Discovery of a Regulatory Motif That Controls the Exposure of Specific Upstream Cyclin-dependent Kinase Sites That Determine Both Conformation and Growth Suppressing Activity of pRb*

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The conformation and activity of pRb, the product of the retinoblastoma susceptibility gene, is dependent on the phosphorylation status of one or more of its 16 potential cyclin-dependent kinase (cdk) sites. However, it is not clear whether the phosphorylation status of one or more of these sites contributes to the determination of the various conformations and activity of pRb. Moreover, whether and how the conformation of pRb may regulate the phosphorylation of the cdk sites is also unclear. In the process of analyzing the function and regulation of pRb, we uncovered the existence of an unusual structural motif, m89 (amino acids 880–900), the mutation of which confers upon pRb a hypophosphorylated conformation. Mutation of this structural domain activates, rather than inactivates, the growth suppressor function of pRb. In order to understand the effect of the mutation of m89 on the phosphorylation of cdk sites, we identified all the cdk sites (Thr-356, Ser-807/Ser-811, and Thr821) the phosphorylation of which drastically modify the conformation of pRb. Mutation of each of these four sites alone or in combinations results in the different conformations of pRb, the migration pattern of which, on SDS-polyacrylamide gel electrophoresis, resembles various in vivo hypophosphorylated forms. Each of these hypophosphorylated forms of pRb has enhanced growth suppressing activity relative to the wild type. Our data revealed that the m89 structural motif controls the exposure of the cdk sites Ser-807/Ser-811 in vitro and in vivo. Moreover, the m89 mutant has enhanced growth suppressing activity, similar to a mutant with alanine substitutions at Ser-807/Ser-811. Our recent finding, that the m89 region is part of a structural domain, p5, conserved antigenically and functionally between pRb and p53, suggests that the evolutionarily conserved p5 domain may play a role in the coordinated regulation of the activity of these two tumor suppressors, under certain growth conditions.

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The product of the retinoblastoma susceptibility gene, RB-1, exists in multiple conformations. The various forms of pRb, even after denaturation, maintain their conformational differences that are readily discerned by SDS-PAGE. The stability of particular localized conformation of certain polypeptides under the denaturing conditions of SDS-PAGE has previously been described (1, 2) and is perhaps a result of hydrophobic intramolecular interaction. At least five different forms of pRb isolated from an asynchronous culture can be discerned by SDS-PAGE. While there is no doubt that these different conformations of pRb are a SDS-PAGE gel artifact, previous studies have clearly showed that they correspond to the phosphorylation status of the pRb.

Multiple forms of heavily phosphorylated pRb appear in late G1 and S phases of the cell cycle. In quiescent and differentiating cells, pRb is hypophosphorylated and migrates fastest in SDS-PAGE (3–5) compared with other forms observed in late G1 and S. This form of pRb is also observed in cells at the M and early G1 phases, when pRb is dephosphorylated (6). There are 16 Ser/Thr-Pro motifs on pRb that are potential cyclin-dependent kinase (cdk) targets. Functional evidence that the phosphorylation of some or all of these cdk sites regulates the conformation and activity of pRb comes from the observation that ectopic expression of cyclins A, D, and E can overcome the growth suppression effect of pRb (7, 8). Other in vitro and in vivo experiments have shown that various viral and cellular proteins bind preferentially to the hypophosphorylated forms of pRb (Refs. 10 and 11; reviewed in Ref. 9). For example, the binding of pRb to the SV40 large T antigen is specifically affected by the phosphorylation status of Thr-821/Thr-826, while the phosphorylation status of Ser-807/Ser-811 controls the assembly of the pRbc-Abl complex (11). Phosphorylation by cdk’s, in turn, leads to the disruption of protein complexes formed between pRb and its targets. Together, these data suggest that the conformation and activity of pRb are regulated by the phosphorylation status of its cdk sites.

