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G_1 arrest and down-regulation of cyclin E/cyclin-dependent kinase 2 by the protein kinase inhibitor staurosporine are dependent on the retinoblastoma protein in the bladder carcinoma cell line 5637

(cell cycle/$p21^{Waf1/Cip1}$/$p27^{Kip1}$)

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ABSTRACT **The protein kinase inhibitor staurosporine has been shown to induce G_1 phase arrest in normal cells but not in most transformed cells. Staurosporine did not induce G_1 phase arrest in the bladder carcinoma cell line 5637 that lacks a functional retinoblastoma protein (pRB^-). However, when infected with a pRB-expressing retrovirus [Goodrich, D. W., Chen, Y., Scully, P. & Lee, W.-H. (1992) *Cancer Res.* 52, 1968–1973], these cells, now pRB^+, were arrested by staurosporine in G_1 phase. This arrest was accompanied by the accumulation of hypophosphorylated pRB. In both the pRB^+ and pRB^- cells, cyclin D1-associated kinase activities were reduced on staurosporine treatment. In contrast, cyclin-dependent kinase (CDK) 2 and cyclin E/CDK2 activities were inhibited only in pRB^+ cells. Staurosporine treatment did not cause reductions in the protein levels of CDK4, cyclin D1, CDK2, or cyclin E. The CDK inhibitor proteins $p21^{(Waf1/Cip1)}$ and $p27^{(Kip1)}$ levels increased in staurosporine-treated cells. Immunoprecipitation of CDK2, cyclin E, and p21 from staurosporine-treated pRB^+ cells revealed a 2.5- to 3-fold higher ratio of p21 bound to CDK2 compared with staurosporine-treated pRB^- cells. In pRB^+ cells, p21 was preferentially associated with Thr160 phosphorylated active CDK2. In pRB^- cells, however, p21 was bound preferentially to the unphosphorylated, inactive form of CDK2 even though the phosphorylated form was abundant. This is the first evidence suggesting that G_1 arrest by 4 nM staurosporine is dependent on a functional pRB protein. Cell cycle arrest at the pRB-dependent checkpoint may prevent activation of cyclin E/CDK2 by stabilizing its interaction with inhibitor proteins p21 and p27.**

The protein kinase inhibitor staurosporine induces both G_1 and G_2 arrests in normal cells but only G_2 arrest in most transformed cells (1–3). Two temporally distinct staurosporine arrest points have been identified in the G_1 phase of normal human diploid fibroblasts that are induced by either 1–4 nM or 20 nM staurosporine (4). At both arrest points, the retinoblastoma protein (pRB) is hypophosphorylated and total cyclin-dependent kinase (CDK) 2 activity is reduced. G_1 arrest was not observed in several virus-transformed tumor cell lines treated with staurosporine, which suggests that the pathway leading to G_1 arrest by staurosporine is deregulated in these cells (3–5). One of the proteins that is a target for viral oncoproteins such as simian virus 40 large T antigen is pRB (6), suggesting that pRB could be responsible for G_1 arrest by staurosporine. Microinjection of pRB leads to G_1 arrest in pRB-lacking cells (7). Probably hypophosphorylated pRB controls the activity of transcription factors such as those of the E2F family that are required for further cell cycle progression (for review, see ref. 8).

The functional state of pRB during the cell cycle is presumably regulated by Ser/Thr phosphorylations at multiple sites. A peak in active hypophosphorylated pRB is observed at early to mid G_1 (9–11). Hyperphosphorylated and, presumably, inactive pRB appear around the G_1/S transition and persist until completion of mitosis. The protein kinases most likely responsible for pRB phosphorylation are the cell cycle-regulated CDK4, CDK6, and CDK2 (12–14). These CDKs are themselves regulated by phosphorylation and by several protein inhibitors (for reviews, see refs. 15–17). The deregulation of CDK4 and CDK2 in virus-transformed and other tumor cells further stresses the importance of pRB in the maintenance of cell cycle control (18). The introduction of pRB-expressing vectors into a variety of pRB^- cells suppresses their tumorigenicity in nude mice, but no consistent growth phenotypes have differentiated between pRB^+ and pRB^- cells (19–24). However, cell cycle arrest induced by depletion of cyclin D1 or inactivation of CDK4 by the CDK4 inhibitor p16 requires pRB (25–28). This suggests that pRB may be regulated by CDK4/CDK6 phosphorylation. To study the effect of extracellular signals on cell cycle progression, various systems have been used, such as growth factor deprivation, addition of negative growth factor (TGFβ) and cell-cell contact inhibition. However, none of these systems has been used to compare isogenic pRB^- with pRB^+ cells. We have tested whether the protein kinase inhibitor staurosporine could induce a different growth response depending on the presence or absence of pRB. A pRB^- bladder carcinoma cell line and a pRB-expressing clone derived from the same cell line (19) were analyzed for cell cycle arrest in G_1 phase by staurosporine, and the mechanism leading to the arrest was further investigated.

MATERIALS AND METHODS

Cells, Culture Conditions, and Flow Cytometric Analysis. BC5637 (pRB^-) cells were originally obtained from American Type Culture Collection. 5637-RB-5 was as described (19). These cells were tested and found free of mycoplasma. Cells were cultured in RPMI 1640 medium (BioWhittaker) containing 10% fetal bovine serum (Sigma), 125 μg/ml of streptomycin, and 63 μg/ml of penicillin (United States Biochemical). Cells were kept under 5% CO_2/air in a 37°C humidified incubator. 5637-RB-5 cells were cultured in 250 μg/ml of geneticin (Sigma). This culture was then maintained in the

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Abbreviations: pRB, retinoblastoma protein; CDK, cyclin-dependent kinase; IP, immunoprecipitation.

