurea (1) and in pseudometaphase with 50 ng of nocodazole (19) per ml in DMEM containing 10% FBS. Synchronized mitotic cells were obtained by the shake-off method upon nocodazole treatment and replated in fresh medium. MC3T3-E1 parental cells were grown in α-MEM Earle's salts medium (Irvine Scientific) plus 10% FBS. Generation of MC3T3-E1 cells expressing wild-type and mutant adenoviral E1A 12S proteins have been previously described (3). These cell lines were maintained as the parental cell line in the presence of 250 μg of G418 (Cellgro) per ml. Inhibition of the proteasome degradation pathway in 293 cells was achieved by growing 293 cells in medium (DMEM–10% FBS) containing 1 μM β-lactone (an inhibitor of the proteasome pathway). β-Lactone was dissolved at the appropriate concentration in dimethyl sulfoxide (DMSO) (vehicle), and 15 μl of solution was added per plate. Control cells received 15 μl of vehicle. Flow cytometric analysis was performed to determine the percentage of cells at different phases of the cell cycle as previously described (18) by using an Epic Elite System (Coulter Electronics, Inc.)

Generation of stable 293 cells conditionally expressing G_1/S cyclins. 293 cells were first transfected with 10 μg of pUHD15-1neo, using the calcium phosphate precipitation method as described earlier (2, 10). Clones were selected in the presence of 500 μg of G418 per ml and tested for the ability to induce tetracycline-sensitive expression from the tetracycline promoter by using the vector pUHD10-3-HA-p130. A 293-derived cell line (293-tet) with the ability of conditionally expressing HA-p130 upon transient transfection of pUHD10-3-HA-p130 in the absence of tetracycline was selected to generate the conditional cyclin cell lines. Then, 293-tet cells were cotransfected with 10 μg of tet-cyclin D3, tet-cyclin A, or tet-cyclin E and 0.5 μg of thymidine kinase-hygromycin plasmid (TK-hygromycin). Clones were selected in the presence of 150 μg of hygromycin, 500 μg of G418, and 3 μg of tetracycline per ml. Stable clones were tested for tetracycline-sensitive expression of the corresponding cyclins by Western blot analysis with specific antibodies. Of the 40 clones analyzed for each cyclin a set of clones was chosen that expressed significant levels of the corresponding exogenous cyclin in the absence of tetracycline: 5 clones for cyclin D3, 16 clones for cyclin A, and 28 clones for cyclin E. Two representative clones for each cyclin were chosen for the experiments shown here.

Transient-transfection experiments. U-2 OS or 293 cells were plated at a density of 60 to 80% in 100-mm plates and grown overnight. Cells were refed 4 h before transfection. Transfections were carried out by using the calcium phosphate DNA precipitation procedure (2, 10). Cells were harvested 48 h after transfection. The total molar amount of vector was kept constant in each transfection by adding the corresponding empty expression vector and adjusting the total amount of DNA with pCAT basic (Promega). See the figure legends for the specific amounts of DNA used in each experiment.

Antibodies. Anti-p130-CT (sc-317), anti-p107 (sc-318), and anti-cyclin D3 (sc-182) rabbit polyclonal antibodies were obtained from Santa Cruz Biotechnology. Anti-pRB mouse monoclonal antibody (MAb; 14001A) was from Pharmingen, and anti-p130 MAb (R27020) was from Transduction Laboratories. Rabbit polyclonal anti-cyclin A antibody was a gift from J. Pines, and anti-cyclin E rabbit polyclonal antibody was a gift from Y. Xiong. M73 anti-E1A and XZ37, XZ77, XZ56, and XZ61 anti-pRb MAbs were a gift from N. Dyson and E. Harlow. Anti-E1B-55K mouse polyclonal antibody and anti-E1B-19K rabbit polyclonal antibody were a gift P. E. Branton and A. Lai.

Western blot analysis. Whole protein lysates were obtained essentially as described previously (9, 18). Briefly, cells were lysed in lysis buffer containing 50 mM Tris-Cl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton X-100, 0.1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg of leupeptin, 4 μg of aprotinin, and 4 μg of pepstatin per ml (lysis buffer). Western blots were performed as previously described (9, 18). Briefly, 25 to 50 μg of protein extract was resolved by 10 or 12.5% SDS-PAGE for cyclins and adenovirus proteins or 6% SDS-PAGE for pocket proteins and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) in 10 mM CAPS (pH 11) containing 10% methanol. The immunoblots were probed with specific primary antibodies and the corresponding horseradish peroxidase-conjugated secondary antibody (Amersham). Bands were visualized by incubating the membranes with enhanced chemiluminescence reagent (NEN) and exposing the membranes to X-ray film.

In vitro kinase assays. Cyclin E- and cyclin A-dependent kinase assays were performed as described earlier with anti-cyclin A and anti-cyclin E immunoprecipitates and histone H1 as exogenous substrate (14, 18). Cyclin D3-dependent kinase assay was carried out essentially as described by Reed et al. (24). Briefly, the cells were lysed in DIP buffer (50 mM HEPES [pH 7.2], 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 0.1% Tween 20) containing freshly added 1 mM dithiothreitol (DTT), 1 mM NaF, 0.5 mM PMSF, 0.5 mM Na_3VO_4, and 1 μg of leupeptin, 1 μg of aprotinin, and 1 μg of pepstatin per ml. Cell lysis was carried out for 30 min at 4°C. The supernatant was then rocked with anti-cyclin D3 for 2 h at 4°C, and the immunocomplex was trapped with protein A-Sepharose beads (Pierce) by rocking the suspension at 4°C for 2 h. Upon four washes with DIP buffer and two washes with kinase buffer (50 mM HEPES, pH 7.2; 10 mM $MgCl_2$; 5 mM $MnCl_2$ containing freshly added 1 mM DTT), the immunoprecipitates were resuspended in 25 μl of kinase buffer containing 20 μM ATP, 10 μCi of [γ-^{32}P]ATP, and 1 μg of GST-pRB-CT (C terminus). The reaction was performed at 37°C for 30 min, resolved by SDS–10% PAGE, and visualized by autoradiography.

Immunoprecipitation and immunodepletion. The association of wild-type and mutant (mt1) E1A 12S with pocket proteins was monitored as follows. A total of 150 to 200 μg of whole protein extracts was incubated with M73 monoclonal anti-E1A at 4°C overnight. The immunocomplexes were trapped with protein A-Sepharose beads (Pierce). After rocking at 4°C for 4 h, the immunoprecipitates were collected by a short spin, and the immunodepleted supernatant was kept. The beads were then washed extensively. Next, 25 μg of whole protein extract, an aliquot of the supernatant corresponding to 25 μg of input protein, and 80% of the total immunoprecipitates were resolved by SDS–6% PAGE. Western blot analysis was performed by using the indicated antibodies (see the figure legends).