While it is clear that the cell cycle-dependent phosphorylation of the 16 potential cdk sites regulates the conformation of pRb, there is surprisingly little information on the relationship between the phosphorylation status of a particular site and a particular conformation. To date, the only cdk sites that have been reported to have an effect on a particular conformation of pRb are those of the combined sites Ser-807/Ser-811 (11–14), or when most cdk sites were mutated (12), although pRb clearly

1 The abbreviations used are: PAGE, polyacrylamide gel electrophoresis; cdk, cyclin-dependent kinase; TGFβ, transforming growth factor-β; GFP, green fluorescent protein; eGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting.
can exist in multiple conformations. It is not known how many of the cdk sites are phosphorylated in any given form of pRb and which of these sites contribute to the determination of a particular conformation. It is also unclear whether these cdk sites act independently or synergistically in determining the conformation of pRb, and whether the resulting conformational changes alter the growth suppressing activity of pRb. There are several lines of evidence which suggest that the phosphorylation of pRb occurs in a stepwise manner as cells progress through the cell cycle. As synchronized cell populations traverse the cell cycle, progressive changes in the phosphorylation of pRb can be discerned by one-dimensional SDS-PAGE analysis (3, 4, 15) as well as by two-dimensional phosphopeptide analyses, in which distinct pRb phosphorylation patterns emerge at the G1 and S phases (4, 14, 16, 17). How this stepwise phosphorylation of particular cdk sites is regulated is unknown at present, but it could be due to a change in the expression of cdk enzyme activities or the conformation of the pRb substrate which may control the exposure of the cdk sites, or both. The fluctuation in the level of cdk-regulating cyclins at different phases of the cell cycle is clearly one mechanism for the stepwise regulation of phosphorylation of particular cdk sites. On the other hand, previous data have shown that the phosphorylation of pRb is also dependent on the conformation of pRb itself. Many naturally occurring mutations within the N terminus, C terminus, and the A/B pocket domains of pRb have been reported to render pRb refractory to phosphorylation (18–21). Naturally occurring point mutations at Ser-567 and Cys-706 have a drastic effect on the conformation of pRb, as shown by the loss of ability to bind various target proteins, or to be phosphorylated. Taken together, these data suggest that the phosphorylation of pRb is also regulated by its conformation.

In our attempts to analyze the function and regulation of pRb, we performed a detailed analysis of the domains that are associated with a particular function and/or regulation by creating a panel of pRb mutants in which certain amino acid residues in a 20-amino acid stretch were mutated. The phosphorylation patterns of the resulting mutants and their ability to suppress cell growth were analyzed. Consistent with previous reports, most mutations in the A/B pocket region resulted in the inactivation of pRb, as measured by the inability of the ectopically expressed mutant to suppress cell growth. In addition to these inactivating mutations, most mutations in other regions have no apparent effect on the growth suppression function of pRb. However, in the course of these analyses, we uncovered the existence of an unusual structural motif, m89 (amino acid residues 880–900), the mutation of which confers upon pRb a hypophosphorylated conformation and an enhanced growth suppressor activity.

To identify the cdk sites affected by m89, each of 15 cdk sites (Ser-567 excluded) was individually mutated and the effect on the phosphorylation status and conformation of pRb analyzed. Our results revealed that there are four cdk sites, Thr-356, Ser-807/Ser-811, and Thr-821, the phosphorylation status of which exerts a drastic change in the conformation of pRb independent of other sites. Moreover, all five different forms of pRb isolated from an asynchronous culture, resolvable by SDS-PAGE, can apparently be accounted for by the phosphorylation status of one or more of these four sites. Data presented below shows that the integrity of the m89 structural motif determines the exposure of two of these four cdk sites, namely Ser-807 and Ser-811 in vitro and in vivo. Bladder carcinoma 5637 cells expressing both a transfected cyclin A and m89 mutant pRb were blocked in cell cycle progression to a greater extent than with the wild type pRb. The m89 motif is therefore a structural domain, other than created cdks sites, the mutation of which activates, rather than inactivates, the function of pRb.

EXPEDIMENTAL PROCEDURES

Construction of Expression Plasmids for pRb cDNA and Its Mutants—Phosphorylation site mutants in which serines and threonines were changed to alanine, were subcloned into pbLuescriptSK+, to generate pBSpRbmS807S811, where XXX is the amino acid residue number of the cdk sites. Double mutants were created at sites Ser-608/Ser-611 and Ser-807/Ser-811. A triple mutant was created at Thr-356/Ser-807/Ser-811, while a quadruple mutant was created by combining the triple mutant with the Thr-821 mutant to create Thr-356/Ser-807/Ser-811/Thr-821. The regulatory site mutant pBSpRbm89 was created by changing the amino acids 880–900 of the m89 region EGS-DEADGSKHLGEGSKFQQK to FAEEVASHILGEGSKFQQK.