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absence of geneticin. Staurosporine (Kamiya Biochemical, Thousand Oaks, CA) was dissolved as 1 mg/ml of dimethyl sulfoxide and then further diluted in RPMI 1640 medium to 1 μg/ml. Cells were plated at a density of 2–3 × 10^5 cells per 100-mm dish and were treated with 4 nM (about 2 ng/ml) staurosporine for 24 hr or left untreated before harvesting. For flow cytometric analysis, cells were fixed with 70% ethanol, cellular DNA was stained with mithramycin, and fluorescence was measured using a flow cytometer at 457 nm (29).

Immunoblot. Cells were lysed by sonication twice for 10 s in CDK4-immunoprecipitation (IP) buffer as described (12) with some modifications [50 mM Hepes, pH 7.5/150 mM NaCl/1 mM EDTA/2.5 mM EGTA/1 mM dithiothreitol/0.1% Tween-20 containing further 10% (vol/vol) glycerol/10 mM β-glycerophosphate/1 mM sodium vanadate/1 mM NaF/1 mM phenylmethylsulfonyl fluoride/1 μg/ml of leupeptin/1 μg/ml antipain/1 μg/ml bestatin/1 μg/ml chymostatin/1 μg/ml pepstatin A/1 μg/ml aprotinin/1 mg/ml benzamidin/20 μg/ml 4-(2-aminoethyl) benzene-sulfonyl fluoride]. The lysate was cleared by centrifugation at 4°C for 5 min at 14,000 rpm. Total protein (30 μg) was separated by 10% or 12% SDS/PAGE (30). Immunoblot analysis was carried out as described (4). Antibodies were added to give a final concentration of 1 μg/ml concentration: anti-CDK4 [C-22, Santa Cruz Biotechnology and Upstate Biotechnology (Lake Placid, NY)], anti-cyclin E (C-19, Santa Cruz Biotechnology), anti-CDK2 (M2, Santa Cruz Biotechnology), anti-cyclin D1 MAB (HD11, Santa Cruz Biotechnology), anti-p21 (C-19, Santa Cruz Biotechnology), anti-p27 (C-19, Santa Cruz Biotechnology; MAB, Pharmingen). Anti-pRB (mAB1, Triton Biosciences) was diluted to 0.4 μg/ml.

IP and Kinase Assays. Cells were lysed in the same way as for immunoblots. Cell lysates were further diluted with CDK4-IP buffer to a final concentration of protein at 0.2 to 0.4 μg per μl. The lysates were preincubated with 20 μl of protein A-Sepharose 4B (Sigma) for 30 min at 4°C and then transferred to protein A Sepharose 4B that was covered with 2 μg antibodies in all cases. When cyclin D1 MAB was used, the beads were precovered with 8 μg of anti-mouse rabbit IgG. The lysates were incubated for 2 to 4 hr with mild shaking and washed 5× with 1 ml cold CDK4-IP buffer. Precipitates were prepared for SDS/PAGE by boiling in 30 μl of SDS-loading buffer. Usually the entire reaction volume was loaded in one lane.

Kinase assays were carried out as described (12) except that for the majority of CDK4 assays 0.5 μg of p56-RB per 30 μl was used as a substrate rather than GST-RB. The final ATP concentrations were 1 mM CDK2, 0.5 mM cyclin E-CDK2, and 20 μM cyclin D1-CDK4. The assay time was 15 min for CDK2 and 30 min for cyclin E-CDK2 and CDK4. All assays were done at 30°C. The reactions were stopped by the addition of 6 μl of SDS sample buffer. The total reaction mix was applied on a gel. Gels were stained with Coomassie blue, destained, and dried. The bands were visualized by autoradiography and cut out for quantitation in a scintillation counter. All assays were performed at least twice.

RESULTS

Staurosporine Induces G_1 Arrest in pRB$^+$ Cells. The bladder carcinoma cell line 5637 lacks pRB and induces tumors in nude mice. When 5637 cells were infected with a pRB-expressing retrovirus, the tumorigenicity of these cells was reduced (19). We compared the responses of pRB$^-$ 5637 and the pRB$^+$ clone 5637-RB-5 with the protein kinase inhibitor staurosporine. We have shown previously that treatment with 2–4 nM staurosporine for 24 hr induces cell cycle arrest in G_1 phase (4). With the same treatment, G_1 arrest was not observed in 5637 cells lacking pRB. However, pRB$^+$ cells undergo cell cycle arrest, with an almost complete disappearance of S phase cells in response to 4 nM staurosporine (Fig. 1*A*). It should be noted that the second peak, which was observed