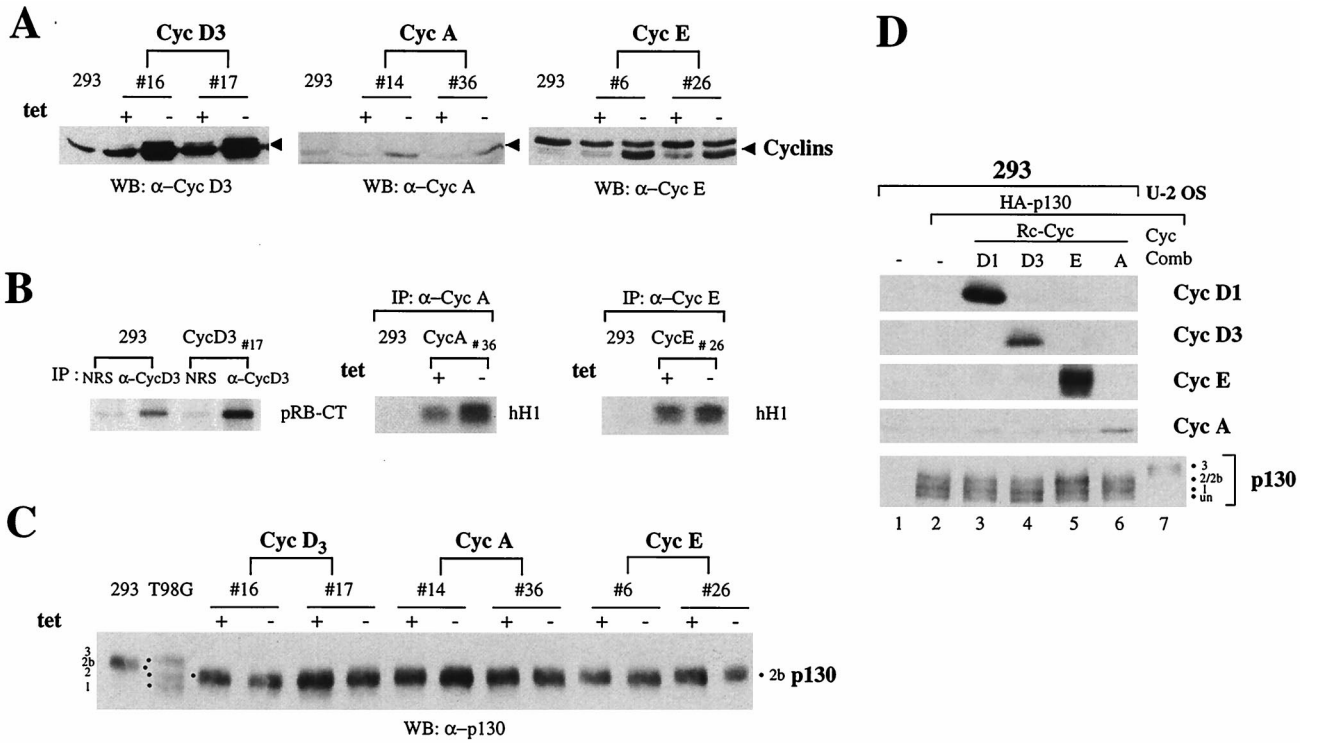


FIG. 1. Ectopic expression of individual G_1/S cyclins in 293 cells does not alter the phosphorylation status of p130. 293 cells conditionally expressing cyclin D3, cyclin A, or cyclin E were obtained as described in Materials and Methods. (A) Ectopic expression of cyclins in the presence (+) and absence (−) of tetracycline (tet). Whole protein extracts from the indicated stable clones were resolved by SDS–10% PAGE and analyzed by Western blot with the corresponding antibodies (indicated below each panel). Whole protein extracts from parental 293 cells were used as controls. (B) The kinase activity associated with each ectopically expressed cyclin was determined by immunoprecipitation with the corresponding anti-cyclin antibody, followed by kinase assay with the indicated exogenous substrates: histone H1 (hH1) or GST-pRB-CT (pRB-CT). 293 cells were used as control of endogenous cyclin activity and normal rabbit serum (NRS) as a negative control antiserum. (C) The phosphorylation status of p130 in the cell lines conditionally expressing cyclin D3, A, and E was determined by resolving p130 forms by SDS–6% PAGE, followed by Western blot analysis. Whole protein extracts of 293 and T98G cells were used as controls of the migration of p130 forms (forms 3, 2b, 2, and 1). (D) 293 cells were transiently cotransfected with vectors encoding HA-p130 (ptTAs-HA-p130) or HA-p130 in combination with cyclins D1, D3, E, and A vectors as indicated (Rc-cyclin expression vectors). The phosphorylation status of ectopically expressed HA-p130 and the expression of exogenous cyclins was determined by Western blot analysis. Note that p130 forms 2 and 2b cannot be distinguished in these transient-transfection experiments, presumably due to the high levels of expression of exogenous HA-p130. A control of migration of exogenous HA-p130 form 3 is shown in lane 7 (U-2 OS cells cotransfected with HA-p130 and a combination of G_1/S cyclin vectors, cyclins D1, D3, E, and A, to ensure complete phosphorylation of the ectopically expressed HA-p130). Relevant proteins are indicated.

RESULTS

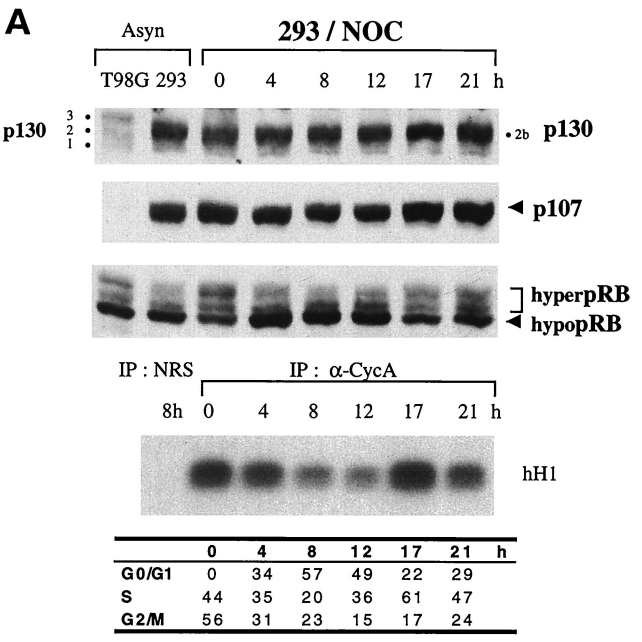
Normal cells growing asynchronously exhibit a pattern of p130 forms consisting of unphosphorylated p130 and p130 phosphorylated forms 1, 2, and 3 as determined by Western blot analysis (18). The abundance of each p130 form depends on the number of cells in G_0 as well as the number of cells at particular stages of the cell cycle for each individual cell population. In G_0, the major form of p130 is form 2 (form 1 is also detected in a number of cell types). As synchronous cells progress through mid-G_1, p130 is hyperphosphorylated to form 3. This form remains through S phase and mitosis and shifts to form 1 as cells exit mitosis and reenter G_1. In cell lines exhibiting very few cells in G_0 in conditions that allow for exponential growth, p130 is detected primarily as p130 form 3 (9, 18, 19). However, in the same conditions, 293 transformed cells, which express the adenoviral E1A and E1B gene products, exhibit an abnormal pattern of phosphorylation of the p130 protein (18).