The plasmids pbGFP/CycA/pRb, pbGFP/CycA/pRbm89, peGFP/CycA/pRbmS807S811, peGFP/CycA/pRbmThr-356S807S811, and peGFP/CycA/pRbmThr-356S807S811/Thr-821 were constructed by subcloning the pRb cDNAs under the control of the immediate early promoter of cytomegaviruses (pCMV) into peGFP/CycA containing a pCMV-cyclin A. The coding sequence for the enhanced green fluorescence protein (eGFP) was linked to pRb cDNA via an internal ribosomal entry site element (IRES, CLONTECH).

Cell Cycle Analysis—Human bladder carcinoma 5637 cells were seeded into T25 flasks at 20% confluence. The following day, cells were transfected with 5 μg of the selected plasmid, together with LipofectAMINE, according to manufacturer’s instructions (Life Technologies, Inc.). Control cells were mock-transfected with LipofectAMINE alone. After 6 h, leftover DNA and LipofectAMINE were removed by washing the cells with phosphate-buffered saline. Cells cultured in RPMI 1640 medium in the presence or absence of serum for 56 h at 37 °C were harvested by trypsinization, fixed in 4% paraformaldehyde in phosphate-buffered saline, and stained with propidium iodide. The cells were then FACs sorted into G1-positive and -negative populations using a Becton Dickinson FACScan equipped with a 488-nm argon laser (argon source and laser). CellQuest software. For these experiments, 100,000 events were acquired and the non-clumped cells were gated for analysis on a second dual parameter display with DNA (linear red fluorescence) on the x axis and FITC-eGFP (log green fluorescence) on the y axis. Negative and positive parameters were set with cells transfected with eGFP plasmid or exposed to LipofectAMINE alone. For cells transfected with other plasmids, G1-negative events were analyzed for DNA content and cell cycle positions using ModFit LT 2.0 DNA analysis software (Verity House Software, Topsham, ME).

Phosphorylation Studies—Immunoprecipitations were performed as described previously (3). 32P-Labeled wild type and mutant pRb proteins were produced in vitro transcription and coupled translation of the pbLuescript/pRb plasmids using the TNT T7 coupled reticulocyte lysate system (Promega), together with Expre3SS-S (labeled at the methionine and cysteine residues). For immunoprecipitation, reticulocyte lysate was diluted in EBC buffer (125 mM NaCl, 40 mM Tris base, pH 8.0, 0.5% Nonidet P-40), antibody RB1-Ab 2–3 (3), and proteinase inhibitors (100 mM phenylmethylsulfonyl fluoride, 10 mM each of NaF, and 0.1 mM NaVO4) to a final concentration of 100 μm. For two-dimensional phosphopeptide analysis, pHuAcprpRbPQ, pHuAcprpRbm89, and pHuAcprpRbmS807S811 were separately transfected into Saos-2-AT (14, 22). Negative control events were also performed in G183 and growth arrested in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics at 37 °C. pRb phosphorylated in vivo was immunoprecipitated from Saos-2 transfectants metabolically labeled with [32P]H3PO4. Immunoprecipitates were resolved by 8% SDS-PAGE. Chromotryptic digestion of pRb and analysis of phosphopeptides were performed as described previously (14). Briefly, proteins were electrophoretically transferred to a nitrocellulose filter,
which was exposed to x-ray film. Bands corresponding to pRb were excised, and the protein, extracted and immunoprecipitated from 5 × 10⁶ cells, was digested with 20 μg of chymotrypsin (Sigma) in 200 μl of 50 mM ammonium bicarbonate, pH 7.3, at 37 °C for 18 h. Chymotryptic peptides were dried, washed, and oxidized using performic acid. Following oxidation, peptides were washed extensively, first with water, then with thin layer electrophoresis buffer (TLE buffer: 2.2% formic acid, 7.8% acetic acid, pH 1.9). For an accurate comparison between samples, the same number of cpm of each sample was applied. Peptides were spotted onto a thin layer cellulose plate (Kodak) and electrophoresed in the first dimension in TLE buffer, for 1.5 h, using a Hunter thin layer electrophoresis apparatus (HTLE-7000, C.B.S. Scientific, San Diego, CA). Plates were dried, then chromatographed in the second dimension using phosphochromatography buffer (n-butanol:pyridine:acetic acid:water, 5:3:1:4 by volume). Dried plates were exposed to x-ray film.