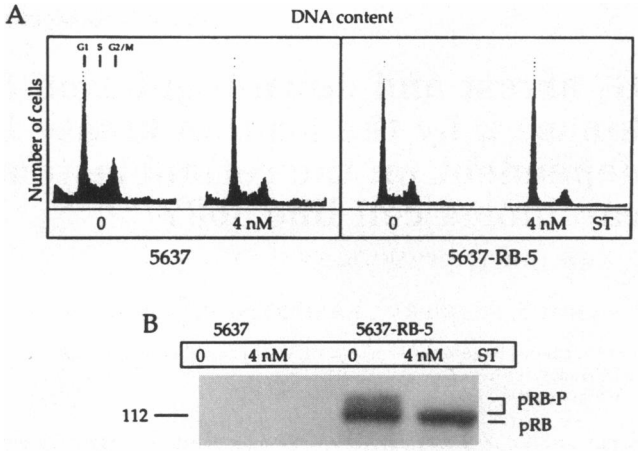

FIG. 1. Cell cycle and pRB analysis of 5637 and 5637-RB-5 cells in response to staurosporine (ST). Cells were treated with 4 nM staurosporine for 24 hr or left untreated (0). (*A*) Flow cytometry analysis. The G_1:S:G_2/M ratios are for untreated 5637 cells 60:12:28, for staurosporine-treated 5637 cells 63:15:22, for untreated 5637-RB-5 cells 62:12:26, and for staurosporine-treated 5637-RB-5 cells 81:3:16. (*B*) Immunoblot with anti-pRB antibodies. pRB-P, hyperphosphorylated form; pRB, hypophosphorylated form.

in the staurosporine-treated cells, may represent both G_2/M phase cells and cells arrested in G_1 that contain a polyploid DNA content. Treatment with 4 nM staurosporine for 24 hr did not cause a higher rate of cell death than for the control cells, because the amount of fragmented DNA did not increase as shown by the flow cytometry analysis. We also did not observe a higher rate of cells detached from the dish as occurs frequently with higher staurosporine concentrations. The state of pRB phosphorylation in staurosporine-treated cells was analyzed by immunoblot (Fig. 1*B*). As expected, we were unable to detect any pRB in 5637 cells. In contrast, 5637-RB-5 cells containing the pRB cDNA expressed both hypo- and hyperphosphorylated pRB. However, in staurosporine-treated 5637-RB-5 cells, pRB accumulated in its hypophosphorylated form, which is characteristic of pRB expressing G_1 cells. These results suggest that pRB is required for the G_1 arrest induced by 4 nM staurosporine in the bladder carcinoma cell line 5637.

Cyclin D1-CDK4/CDK6 Activity Is Reduced in Staurosporine-Treated pRB$^+$ and pRB$^-$ Cells. Because both CDK4 and CDK6 can phosphorylate pRB (12, 13), their activity was determined after 4 nM staurosporine treatment. We analyzed the CDK4/CDK6 kinase activities associated with cyclin D1, but not cyclins D2 and D3, because cyclin D1 has been reported to be essential for G_1 phase progression in cells with a functional pRB (25). Cyclin D1/CDK4/CDK6 from asynchronously growing cells and from staurosporine-treated cells were immunoprecipitated and the kinase activities were determined using p56-RB as a substrate (Fig. 2, lanes 3–6). To determine the level of nonspecific kinase activity, control immunoprecipitates from both cell lines were prepared by the use of protein A-Sepharose 4B beads containing anti-mouse IgG without anti-cyclin D1 antibodies. After subtraction of the background, we found about 50% reduction in the activity of cyclin D1/CDK4/CDK6 in both pRB$^-$ and pRB$^+$ cells on staurosporine treatment. Interestingly, the kinase activities were higher in pRB$^-$ than in pRB$^+$ cells regardless of staurosporine treatment. Conceivably, the presence of pRB has some influence on the kinase activities. However, a 2-fold reduction in cyclin D1/CDK4/CDK6 activity, in particular in pRB$^+$ cells, is not sufficient to explain the accumulation of hypophosphorylated pRB. One possible explanation could be that in untreated asynchronously growing cells, only a fraction of the cells contain active CDK4/CDK6, because this activity is strongly dependent on the cell cycle and highest in early to