Since G_1/S cyclin-CDK complexes are the most likely kinases involved in hyperphosphorylation of p130 and the other pocket proteins, we generated 293 cells conditionally expressing a variety of G_1/S cyclins, namely, cyclins A and E and a D-type cyclin (cyclin D3). 293 cells conditionally expressing individual cyclins were obtained in two steps. First, 293 cells were transfected with the vector pUH15-1-neo, and several clones were obtained with the ability to support tetracycline-dependent repression of the expression of a reporter vector in transient-transfection experiments (see Materials and Methods). One of these clones, designated here 293-tet, was used to generate the cyclin conditional cell lines. 293-tet cells were transfected with pUH10-3-cyclin D3, pUH10-3-cyclin E, or pUH10-3-cyclin A, and stable clones were obtained as described in Materials and Methods. Figure 1A shows the conditional ectopic expression of cyclins D3, E, and A upon tetracycline removal. Kinase assays were performed to demonstrate that ectopic expression of each cyclin (rate-limiting regulatory subunit) was sufficient to upregulate the kinase activity of the corresponding cyclin-CDK complex (Fig. 1B). We compared the levels of kinase activity in the clones grown in the absence of tetracycline with the levels of kinase activity in parental cells. However, while the corresponding cyclin-CDK kinase activity was upregulated in each clone, the phosphorylation status of endogenous p130 did not change (Fig. 1C). (See also lanes 1 to 3 in Fig. 5A, which exhibit a higher resolution of p130 forms 1, 2, 2b, and 3.) These results suggested that the presence of a p130 phosphorylated form with an intermediate migration (p130 form 2b) in 293 cells was not the result of limiting levels of the activity of individual G_1/S cyclin-CDK complexes. In

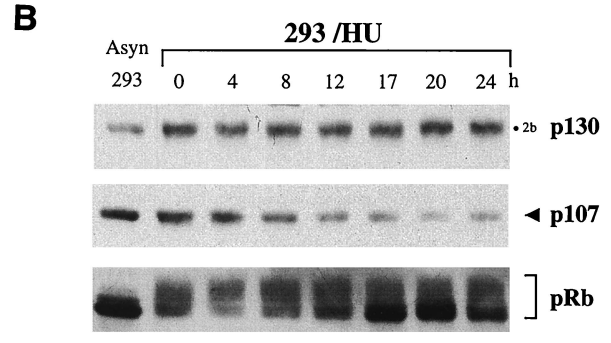
order to rule out the possibility that cyclin D1 could be the rate-limiting cyclin regulatory subunit for hyperphosphorylation of p130 to form 3 in 293 cells, we performed the following transient-cotransfection analysis. Vectors encoding cyclins D1, D3, E, and A were cotransfected with vectors encoding p130 in 293 cells. The phosphorylation status of the exogenous p130, as well as the expression of the exogenous cyclins, was determined by Western blot analysis (Fig. 1D). Transient transfection of p130 in 293 cells results in the detection of unphosphorylated p130 and of p130 phosphorylated forms 1 and 2 or 2b. As expected, p130 form 3 is not detected. In agreement with the results shown in Fig. 1C, coexpression of exogenous cyclins D1, D3, E, and A did not significantly affect the phosphorylation status of the ectopically overexpressed p130 (Fig. 1D, compare lane 2 with lanes 3, 4, 5, and 6). Thus, ectopically expressed p130 is not hyperphosphorylated to form 3 even when coexpressing high levels of individual G_1/S cyclin regulatory subunits in 293 cells.

We then analyzed whether the phosphorylation status of p130 is regulated during the cell cycle of 293 cells. 293 cells were synchronized in mitosis (mitotic pseudometaphase) and at the G_1/S transition with nocodazole and hydroxyurea, respectively, as described in Materials and Methods. Synchronized mitotic cells were obtained by the shake-off method upon nocodazole treatment and replated in fresh medium (see Materials and Methods). Hydroxyurea-treated cells were also washed and replenished with fresh medium. Cells were then harvested at the indicated time points and processed for fluorescence-activated cell sorting (FACS) and Western blot analysis. Figure 2 shows that p130 phosphorylation status is not modulated in these transformed cells. p130 form 2b (18) is the primary form of p130 at each stage of the cell cycle (Fig. 2A and B, upper panels). Similarly, p107 phosphorylation status is also not modulated. However, downregulation of p107 levels was detected in 293 cells progressing throughout the cell cycle upon hydroxy urea-block release. The significance of this effect on p107 is currently under investigation. In contrast to p130 and p107, the phosphorylation status of pRB changes in a cell cycle-dependent manner as in normal cells (Fig. 2). The modulation of pRB phosphorylation status is evident from the change in the proportion of various pRB forms throughout the cell cycle. Note that maximum accumulation of hypophosphorylated pRB occurs as cells reenter G_1 from mitosis (Fig. 2A, 4 h; Fig. 2B, 17 h). In addition, we also measured the kinase activity associated with cyclin A in protein extracts from the nocodazole-treated cells. We found that cyclin A-associated kinase activity was modulated as in normal cells (Fig. 2A, lower panel). Cyclin A-associated kinase activity decreases as cells exit mitosis and increases as cell progress through S phase. These results demonstrate that in 293 cells the pathway leading to cell cycle-dependent hyperphosphorylation of both p130 and p107 is disrupted, while cell cycle-dependent inactivation of pRB by phosphorylation is still taking place in these cells.

Since 293 cells express the products of two adenoviral early genes, E1A and E1B, we hypothesized that one or more of the expressed products could block the phosphorylation of p130 and, perhaps, p107 in these cells. Thus, we cotransfected human U-2 OS osteosarcoma cells with vectors encoding p130 in combination with E1A 12S, E1B55K, E1B19K, E1B55K and E1B19K combined, or empty vectors. Figure 3A shows that E1A 12S, but not E1B55K or E1B19K prevents hyperphosphorylation of p130. Interestingly, while E1A 12S also seems to impair hyperphosphorylation of p107 (see below), it does not affect the phosphorylation status of pRB (Fig. 3B and C). Thus, introduction of E1A 12S in U-2 OS cells is sufficient to block p130 and p107 hyperphosphorylation, a finding which is in agreement with our observations in 293 cells. From these experiments we hypothesized that one possibility is that E1A binding to p130 or to p107 physically blocks a set of phosphorylation sites in these pocket proteins. In support of this hypothesis, it has been suggested that binding of an LXCXE containing protein to a pocket protein may block phosphorylation of certain sites by cyclin-CDK pairs (32). Alternatively,



	0	4	8	12	17	21	h
G0/G1	0	34	57	49	22	29	
S	44	35	20	36	61	47	
G2/M	56	31	23	15	17	24	



	Asyn	0	4	8	12	17	20	24	h
G0/G1	46	60	54	17	23	55	55	47	
S	40	32	40	67	51	29	28	36	
G2/M	14	8	6	16	27	16	17	17	

FIG. 2. pRB, but not p130 nor p107 phosphorylation status is modulated during the cell cycle of 293 cells. (A) 293 cells were incubated with nocodazole for 18 h. Loosely attached cells were detached by shaking the tissue culture dishes and then collected, washed, and placed in fresh medium. Cells were then harvested at the indicated time points. The phosphorylation status of the three pocket proteins was assessed by Western blot analysis by using antibodies to p130, p107, and pRB (three upper panels). The kinase activity associated with cyclin A was also determined from the same whole protein extracts (lower panel) as in Fig. 1B. (B) 293 cells were incubated with hydroxyurea for 16 h and then washed and incubated with fresh medium. The phosphorylation status of the three pocket proteins was determined by Western blot analysis as in panel A. The cell cycle distribution of cells collected at each time point was determined by FACS (both panels). The percentage of cells at each stage of the cell cycle is indicated below. The differently phosphorylated forms of each pocket protein are indicated.