RESULTS

Discovery of the m89 Motif Which Confers upon pRb a Hypophosphorylated Conformation—To understand the function and regulation of pRb, we performed in vitro mutagenesis throughout the RB-1 CDNA. Forty-seven different amino acid substitution mutants, each covering 20 amino acid residues, were created, and the effect of the mutation on the function and regulation of phosphorylation of pRb analyzed using different cell lines. As expected, mutations in the pocket region resulted in the inactivation of pRb function.

In order to understand the effect of the mutations on the regulation of pRb phosphorylation, we made use of a Saos2 variant cell line (Saos-2AT), which is resistant to growth inhibition by ectopically expressed pRb (22–24). Cell lines harboring the transfected mutants were isolated, and the in vivo phosphorylation status of the ectopically expressed pRb in each was analyzed. In the process, we encountered a mutant, pRbm89, which exhibited a different mobility by SDS-PAGE than wild type pRb. As is shown in Fig. 1A, wild type pRb isolated from an asynchronous cell population of Saos2pRbPQ (lane 1) multiple forms in a one-dimensional SDS-PAGE. In contrast, the mutant pRb isolated from an asynchronous cell population of Saos2pRbm89 exhibited only hypophosphorylated forms (lane 2). Since pRbm89 protein was isolated from an asynchronous population, this observation suggests that the mutant form of pRb assume a hypophosphorylated conformation, even at the S phase of the cell cycle when wild type pRb is normally fully phosphorylated. Indeed, pRbm89 protein isolated from a cell population growth arrested at G1/S with alphadicolin remains in the same hypophosphorylated conformation (data not shown).

To understand if the hypophosphorylated conformation of the pRbm89 mutant is due to mutation at the m89 region or due to a peculiar lack of a specific cdk activity in the Saos2pRbm89 cell line, we analyzed the protein in vitro phosphorylation pattern of the pRbm89. In vitro translated mutant and wild type pRb were kinase with either cyclin A/cdk2, cyclin D2/cdk4, or cyclin E/cdk2. As is shown in Fig. 1B, under optimized kinasing conditions where unphosphorylated wild type pRb (lane 1) became fully phosphorylated (lane 3), the kinase pRbm89 still maintain the hypophosphorylated conformation (Fig. 1B, lane 2) with an apparent molecular mass midway between the unphosphorylated and the hyperphosphorylated forms. The fact that the hypophosphorylated conformation of pRbm89 is observed both in vivo and in the S phase, and in vitro under optimized conditions when wild type pRb is fully phosphorylated, suggests that it is an intrinsic property of the m89 mutant and not the result of suboptimal cdk activity.

There are several possible explanations for the observed conformation of pRbm89. One is that all sites on the protein are fully phosphorylated, but that mutation at m89 has somehow altered conformation of the phosphorylated form of pRb. Alternatively, it is possible that the mutation at m89 has caused a conformational change in pRb, such that particular phosphorylation sites, critical for the determination of the conformation of pRb, are not exposed to the kinase. To distinguish between these two possibilities, it is necessary to have an understanding of the relationship between the conformation of pRb and the phosphorylation status of the cdk sites. By identifying the cdk sites that control the migration pattern of the various forms of pRb (Fig. 2A, lane 1), it should be possible to determine if the mutation at m89 has altered the exposure of the cdk sites that confer the particular conformation of pRbm89. A detailed analysis of the cdk sites and their contribution to the conformations of pRb was therefore performed.

Detailed Analysis of the Relationship between the Phosphorylation Status of Individual cdk Sites and the Conformation of pRb—To understand the status of phosphorylation of each of the cdk sites in the various forms of pRb, we mutagenized the serine or threonine residues of individual cdk sites into alanine. In choosing the cdk sites for detailed analysis, Thr-5, Ser-230, and Ser-780, which have been shown not to be phosphorylated in vivo (25), and Ser-567, the mutation of which reportedly inactivates pRb (19), were excluded from analysis. The remaining 12 sites: Ser-249, Thr-252, Thr-356, Thr-373, Ser-608, Ser-612, Ser-788, Ser-795, Ser-807, Ser-811, Thr-821, and Thr-826, which have been demonstrated to be phosphorylated in vivo (25–27), were analyzed. To show that mutations at these 12 cdk sites do not inactivate the function of pRb, corresponding mutants were subcloned into both the pHU4-Acpr-1-neo and the pAcyYM1 vectors for growth suppression and protein binding analysis. Ectopic expression of these mutants driven by the human β actin promoter resulted in suppression of the growth of Saos-2 cells (ATCC HTB-85) (data not shown). In addition, all of these mutants, expressed as recombinant baculoviruses in S9 cells, retain their ability to bind the SV40 large T antigen and the cellular proteins c-Abl and E2F-1 in vivo (data not shown). These results indicated that, unlike the mutation at Ser-567, none of these mutations were inactivating.