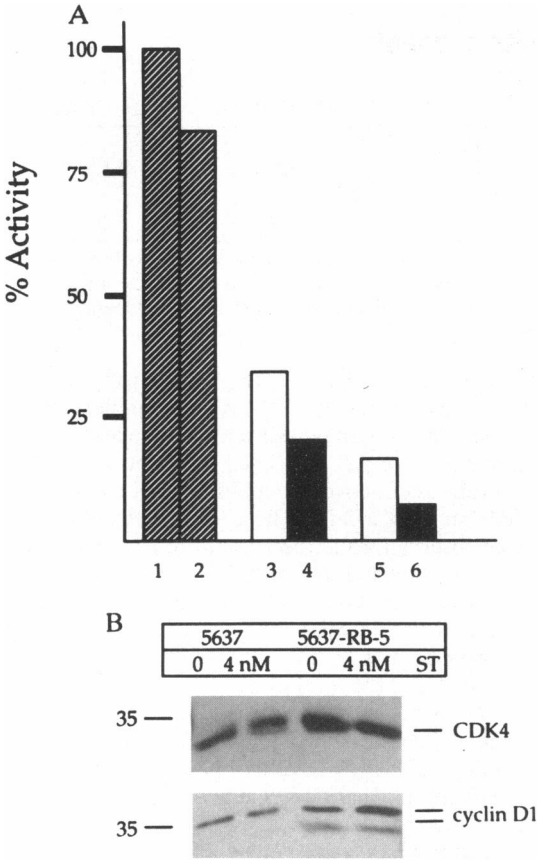

FIG. 2. CDK4 analysis. (*A*) Kinase assay: 2 μg anti-cyclin D1 antibodies were used for IPs with about 100–120 μg of total protein and CDK4/CDK6 activity was determined with p56-RB as substrate. Control assays were done with 5637 or 5637-RB-5 cell extracts with only anti-mouse IgG and the background activity was subtracted. The 100% activity corresponds to a normalized value of 3528 cpm. Lanes 1 and 2: 5637 cells were treated with 100 ng/ml nocodazole for 20 hr and released into drug-free medium. Samples for cyclin D1 IP were taken 3 (lane 1) and 6 (lane 2) hr later. Lanes 3 and 4: CDK4/CDK6 kinase activity in asynchronously growing (lane 3) and staurosporine-treated (lane 4) 5637 cells. Lanes 5 and 6: CDK4/CDK6 activity in asynchronously growing (lane 5) and staurosporine-treated (lane 6) 5637-RB-5 cells. The assays in lanes 3–6 were done three times. (*B*) Immunoblot of cyclin D1 and CDK4.

mid/late G_1 phase. On the other hand, staurosporine-treated pRB^+ cells represent a mostly synchronized cell population with the majority of cells in G_1 phase, where the cyclin D1/CDK4/CDK6 activity is high. Further, there could be some influence of staurosporine on the distribution of pRB^- cells within G_1 phase, resulting in a relatively higher cyclin D1/CDK4/CDK6 activity in these cells. To determine the cyclin D1/CDK4/CDK6 activity in early-to-mid G_1 phase, 5637 cells were synchronized with nocodazole in mitosis and released into a drug-free medium. Samples were taken at 3 and 6 hr after release and cyclin D1 IP was performed followed by kinase assays (Fig. 2, lanes 1 and 2). Compared with asynchronously growing 5637 cells, the cyclin D1/CDK4/CDK6 activity in synchronized cells was about 3-fold higher. This would imply that in pRB^+ cells the cyclin D1/CDK4/CDK6 activity is most probably reduced to about 20% by treatment with 4 nM staurosporine for 24 hr.

When CDK4 and cyclin D1 proteins were analyzed by immunoblotting, the levels of both proteins were largely unaffected by staurosporine treatment (Fig. 2*B*). We noticed two bands for CDK4 and for cyclin D1. The exact identities of the multiple bands are not understood but may represent different posttranslationally modified forms. These results suggest that the inhibition in CDK4/CDK6 activities on staurosporine treatment was not caused by a change in protein levels per se. It is also unlikely that staurosporine directly inhibited CDK4/CDK6, because staurosporine would have been removed during the IP, and the binding of staurosporine to protein kinases *in vitro* is reversible (31–33). This was confirmed by addition of 4 nM staurosporine to cell extract before cyclin D1 IP, which did not result in any kinase activity reduction (data not shown). Therefore, our results indicate that staurosporine affects upstream events regulating CDK4/CDK6 activity resulting in a reduction in kinase activity. Our results are also consistent with the hypothesis that the primary substrate for CDK4/CDK6 is pRB and that inhibition of CDK4/CDK6 activity results in accumulation of underphosphorylated pRB causing cell cycle arrest.

Cyclin E/CDK2 Activity Is Reduced in Staurosporine-Treated pRB^+ but not pRB^- Cells. Inactivation of CDK4 by TGFβ in mink lung cells results in the inactivation of cyclin E-CDK2 (34). We tested cyclin E/CDK2 and total CDK2 activities, using histone H1 as a substrate, in staurosporine-treated pRB^- and pRB^+ cells (Fig. 3). In pRB^+ cells, staurosporine treatment resulted in a 70% reduction in the cyclin E/CDK2 activity (Fig. 3*A*). In contrast, in pRB^- cells staurosporine increased kinase activity by about 15–20%. This increase could again be caused by partial synchronization of cells at a point when cyclin E/CDK2 activity is high and again suggests that even in pRB^- cells staurosporine causes some redistribution of cells in the cell cycle. The analysis of cyclin E immunoprecipitates showed that staurosporine did not disrupt cyclin E/CDK2 complex formation, but in both cell lines there was an increase in the inactive unphosphorylated form of CDK2, which migrates more slowly in SDS/PAGE (35). Staurosporine treatment had little or no effect on the total cyclin E protein level. In this particular experiment, we observed an increase in cyclin E in pRB^+ cells. However, this was also observed in pRB^- cells in another experiment, and had no influence on the effect of staurosporine on CDK2 activity. As expected, total CDK2 activity, which also includes cyclin A/CDK2, was significantly reduced in pRB^+ cells on staurosporine treatment but not in pRB^- cells (Fig. 3*B*, Table 1). Immunoblot analyses of total (data not shown) and immunoprecipitated CDK2s showed that the reduction in kinase activity was not due to differences in the CDK2 protein level. In contrast to cyclin D1/CDK4/CDK6 activities, which were