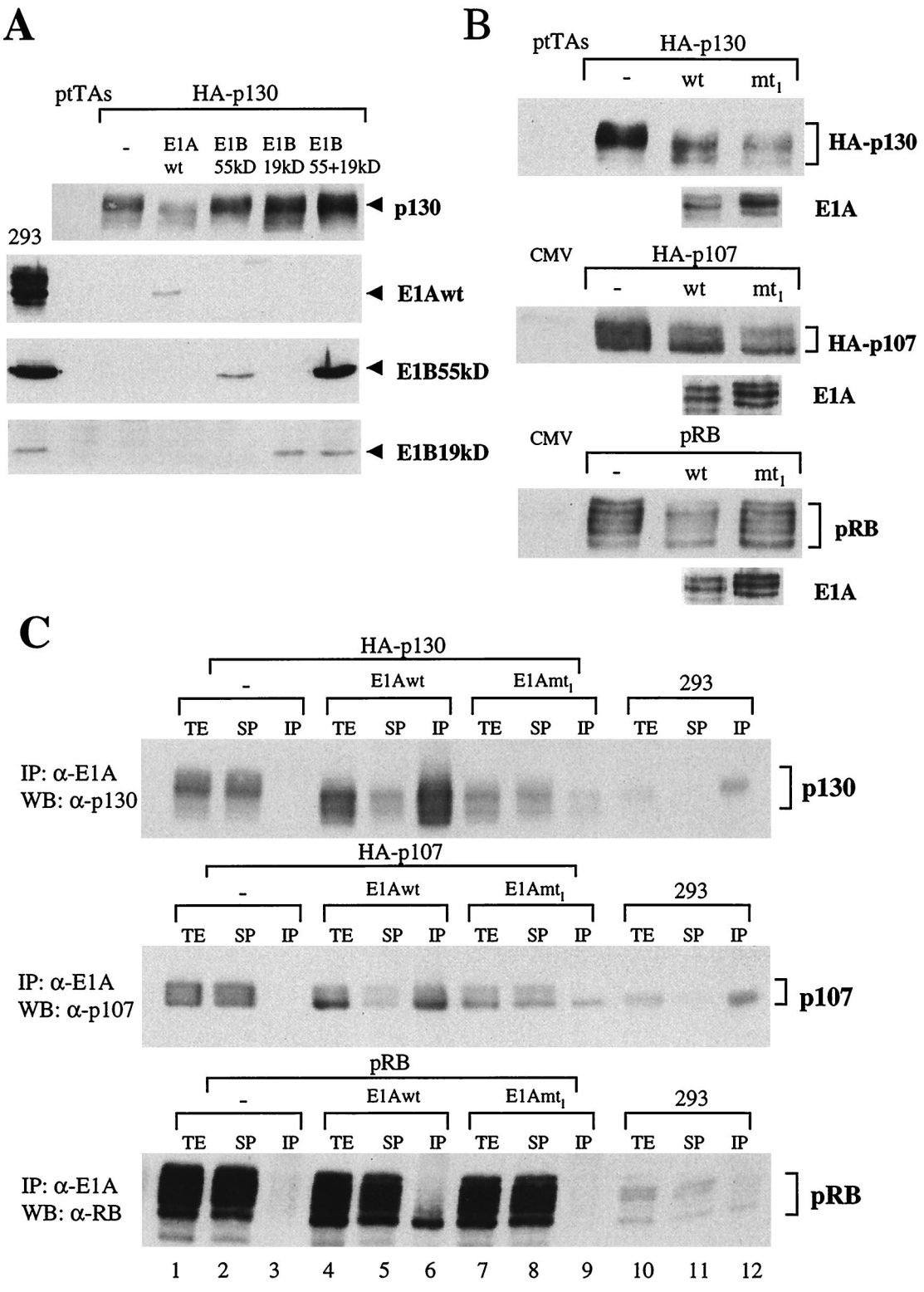


FIG. 3. Transient transfection of E1A 12S in U-2 OS cells blocks hyperphosphorylation of p130 and p107, but does not alter the phosphorylation of pRB. (A) Cotransfection of ptTAs-HA-p130 and pcDNAI-E1A, but not pCMV-E1B19K or -55K, blocks hyperphosphorylation of p130 to form 3. U-2 OS cells were transfected with the indicated combination of vectors. A total of 20 μg of ptTAs-HA-p130 was cotransfected with 5 μg of the following plasmids or combinations as indicated in the figure: pcDNAI-E1A wild-type, pCMV-E1B19K, and pCMV-E1B55K. Equal molar amounts of plasmid were kept by adding empty expression vector. The total amount of DNA was kept to 30 μg by adjusting with pCAT basic plasmid (Promega). The phosphorylation status of transfected HA-p130 was determined by Western blot analysis with anti-p130 antibodies (upper panel). With the exposure time shown here, only the exogenous p130 protein is detected. The expression of E1A 12S and E1B proteins was also determined by Western blot analysis by using specific antibodies (lower panels). A total of 25 μg of whole protein lysate of 293 cells was included as a control of the expression of adenoviral proteins. (B) 12S Wild-type E1A (wt) and 12S E1A mt 928-961(mt_1) block hyperphosphorylation of both p130 and p107 but do not affect the phosphorylation status of pRB. U-2 OS were transfected with 20 μg of ptTAS-HA-p130, pCMV-pRB, or pCMV-HA-p107 plasmids in

a hereto-unnoticed function of E1A, independent of binding to pocket proteins, could be responsible for the block in hyperphosphorylation of both p130 and p107. To test these possibilities, we also cotransfected p130 vectors with a vector encoding an E1A 12S mutant, E1A-mt 928-961(mt1), with an impaired ability for binding to the pRB family of proteins. Figure 3C shows that E1A-mt 928-961(mt1) does not associate with pRB, although it weakly associates with both p130 and p107 (Fig. 3C, compare lanes 6 and 9). Of note, wild-type E1A 12S associates with all the forms of p130 and p107 present in these cells but, as expected, only associates with hypophosphorylated pRB. Interestingly, E1A-mt 928-961 also blocks hyperphosphorylation of both p130 and p107, without affecting the phosphorylation status of pRB (Fig. 3B). Since most p130 or p107 is not physically associated with E1A-mt 928-961 but their phosphorylation status is still modulated by the presence of this E1A 12S mutant, our data indicate that the block in hyperphosphorylation is not due to E1A physically masking phosphorylation sites in these pocket proteins (see below).