FIG. 1. Analysis of the effect of mutation of m89 on the phosphorylation status of pRb. Panel A, SDS-PAGE profiles of immunoprecipitated wild type pRb (lane 1) and m89 mutant pRbm89 (lane 2), extracted from [35S]methionine metabolically labeled randomly growing cultures of transfected Saos2 (AT) cells. Panel B, SDS-PAGE profiles of immunoprecipitated wild type pRb (lanes 1 and 3) and m89 mutant pRbm89 (lane 2) proteins generated by in vitro translation with [35S]methionine. The pRb were either not phosphorylated (lane 1) or phosphorylated in vitro (lanes 2 and 3) using a kinase preparation from S9 cells co-infected with baculoviruses expressing cyclin A and cdk2.
To understand the effect of the mutation of the cdk sites on the conformation of pRb, we compared the migration pattern of \textit{in vitro} translated pRb mutants to the various forms of pRb extracted from cell culture, on SDS-PAGE. As is shown in Fig. 2A (lane 1), wild type pRb protein extracted from an asynchronous cell culture can be fractionated into five distinct forms. We designated these as forms 1–5, with form 1 being the most underphosphorylated, fastest migrating form and form 5 being the fully phosphorylated and slowest migrating form. As expected, form 1 pRb is the only form extracted from cells growth-arrested by serum starvation (Fig. 2A, lane 2).

For \textit{in vitro} analysis, wild type and cdk site mutants of pRb, generated by \textit{in vitro} translation, were kinased under optimized conditions and their migration analyzed by one-dimensional SDS-PAGE. As expected, unphosphorylated wild type pRb migrates as form 1 (Fig. 2B, lanes 1 and 14) and is converted into the slowest migrating form 5 (lanes 2 and 13) by the kinasing reaction. Differences in the migration pattern of the optimally phosphorylated cdk site mutants were reproducibly observed (Fig. 2B, lanes 3–12). Of these, mutations at Thr-356 (lane 5), Ser-807/Ser-811 (lane 10), and Thr-821 (lane 11) resulted in the most noticeable difference in the migration pattern. It should be noted that these pRb mutants were otherwise fully phosphorylated at all the other cdk sites, as determined by two-dimensional peptide analysis using the wild type pattern as a standard (Fig. 4B). Thus, if either Thr-356 (lane 5) or Thr-821 (lane 11) is not phosphorylated, the otherwise fully phosphorylated pRb mutant migrates to a slightly faster position, corresponding to form 4 in Fig. 2A. Mutation of Ser-807/Ser-811 causes a more drastic effect on pRb conformation (Fig. 2B, lane 10), resulting in a migration pattern similar to that of form 3. Thus, mutation at Thr-356, which is N-terminal to the pocket region, or Ser-807/Ser-811 or Thr-821, which are C-terminal to the pocket region, can individually cause conformational changes independent of all other sites. While Thr-356 was previously reported to play no role in the determination of pRb conformation (12), the data provided here clearly show that Thr-356 has a drastic effect on the conformation of the N-terminal domains upstream of the pocket region. The slight but reproducible difference in the migration of these mutants is consistent with changes being individual and local.

Mutation of serine/threonine to alanine at Ser-249 (lane 3), Thr-252 (lane 4), Thr-373 (lane 5), Ser-608/Ser-612 (lane 7), Ser-788 (lane 8), Ser-795 (lane 9), Ser-807/Ser-811 (lane 10), Thr-821 (lane 11), and Thr-826 (lane 12) were kinased under identical conditions and the immunoprecipitated proteins resolved by SDS-PAGE. Panel C, schematic representation of pRb, showing the location of 16 putative cdk target sites with the four cdk sites, which affect pRb conformation shown in black. Pocket regions A and B, including the inactivating point mutation site Ser-567, are shaded in gray, and the site of the m89 mutation is marked in black.