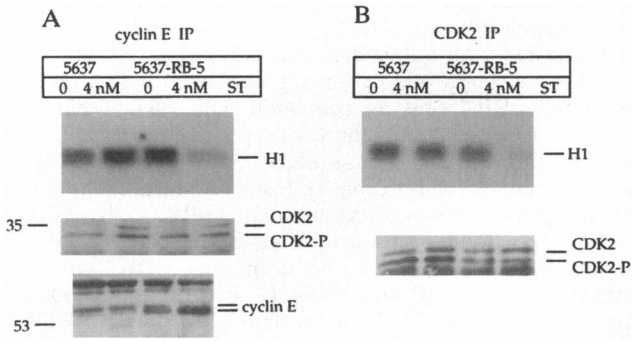

FIG. 3. CDK2 kinase assays. (*A*) Cyclin E/CDK2 was immunoprecipitated with 2 μg of anti-cyclin E antibodies from 100 μg of total cell extract followed by kinase assay with histone H1 as substrate. The exposure time for autoradiography was 6 hr. A second immunoprecipitate with the same cell extract was analyzed by immunoblot with anti-CDK2 antibodies. Cyclin E immunoblot was done only with total cell extract. A higher molecular weight unidentified band crossreacted with the cyclin E antibodies. (*B*) CDK2 was immunoprecipitated with 2 μg of anti-CDK2 antibodies followed by kinase assay with histone H1. The exposure time for autoradiography was 2 hr. In a second IP with anti-CDK2 antibodies, the IP was analyzed for CDK2. CDK2, Thr160 unphosphorylated form; CDK2-P, Thr160 phosphorylated form (35).

Table 1. Quantitation of immunoblots and CDK2 kinase assays

Cell line	ST, nM	CDK2 IP kinase activity, %	Ratio p21/CDK2	cycE IP kinase activity, %	Ratio p21/CDK2	p21 IP ratio p21/CDK2
5637	0	100	0.053	100		
	4	86	0.127	117	0.077	0.126
5637-RB-5	0	100	0.217	100		
	4	18	0.382	30	0.183	0.369

The band intensity from immunoblots was determined and the p21/CDK2 ratios were calculated. The values were obtained from two independent experiments in case of CDK2 IP. The ratios are relative and reflect only the band intensities but not the absolute p21/CDK2 ratios. The values for kinase assays were obtained from two independent experiments. Independent experiments were performed with different culture batches. ST, staurosporine.

reduced by staurosporine treatment in both cell lines, a reduction in CDK2 activity was only observed in pRB$^+$ cells, thereby correlating the presence of pRB with the reduction in CDK2 activity by staurosporine. Since cyclin E is essential for entry into S phase independent of pRB (36), it appears that the reduction in cyclin E/CDK2 activity after the CDK4/CDK6 inactivation was the proximal cause for G_1 arrest in pRB$^+$ cells. These results are consistent with a CDK4/CDK6 dependent cell cycle checkpoint lying upstream of a cyclin E/CDK2 checkpoint. The CDK4/CDK6 checkpoint would require pRB for its creation.

p21$^{(Waf1/Cip1)}$-CDK2 and p27$^{(Kip1)}$-CDK2 Levels Change in Staurosporine-Treated Cells. Our results suggested that neither the disruption of the cyclin E/CDK2 complex nor direct inhibition of CDK2 by staurosporine were likely to be the cause of the reduced CDK2 activity in pRB$^+$ cells. An alternative mechanism of CDK regulation involves the inhibitor proteins. There are at present three inhibitor proteins, p21$^{(Waf1/Cip1)}$, p27$^{(Kip1)}$, and p57$^{(Kip2)}$ known to bind and inhibit CDK2. Whereas p21 and p27 expression seems to be ubiquitous, p57 expression is tissue specific and related to terminally differentiated cells (37–43). Therefore, we examined the effect of staurosporine on p21 and p27 expression and their association with CDK complexes. Immunoblot analysis showed that p21 accumulated in staurosporine-treated pRB$^-$ cells and, to a much lesser extent, in staurosporine-treated pRB$^+$ cells (Fig. 4*A*). It is known that p21 is induced by γ-irradiation in the presence of wild-type p53 protein (44). However, the increase that we observed here is likely to be independent of p53, because both cell lines express stable p53 proteins characteristic of mutant forms (data not shown). To estimate the amount of p21 that is associated with CDK2, we performed IP experiments with anti-CDK2 antibodies followed by immunoblot analyses with anti-CDK2 and anti-p21 antibodies (Fig. 4*B*). We detected little or no p21 associated with CDK2 in untreated pRB$^-$ cells as compared with pRB$^+$ cells. On staurosporine treatment, there was a 2-fold increase in p21 coprecipitated with CDK2 in pRB$^+$ cells and a 2- to 3-fold increase in pRB$^-$ cells (Table 1). However, the amount of p21 bound to CDK2 in staurosporine-treated pRB$^-$ cells was 2- to 3-fold lower than in staurosporine-treated pRB$^+$ cells. A similar difference in the ratio of p21 to CDK2 was also confirmed by p21 IP and cyclin E IP from staurosporine-treated cells (Table 1). To determine which form of CDK2, Thr160 phosphorylated or unphosphorylated, was associated with p21 in staurosporine-treated cells, we performed IP with anti-p21 and immunoblotted for CDK2, CDK4, and p21 (Fig. 4*C*). About equal amounts of p21 from both cell lines was immunoprecipitated. In pRB$^-$ cells, p21 associated equally well with both forms of CDK2. In contrast, in pRB$^+$ cells the active Thr160 phosphorylated form of CDK2 was predominant in coprecipitates with p21. As expected, there was less CDK2 coprecipitated with p21 in pRB$^+$ as compared with pRB$^-$ cells. CDK4 was also present in coprecipitates with p21. But, in comparison with CDK2, the amount of CDK4 was nearly equal in pRB$^-$ and pRB$^+$ cells. This confirmed that there was more p21 associated per CDK2 in pRB$^+$ as compared with pRB$^-$ cells. The stoichiometry of p21/CDK2 complexes has been shown to be important in determining kinase activity. While a 1:1 or lower ratio of p21 to CDK2 does not inhibit CDK2 a higher 2:1 ratio is effective in inhibiting CDK2 activity (45). In pRB$^+$ cells, at least a 2-fold increase in p21 associated with CDK2 was observed while the amount of p21 associated with CDK2 in staurosporine-treated pRB$^-$ cells was less than in untreated pRB$^+$ cells. Our results show that the amount of p21 that associated with CDK2 in staurosporine-treated pRB$^-$ cells was probably not sufficient to inhibit CDK2 activity.