While these results strongly suggest that E1A blocks the phosphorylation of p130, we wanted to rule out the possibility that E1A could induce the selective degradation of p130 form 3. In this regard, it has been shown previously that SV40 TAg induces the degradation of p130, leading to a pattern of p130 forms consisting of low levels of hypophosphorylated p130 (27). TAg promotes the degradation of p130 through the proteasome system. Therefore, we treated 293 cells with the proteasome inhibitor β-lactone for the indicated periods of time and monitored the levels and patterns of phosphorylation of p130 by Western blot analysis (Fig. 4). Figure 4 shows that the levels of p130 increase significantly at the longer time point, which is in agreement with data reported by others suggesting that p130 might be degraded through the proteasome pathway (26). However, we did not detect accumulation of hyperphosphorylated p130 form 3. Thus, these results demonstrate that the absence of p130 form 3 in 293 cells is not due to selective degradation of p130 form 3 through the proteasome pathway. In addition, we have found that the half-life of the p130 protein is not affected by the expression of E1A (unpublished data). This demonstrates that the mechanism by which E1A affects the patterns of phosphorylation of p130 is completely different from the mechanism used by SV40 TAg.

Since the experiments shown in Fig. 3 were performed by transient cotransfection, which often relies on the expression of high levels of the ectopically expressed proteins in a limited number of cells, we also analyzed the endogenous phosphorylation status of p130 and p107 in normal cells. MC3T3-E1 cells are a murine preosteoblast cell line used as a model for the study of cell cycle and differentiation controls. We have constructed a series of MC3T3-E1 cells stably transfected with various E1A 12S mutants (3).

Stable expression of E1A 12S in MC3T3-E1 cells results in a block in hyperphosphorylation of both p130 and p107, as determined by Western blot analysis (Fig. 5A, compare lane 4 with lane 5). p130 and p107 forms detected in MC3T3-E1 cells expressing E1A 12S migrate similarly to the forms observed in 293 cells (Fig. 5A, compare lanes 3 and 5). We also analyzed the phosphorylation status of p130 and p107 in MC3T3-E1 stably expressing an E1A 12S mutant protein (E1A-mt YH47/928, mt2), which bears two point mutations that completely abolish binding to pocket proteins (Fig. 5A, compare lanes 4, 5, and 6; see also Fig. 5B). This mutant shows no impairment in the activity that blocks hyperphosphorylation of p130 and p107. These experiments demonstrate unequivocally that physical binding of E1A to p130 and p107 is not required for the E1A-mediated block on hyperphosphorylation of these two proteins. We also determined the phosphorylation status of p130 and p107 in a clone of MC3T3-E1 cells expressing severalfold-lower levels of wild-type E1A 12S. Figure 6A shows that, at lower levels, E1A 12S is less efficient in blocking p107 hyperphosphorylation, suggesting that the effect of E1A 12S is dose dependent (Fig. 6A, intermediate panel; compare lane 2, E1A 12S clone G1, and lane 6, E1A 12S clone G2). The effect on p130 phosphorylation is more complex. p130 hyperphosphorylation to form 3 is effectively blocked by low levels of E1A 12S (Fig. 6A, upper panel, lanes 5 and 6). However, while a p130 form with a mobility similar to form 2b is the major form at high levels of E1A 12S, unphosphorylated p130 and p130 form 1 are clearly detectable in cells expressing lower levels of E1A 12S (Fig. 6A, compare lanes 2 and 6). This suggests that in addition to blocking hyperphosphorylation of p130 to form 3, E1A induces phosphorylation of p130 to an intermediate form with a mobility similar to p130 form 2b, this effect being dose dependent. To assess this possibility directly, we cotransfected U-2 OS cells with p130 vectors and increased amounts of E1A-wt or E1A-mt 928-961 vectors and then analyzed the modulation of p130 phosphorylation status by Western blot (Fig. 6B).

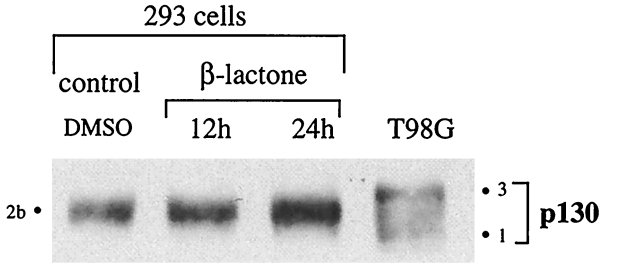


FIG. 4. The absence of p130 form 3 in 293 cells is not due to E1A-mediated selective degradation of this p130 form through the proteasome pathway. 293 cells were grown for the indicated periods of time in DMEM–10% FBS containing 1 μM β-lactone (an inhibitor of the proteasome pathway). β-Lactone was dissolved at the appropriate concentration in DMSO (vehicle), and 15 μl of solution was added per plate. Control cells received 15 μl of DMSO. Cells were collected at the indicated times, and the levels and phosphorylation status of endogenous p130 were determined by Western blot analysis.

combination with 5 μg of pcDNAI-E1A wild-type or pcDNAI-E1A-mt 928-961(mt1). Where ptTAs-HA-p130, pCMV-pRB, pCMV-HA-p107, and pcDNA-E1A were omitted they were replaced by an equal molar ratio of corresponding empty cytomegalovirus promoter vector. The total amount of DNA was adjusted to 25 μg with pCAT basic vector. Changes in the pattern of phosphorylation of pocket proteins were assessed by Western blot analysis. With the exposure time shown here the endogenous pocket proteins were not detected (see empty vector lanes). The expression of wild-type and mutant E1A 12S was also determined by Western blot. Note that cotransfection of E1A 12S wild-type and mutant vectors appears to affect the expression of exogenous p130. It has been previously reported that E1A 12S represses certain promoters (4, 22). (C) The E1A-mt 928-961(mt1) does not associate with pRB but weakly associates with p130 and p107. U-2 OS cells were transfected as in panel B. Whole protein lysates were immunoprecipitated with the M73 monoclonal anti-E1A antibody. Then, 25 μg of input whole protein lysate (TE), an aliquot of supernatant after immunoprecipitation corresponding to 25 μg of input protein (SP) and the immunoprecipitates (IP), was resolved by SDS–6% PAGE, followed by Western blot analysis with the indicated antibodies. Note that low levels of p130 and p107 are detected in the supernatants of cells transfected with wild-type E1A (lanes 5). This is likely to be due to an increased molar content of ectopic p130 and p107 with respect to ectopic E1A 12S in these transfection experiments. Alternatively, the M73 antibody might not completely preclear the lysates of E1A 12S-pocket protein complexes. The mobility of the pocket proteins is indicated.

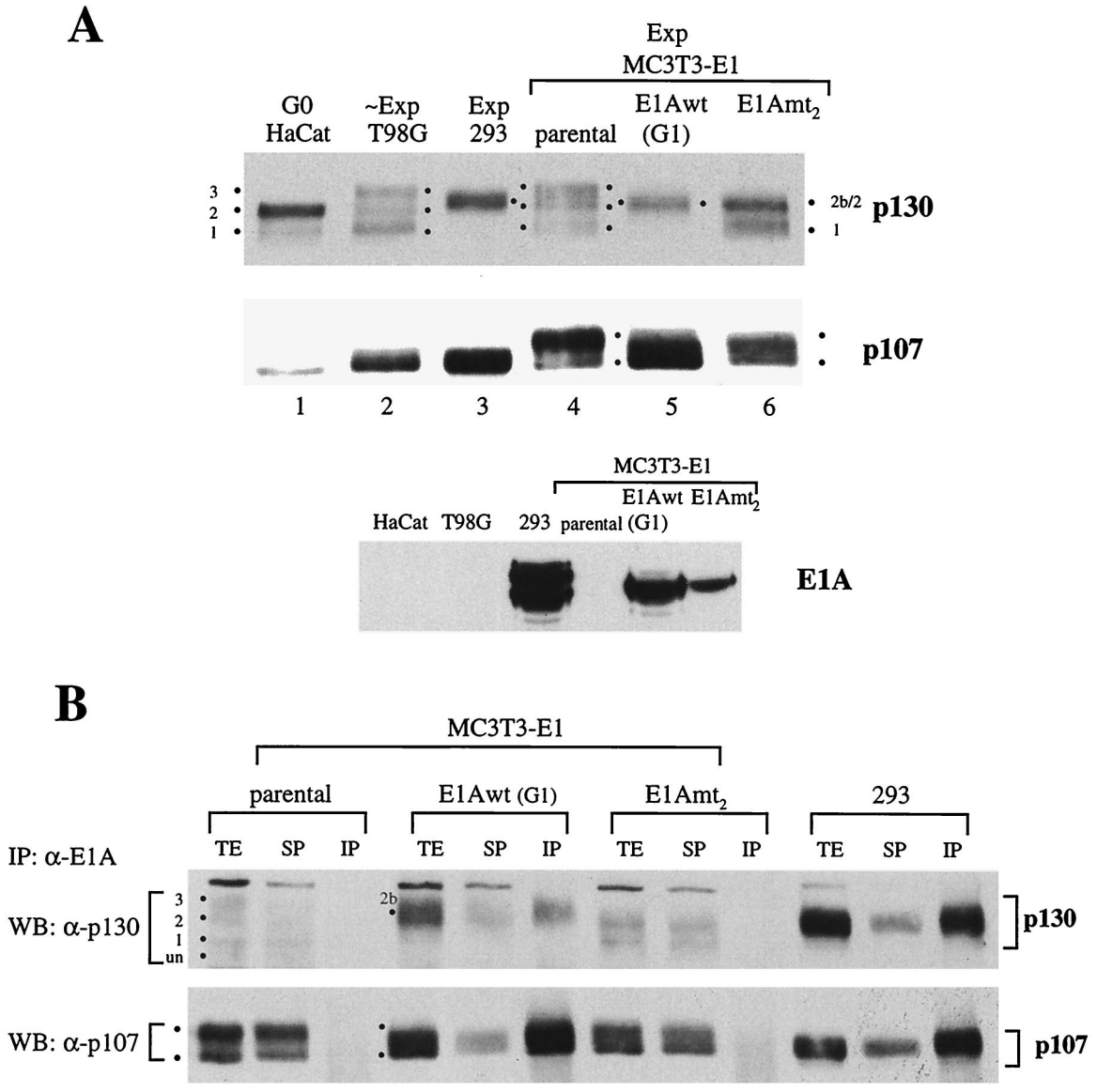


FIG. 5. Stably expression of E1A 12S and an E1A 12S mutant defective in binding to the pRB family in murine MC3T3-E1 preosteoblasts blocks hyperphosphorylation of p130 and p107 similarly as occurs in 293 cells. (A) The phosphorylation status of p130 and p107 in parental MC3T3-E1 cells and MC3T3-E1 cells stably expressing wild-type E1A 12S or the E1A 12S mutant YH47/928 (mt_2) was determined by Western blot analysis. Whole protein extracts of high-density-arrested HaCat cells (G_0) and exponentially growing 293 cells (Exp) and nearly exponential T98G cells (~Exp) were also loaded as controls of migration of differently phosphorylated forms of p130. The expression of E1A was also monitored by Western blot (lower panel). (B) E1A does not require physical binding to p130 or p107 to block their hyperphosphorylation. Whole protein lysates were immunoprecipitated with the M73 monoclonal anti-E1A antibody. A total of 35 μg of input whole protein lysate (TE), an aliquot of supernatant after immunoprecipitation corresponding to 35 μg of input protein (SP) and the immunoprecipitates (IP), was resolved by SDS–6% PAGE, followed by Western blot analysis with the indicated antibodies as in Fig. 3C. The mobility of pocket proteins is indicated by dots.

While low levels of expression of E1A 12S were sufficient to block hyperphosphorylation of p130, higher levels of expression of E1A 12S resulted in a pattern of forms more closely resembling the form of p130 observed in 293 cells and in MC3T3-E1 expressing high levels of E1A 12S (p130 form 2b). In addition, E1A-wt and E1A-mt 928-961 were similarly effective in blocking p130 hyperphosphorylation. Thus, the E1A-mediated modulation of p130 phosphorylation is dose dependent.

One might argue that E1A-induced changes in cell cycle distribution are responsible for the observed changes in the modulation of p130 and p107 phosphorylation status. To address this possibility, parental MC3T3-E1 and stable E1A 12S-expressing cell lines were grown in conditions allowing for exponential growth, and the relative proportion of cells at different phases of the cell cycle was analyzed by FACS (Fig. 7). The cell cycle phase distribution is similar in parental and E1A 12S-expressing cells. Therefore, the effect of E1A 12S on p130 and p107 is not due to an E1A-mediated increase in the proportion of cells in the S phase. The effect of E1A 12S was also assayed in conditions leading to cell cycle exit. At high density MC3T3-E1 cells exit the cell cycle, and the relative number of cells in G_0 increases. To this end, cells were kept growing for five additional days after they reached confluence. As expected, cell cycle exit of parental MC3T3-E1 cells results in the accumulation of p130 forms 1