We also analyzed the level of p27$^{(Kip1)}$ by immunoblot. The level of p27 increased on staurosporine treatment in both pRB$^-$ and pRB$^+$ cells (Fig. 5*A*). We further analyzed the association of p27 with CDK2 by immunoblotting for CDK2 after IP with anti-p27 antibodies (Fig. 5*B*). In untreated pRB$^-$ cells, there was no detectable p27 associated with CDK2, whereas in untreated pRB$^+$ cells CDK2 was associated with p27. The amount varied depending on the culture batch. On

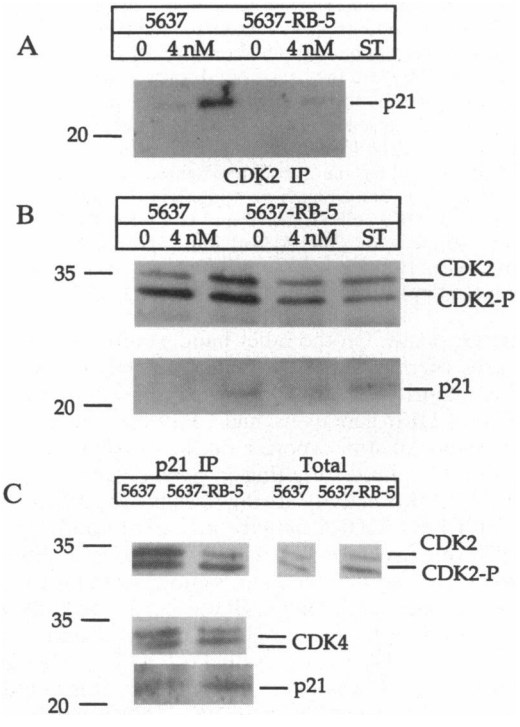

FIG. 4. Analysis of p21$^{(Waf1/Cip1)}$. (*A*) p21 immunoblot. (*B*) IP with 2 μg of anti-CDK2 antibodies was performed with 200 μg of total protein. The precipitates were separated by SDS/PAGE and immunoblotted with anti-CDK2 and anti-p21 antibodies. (*C*) IP with 2 μg of anti-p21 antibodies was performed with 150 μg of total cell extract. After separating proteins by gel CDK2, CDK4 and p21 were immunoblotted. CDK2, Thr160 unphosphorylated form; CDK2-P, Thr160 phosphorylated form.

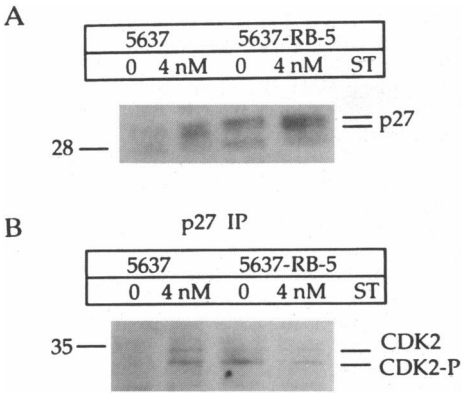

FIG. 5. Analysis of $p27^{(Kip1)}$. (*A*) p27 immunoblot. The lower band is unspecific, which was confirmed with another antibody (MAB from Pharmingen) (*B*) IP with 2 μg of anti-p27 antibodies was performed with 200 μg total protein. The immunoprecipitates were separated by SDS/PAGE and immunoblotted with anti-CDK2 antibodies.

staurosporine treatment, both pRB^- cells and pRB^+ cells showed CDK2 association with p27. The amount of CDK2 associated with p27 in pRB^+ cells did not further increase but actually decrease indicating that as with p21 the ratio of p27 to CDK2 may change and the p27 level increase over CDK2. These results suggest that the p27-CDK2 association in addition to p21-CDK2 association may be responsible for the reduction of CDK2 activity in pRB^+ cells.

DISCUSSION

It has been shown that the kinase inhibitor staurosporine arrests normal cells in both G_1 and G_2 phases of the cell cycle (1–3). However, virus-transformed mammalian cells are resistant to G_1 arrest but not G_2 arrest by staurosporine (3, 5). One of the effects of a viral oncogene such as simian virus 40 T antigen is the inactivation of pRB protein (6). The analysis of tumor cell lines of various origins revealed a correlation between staurosporine arrest points in G_1 phase and the absence or presence of a functional pRB (unpublished results). Through the use of an isogenic pair of cell lines, we have addressed the question whether pRB plays an essential role in the G_1 arrest induced by 4 nM staurosporine. We found that while a pRB^- bladder carcinoma cell line was not arrested in G_1 phase by 4 nM staurosporine, a pRB-reconstituted isogenic cell line was arrested and this arrest was accompanied by an accumulation of hypophosphorylated pRB. Our results suggest that the staurosporine-induced G_1 arrest results from the accumulation of hypophosphorylated pRB.