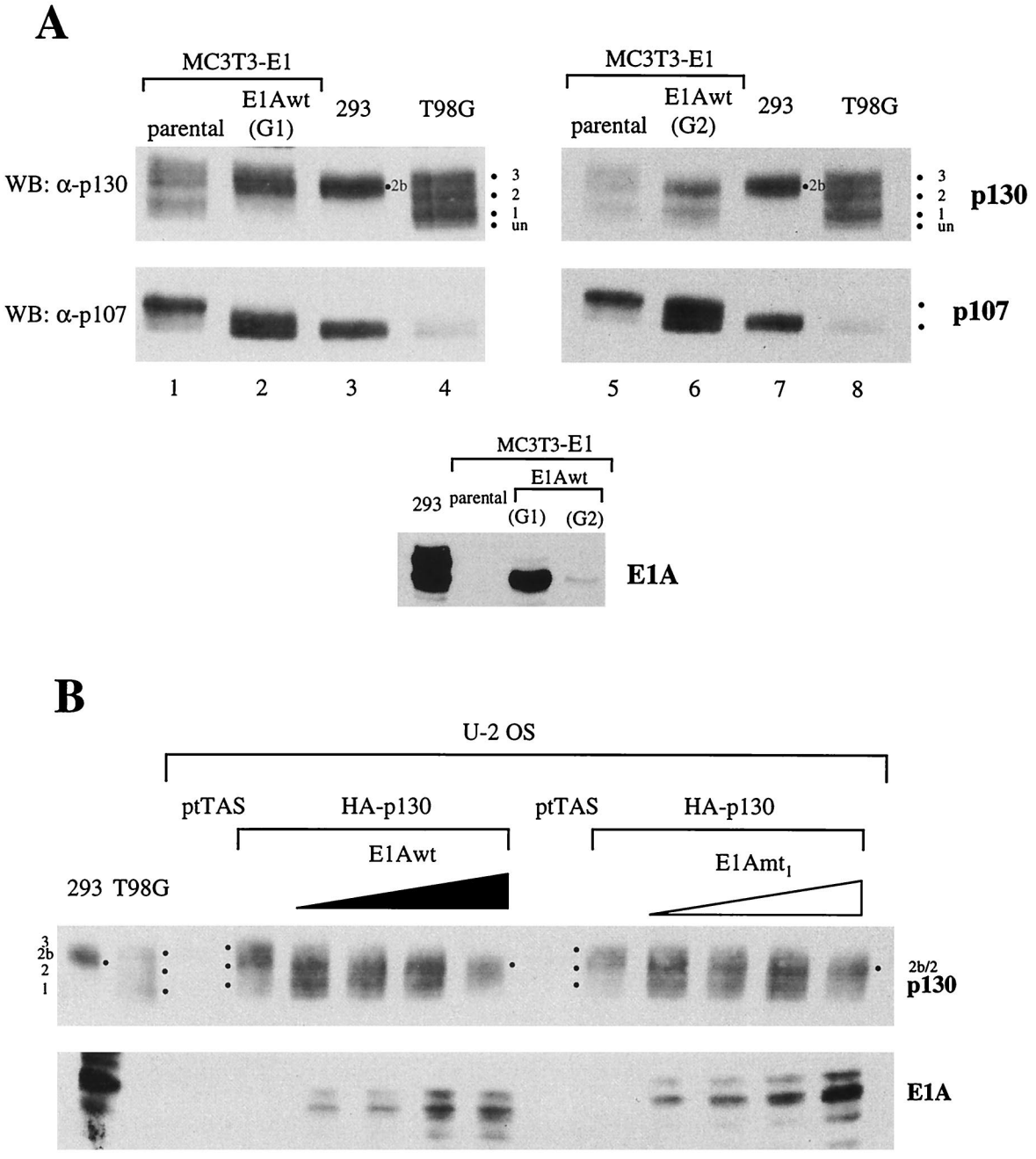


FIG. 6. E1A effect on p130 and p107 phosphorylation status is dose dependent. (A) The phosphorylation patterns of p130 and p107 were compared in two different clones of MC3T3-E1 cells stably expressing significantly different levels of wild-type E1A 12S. MC3T3-E1 clone G1 expresses severalfold-higher levels of E1A 12S than clone G2 (lower panel). The patterns of phosphorylation of p130 and p107 in these clones were assessed by Western blot analysis (upper panels). (B) A total of 20 μg of ptTAs-HA-p130 was cotransfected with increased amounts of pcDNAI-E1A wild-type or pcDNAI-E1A mt 928-961(mt1) vectors (0 to 10 μg). Changes in the pattern of p130 phosphorylation were monitored by Western blot analysis. The increased levels of E1A 12S were also monitored by Western blot analysis. The mobility of pocket proteins is indicated by dots.

and 2 and the disappearance of p130 form 3 (Fig. 7, compare lanes 4 and 9) as we described previously for normal cells (19). However, p130 phosphorylation status did not change in E1A 12S-expressing MC3T3-E1 cells grown to high density (Fig. 7, compare lanes 5 and 10), which exhibited an increased number of cells with G_0/G_1 DNA content when compared to exponentially growing cells. Thus, E1A 12S induces phosphorylation of p130 to form 2b even in conditions leading to cell cycle exit. As seen with p130, the phosphorylation status of p107 did not change in cells expressing E1A 12S in both growth conditions (Fig. 7, lower panel).

DISCUSSION

In this report we show that the adenoviral oncoprotein E1A modulates the phosphorylation status of p130 and p107 without affecting the phosphorylation patterns of pRB. Hyperphosphorylated p107 and p130 form 3 are the forms lacking in cells

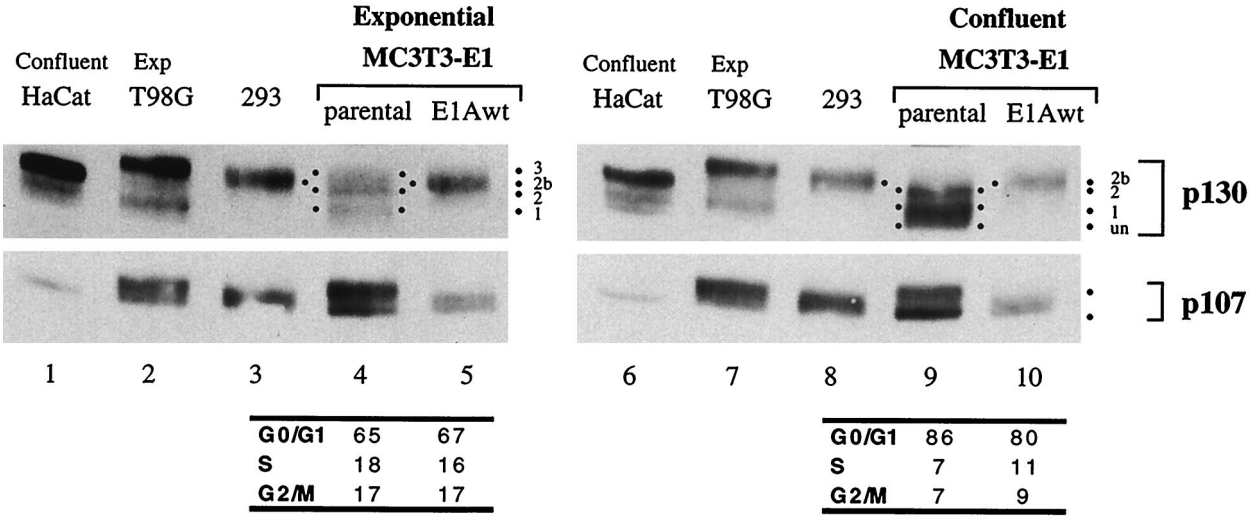


FIG. 7. E1A-dependent block on p130 and p107 hyperphosphorylation is not due to an E1A-induced change in cell cycle phase distribution. The patterns of phosphorylation of p130 and p107 in parental MC3T3-E1 cells and MC3T3-E1 cells expressing wild-type E1A 12S were compared in exponential and confluent cultures. The phosphorylation status of p130 and p107 was determined by Western blot analysis. The percentage of cells at different phases of the cell cycle was determined by FACS. Whole protein extracts of density-arrested HaCat cells and exponentially growing T98G and 293 cells were also loaded as controls of migration of differently phosphorylated forms of p130. Differently phosphorylated forms are indicated (p130 forms: unphosphorylated [un] and phosphorylated forms 1, 2, 2b, and 3).

expressing E1A. We demonstrate that direct binding of E1A to either p130 or p107 is not required for E1A to modulate the phosphorylation status of these proteins. This clearly shows that E1A binding does not mask phosphorylation sites in these proteins, suggesting that a domain in E1A different from the domain required for binding to pocket proteins is responsible for this hereto-unnoticed function of E1A. The E1A domains required for this hereto-unnoticed E1A function are under further investigation.