To determine the molecular events involved in pRB-dependent cell cycle arrest on staurosporine treatment, we first determined the activity of known pRB kinases. We found that cyclin D1-associated kinase activities (CDK4/CDK6) were reduced by staurosporine treatment of both pRB^- and pRB^+ cells, although we noted a lower activity in pRB^+ compared with pRB^- cells. It is unlikely that staurosporine inhibits CDK4/CDK6 directly because staurosporine addition to total cell extract before IP did not result in a reduction in activity. We also tested CDK4/CDK6 activity in cells treated for 3 and 6 hr with staurosporine. Only in pRB^+ and pRB^- cells treated for 6 hr with staurosporine was there a reduction in enzyme activity (data not shown). This indicates that the CDK4/CDK6 regulatory system was most probably affected by staurosporine. The lack of cell cycle arrest in pRB^- cells despite CDK4/CDK6 reduction is in agreement with previous reports that inactivation of CDK4/CDK6 by overexpression of $p16^{ink}$ did not cause cell cycle arrest in cells derived from pRB^- knockout mice (26–28). Thus the question arises as to why the CDK4/CDK6 down-regulation results in cell cycle arrest only in the presence of pRB. In contrast to cyclin D1/CDK4/CDK6, staurosporine caused cyclin E/CDK2 inactivation only in pRB^+ cells. This observation allowed us to speculate that the reduction of CDK4/CDK6 activities by staurosporine resulted in an accumulation of hypophosphorylated active pRB. Cell cycle-arrest at the pRB-dependent checkpoint may preclude the activation of CDK2 that normally occurs before cells start DNA replication. Thus, the inactivation of cyclin E/CDK2 resulting from the accumulation of hypophosphorylated pRB is the most likely cause for cell cycle arrest.

How does pRB cause the inactivation of CDK2? CDK2 activity is regulated by several mechanisms including cyclin binding (for review, see ref. 46), phosphorylation of Thr160 and Tyr15 (35), and the binding of inhibitor proteins p21, p27, and p57 (37–42). The level of cyclin E and its association with CDK2 in pRB^- and pRB^+ cells was analyzed and found to be unchanged on staurosporine treatment. We also analyzed Thr160 phosphorylation, which activates CDK2. Previously, we found that staurosporine caused the accumulation of Thr160 unphosphorylated CDK2 in normal diploid fibroblasts (4). Here we also observed a higher ratio of unphosphorylated to phosphorylated CDK2, but this was independent of the presence of pRB. This suggested an involvement of inhibitor proteins in CDK2 inactivation by staurosporine. We analyzed p21 and p27 protein levels and their association with CDK2 in pRB^- and pRB^+ cells. p21 levels increased on staurosporine treatment of both pRB^+ and pRB^- cells. In exponentially growing pRB^+ cells, the ratio of p21 to CDK2 was 3- to 4-fold higher than in pRB^- cells and increased further on staurosporine treatment. In pRB^- cells, although the level of p21 increased, the p21 to CDK2 ratio remained always 2- to 3-fold lower compared with pRB^+ cells. Probably a drastic exogenous overexpression of p21 may partly or completely inhibit CDK2 in pRB^- cells as well because exogenously produced p21 caused slow growth in other cell lines with functionally inactive pRB (38). We also found an increase of p27 by staurosporine in both pRB^- and pRB^+ cells. As in the case of p21, our data suggest that there is more p27 associated with CDK2 in pRB^+ cells than in pRB^- cells resulting in CDK2 inhibition. Therefore, it seems that the p21 and p27 to CDK2 complex formations do not occur efficiently in pRB^- cells. It was reported that a simian virus 40 T antigen transformed cell line contained altered CDK2 complex compared with normal fibroblasts. Instead of p21 other proteins were found to associate with CDK2 (18, 47). It is tempting to speculate that in pRB^- cells, CDK2 associates with a protein that is not inhibitory, but occupies the same site on CDK2 as p21 and p27. Thus, in staurosporine-treated pRB^- cells, instead of two molecules of p21 that are necessary for CDK2 inhibition, only one molecule of p21 may bind to CDK2. Possibly pRB regulates the level of CDK2-associated proteins and thereby influences the composition and activity of CDK2 complexes. pRB may negatively regulate the level of a p21 or p27 competitor protein for CDK2, thus allowing more p21 and p27 to bind to CDK2.

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1. Abe, K., Yoshida, M., Usui, T., Horinouchi, S. & Beppu, T. (1991) *Exp. Cell Res.* **192,** 122–127.
2. Bruno, S., Ardelt, B., Skierski, J. S., Traganos, F. & Darzynkiewicz, Z. (1992) *Cancer Res.* **52,** 470–473.