While the hyperphosphorylated forms of pRB and p130 do not associate with E1A (18), hyperphosphorylated p107 seems to efficiently associate with E1A (Fig. 3C). Thus, E1A cannot inactivate the hyperphosphorylated forms of pRB and p130 by direct binding. The fact that E1A selectively blocks hyperphosphorylation of p130 and p107 without blocking the phosphorylation of pRB strongly suggests that hyperphosphorylated p130 carries out a cellular function in normal cells not shared by pRB, whose inactivation is important for the biological effects of E1A. Thus, it is likely that the purpose of the E1A-mediated block in the phosphorylation of p130 is to ensure that E1A associates with and inactivates all p130 forms present in the cell. We and others have shown that hyperphosphorylated p130 and p107 do not associate with E2F transcription factors (5, 19, 31). However, impeding the hyperphosphorylation of these pocket proteins is not likely to affect E2F activity in cells expressing E1A since E1A itself disrupts the interaction between p130 and p107 and the E2F transcription factor. On the other hand, the interaction of p130 and p107 with cyclin E-CDK2 or cyclin A-CDK2 complexes is not disrupted by hyperphosphorylation of these pocket proteins, and there is compelling evidence that both p130 and p107 can act as CDK inhibitors (CKIs) (for a review, see reference 15). Thus, one attractive possibility is that E1A, by blocking hyperphosphorylation of p130, ensures binding to p130, which in turn allows E1A inactivation of a function of p130 perhaps as CKI.

While the E1A block of hyperphosphorylation of p130 and, perhaps p107, might inactivate a function of these proteins, we cannot rule out another possibility. We have shown earlier that p130 is phosphorylated to form 3 starting in mid-G_1 and that this is the primary form detected throughout the remainder of the cell cycle. Interestingly, the levels of p130 drop dramatically as cells enter S phase (18, 19), suggesting that phosphorylation of p130 to form 3 might induce its degradation. Thus, it is conceivable that E1A could block hyperphosphorylation of p130 to form 3 to block its degradation. If this were the case, it would suggest that a particular function of p130 is important for the E1A-mediated cellular reprogramming. Accordingly, Fig. 2 shows that the S-phase-dependent degradation of p130 is suppressed in synchronized 293 cells. However, if E1A were responsible for blocking p130 degradation, an increase in p130 protein levels due to an increased half-life of the p130 could be expected. Our unpublished data show that the half-life of p130 protein in U-2 OS cells is not significantly changed by E1A 12S. Thus, whether the E1A-mediated block in p130 hyperphosphorylation is designed to block p130 degradation remains to be elucidated.

It has been reported earlier that SV40 TAg affects the phosphorylation state of p130 and p107 (28). It is important to note that the mechanism by which TAg modifies the patterns of phosphorylation of these proteins is completely different than the mechanism(s) used by E1A. TAg contains a domain at its N terminus called the J-domain, which is similar to and can be substituted by a domain present in DnaJ proteins. These proteins are a family of molecular chaperones. The J-domain and LXCXE motifs of TAg cooperate to decrease the stability of p130 and to alter the phosphorylation patterns of both p130 and p107 (27, 28). In agreement with these observations, it has been shown that the J-domain of TAg mediates the inactivation of growth inhibitory functions of these two pocket proteins (27). The mechanisms by which TAg and E1A alter the patterns of phosphorylation of p130 and p107 differ in the following critical characteristics. (i) Our data demonstrate that the E1A-mediated alteration of the phosphorylation state of both p130 and p107 does not require E1A binding to these proteins, while TAg requires an intact LXCXE domain to modulate the patterns of phosphorylation of these two proteins. (ii) TAg, unlike E1A, dramatically reduces the half-life of p130 protein by inducing degradation of p130 through the proteasome path-

way. (iii) TAg-induced changes in the patterns of phosphorylation of both p130 and p107 are mediated through the TAg J-domain. However, E1A 12S does not contain a similar domain.

While the mechanisms by which TAg and E1A modulate the patterns of phosphorylation of both p130 and p107 are different, both small DNA viruses, SV40 and adenovirus, seem to accomplish the same objective. Both viruses have evolved toward specifically targeting and inactivating a set of cellular proteins, which are critical for normal cell growth and differentiation. SV40 uses TAg to inactivate p53 and hypophosphorylated pRB, while adenovirus E1B inactivates p53 and E1A the hypophosphorylated form of pRB (4, 22). This report, together with previous work on TAg, demonstrates that, in addition to the ability of both TAg and E1A to associate with p130 and p107, these viral proteins have the ability to modify a critical process that regulates the growth suppressor capabilities of both p130 and p107. Of note, blocking hyperphosphorylation of p107 does not seem to change the ability of E1A or TAg to associate with this protein, since both hypo- and hyperphosphorylated forms of p107 associate with the viral proteins. In contrast, p130 form 3 does not bind E1A (18). Thus, the E1A-mediated block in hyperphosphorylation to form 3 ensures that all p130 present in the cell can be associated with E1A, which conceivably allows E1A to inactivate all p130 in the cell. In an analogous way, TAg inactivates all cellular p130 by inducing its degradation (27). Given that the domains in TAg required to modify p130 function are required for cellular transformation and the fact that the J-domain confers a growth advantage to wild-type mouse embryo fibroblasts (MEFs) but not to MEFs lacking both p130 and p107 (27, 28), it is tempting to speculate that full inactivation of p130 is an important step for the life cycles of SV40 and adenovirus. Interestingly, we have also observed that HeLa cells, which are transformed cells expressing oncogenic proteins of human papillomavirus also lack the hyperphosphorylated forms of p130 (our unpublished data). It will be important to determine whether the E6 and/or E7 oncoproteins from human papillomavirus, which share the pRB and p53 targeting functions of E1A and TAg, also modulate the phosphorylation status of p130. Our results have revealed a hereto-unnoticed function of E1A that selectively targets p130 and p107 but does not target pRB. The consequences of blocking p130 hyperphosphorylation suggest that inactivation of the hyperphosphorylated forms of p130 might be an important step for the adenoviral life cycle.

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