3. Crissman, H. A., Gadbois, D. M., Tobey, R. A. & Bradbury, E. M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 7580–7584.
4. Schnier, J. B., Gadbois, D. M., Nishi, K. & Bradbury, E. M. (1994) *Cancer Res.* **54,** 5959–5963.
5. Kraemer, P. M. & Bradbury, E. M.(1993) *Exp. Cell Res.* **207,** 206–210.
6. Ludlow, J. W., DeCaprio, J. A., Huang, C.-M., Lee, W.-H., Paucha, E. & Livingston, D. M. (1989) *Cell* **56,** 57–65.
7. Goodrich, D. W., Wang, N. P., Qian, Y. W., Lee, E. Y. & Lee, W.-H. (1991) *Cell* **67,** 293–302.
8. Weinberg, R. A. (1995) *Cell* **81,** 323–330.
9. Buchkovich, K., Duffy, L. A. & Harlow, E. (1989) *Cell* **58,** 1097–1105.
10. Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y. J. & Lee, W.-H. (1989) *Cell* **58,** 1193–1198.
11. DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C. M. & Livingston, D. M. (1989) *Cell* **58,** 1085–1095.
12. Matsushime, H., Quelle, D. E., Shurtleff, S. A., Shibuya, M., Sherr, C. J. & Kato, J. Y. (1994) *Mol. Cell. Biol.* **14,** 2066–2076.
13. Meyerson, M. & Harlow, E. (1994) *Mol. Cell. Biol.* **14,** 2077–2086.
14. Akiyama, T., Ohuchi, T., Sumida, S., Matsumoto, K. & Toyoshima, K. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7900–7904.
15. Hunter, T. & Pines, J. (1994) *Cell* **79,** 573–582.
16. Sherr, C. J. & Roberts, J. M. (1995) *Genes Dev.* **9,** 1149–1163.
17. Morgan, D. O. (1995) *Nature (London)* **374,** 131–134.
18. Xiong, Y., Zhang, H. & Beach, D. (1993) *Genes Dev.* **7,** 1572–1583.
19. Goodrich, D. W., Chen, Y., Scully, P. & Lee, W.-H. (1992) *Cancer Res.* **52,** 1968–1973.
20. Huang, H.-J. S., Yee, J.-K., Shew, J. Y., Chen, P.-L., Bookstein, R., Friedmann, T., Lee, E. Y. & Lee, W.-H. (1988) *Science* **242,** 1563–1566.
21. Bookstein, R., Shew, J. Y., Chen, P.-L., Scully, P. & Lee, W.-H. (1990) *Science* **247,** 712–715.
22. Chen, P.-L., Chen, Y., Shan, B., Bookstein, R. & Lee, W.-H. (1992) *Cell Growth Differen.* **3,** 119–125.
23. Sumegi, J., Uzvolgyi, E. & Klein, G. (1990) *Cell Growth Differen.* **1,** 247–250.
24. Zhou, Y., Li, J., Xu, K., Hu, S. X., Benedict, W. F. & Xu, H. J. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4165–4169.
25. Lukas, J., Bartkova, J., Rohde, M., Strauss, M. & Bartek, J. (1995) *Mol. Cell. Biol.* **15,** 2600–2611.
26. Lukas, J., Parry, D., Aagaard, L., Mann, D. J., Bartkova, J., Strauss, M., Peters, G. & Bartek, J. (1995) *Nature (London)* **375,** 503–506.
27. Koh, J., Enders, G. H., Dynlacht, B. D. & Harlow, E. (1995) *Nature (London)* **375,** 506–10.
28. Medema, R. H., Herrera, R. E., Lam, F. & Weinberg, R. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6289–6293.
29. Tobey, R. A. & Crissman, H. A. (1974) *Science* **21,** 1297–1298.
30. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
31. Gross, J. L., Herblin, W. F., Do, U. H., Pounds, J. S., Buenaga, L. J. & Stephens, L. E. (1990) *Biochem. Pharmacol.* **40,** 343–350.
32. Herbert, J. M., Seban, E. & Maffrand, J. P. (1990) *Biochem. Biophys. Res. Commun.* **171,** 189–195.
33. Miyazaki, A., Kitamura, Y. & Nomura, Y. (1993) *Neurochem. Int.* **22,** 455–464.
34. Ewen, M. E., Sluss, H. K., Whitehouse, L. L. & Livingston, D. M. (1993) *Cell* **74,** 1009–1020.
35. Gu, Y., Rosenblatt, J. & Morgan, D. O. (1992) *EMBO J.* **11,** 3995–4005.
36. Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M. & Pagano, M. (1995) *Mol. Cell. Biol.* **15,** 2612–2624.
37. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. & Elledge, S. J. (1993) *Cell* **75,** 805–816.
38. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. & Vogelstein, B. (1993) *Cell* **75,** 817–825.
39. Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. & Beach, D. (1993) *Nature (London)* **366,** 701–704.
40. Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P. & Massague, J. (1994) *Cell* **78,** 59–66.
41. Toyoshima, H. & Hunter, T. (1994) *Cell* **78,** 67–74.
42. Lee, M. H., Reynisdottir, I. & Massague, J. (1995) *Genes Dev.* **9,** 639–649.
43. Matsuoka, S., Edwards, M. C., Bai, C., Parker, S., Zhang, P., Baldini, A., Harper, J. W. & Elledge, S. J. (1995) *Genes Dev.* **9,** 650–662.
44. Dulic, V., Kaufmann, W. K., Wilson, S. J., Tlsty, T. D., Lees, E., Harper, J. W., Elledge, S. J. & Reed, S. I. (1994) *Cell* **76,** 1013–1023.
45. Zhang, H., Hannon, G. J. & Beach, D. (1994) *Genes Dev.* **8,** 1750–1758.
46. Sherr, C. (1993) *Cell* **73,** 1059–1065.
47. Zhang, H., Kobayashi, R., Galaktinov, K. & Beach, D. (1995) *Cell* **82,** 915–925.