![Image](Image 150x407 to 454x729)

**FIG. 2.** Detailed analysis of the relationship between the phosphorylation status of individual cdk sites and the conformation of pRb. Panel A, SDS-PAGE profiles of immunoprecipitated wild type pRb extracted from a random culture of Saos-2 (AT) cells expressing a transfected wild type pRb plasmid (lane 1). The five most easily identifiable forms were designated forms 1–5, with form 1 being the fastest migrating, least phosphorylated form and form 5 being the most heavily phosphorylated, slowest migrating form. Wild type pRb extracted from cells growth-arrested by serum starvation is included for comparison (lane 2). Panel B, SDS-PAGE profiles of immunoprecipitated wild type and cdk site mutant pRb, labeled with \[^{35}\text{S}\]methionine during \textit{in vitro} translation, and kinased using a combination cyclin A/cdk2 kinase extracted from recombinant baculovirus-infected Sf9 cells. Wild type unphosphorylated pRb (lanes 1 and 14) and fully phosphorylated pRb (lanes 2 and 13) pRb cdk mutants at Ser-249 (lane 3), Thr-252 (lane 4), Thr-356 (lane 5), Thr-373 (lane 6), Ser-608/Ser-612 (lane 7), Ser-788 (lane 8), Ser-795 (lane 9), Ser-807/Ser-811 (lane 10), Thr-821 (lane 11), and Thr-826 (lane 12) were kinased under identical conditions and the immunoprecipitated proteins resolved by SDS-PAGE. Panel C, schematic representation of pRb, showing the location of 16 putative cdk target sites with the four cdk sites, which affect pRb conformation shown in black. Pocket regions A and B, including the inactivating point mutation site Ser-567, are shaded in gray, and the site of the m89 mutation is marked in black.
As these changes appear to be independent of each other, we investigated the possibility that the conformational changes are additive by performing an analysis on pRb mutated at more than one site. The resulting data showed that the conformational changes brought about by mutation at Thr-356 and Ser-807/Ser-811 appear to be additive, such that a mutant encompassing all three sites (Fig. 3A, lane 3) migrates faster than either of the mutants alone (lane 2), to a position similar to that of form 2. Thus, the conformation of pRb lacking phosphorylation at Thr-356, located in front of the pocket region, appears to be independent and additive to the conformation caused by mutation at Ser-807 and Ser-811, located behind the pocket region. The conformational change brought about by mutation at Thr-821 is also additive, in that mutation at all four critical sites (Thr-356, Ser-807/Ser-811, and Thr-821) produces a form of pRb with a migration pattern similar to that of the unphosphorylated form (form 1) (Fig. 3A, lane 2; see also Fig. 4A, lanes 1 and 2). It should be noted that although there is an additive change in the conformation in pRb mutated at both Thr-356 and Thr-821 (Fig. 3B, lanes 1 and 2) or Ser-807/Ser-811 and Thr-821 (Fig. 3B, lanes 5, 6, and 7), the change is difficult to be resolved satisfactorily by one-dimensional SDS-PAGE. Thus, what we called form 3 pRb, isolated from an asynchronous cell population could, in fact, be a mixed pRb population consisting of pRb unphosphorylated at Ser-807/Ser-811 with or without phosphorylation at Thr-821. Likewise, form 4 could be composed of a mixture of pRb unphosphorylated Thr-356, Thr-821, or a combination of the two. In summary, the five forms of pRb observed in vivo can be accounted for by the lack of phosphorylation at one or more of these four cdk sites, even when all other sites are phosphorylated (summarized in Fig. 3C).

The m89 Region Regulates the Exposure of the Ser-807/Ser-811 cdk Sites—To determine if mutation at the m89 region resulted in a conformational change that blocks cdk site exposure, we compared the migration pattern of \emph{in vitro} phosphorylated pRbm89 to those of the cdk site mutants (Fig. 4A). Our results showed that the migration pattern of pRbm89 matched that of pRb mutated at Ser-807/Ser-811 (Fig. 4A, lanes 3 and 4).

To determine if the Ser-807/Ser-811 sites are indeed affected by the m89 mutation, we performed two-dimensional phosphopeptide mapping analysis of the \emph{in vivo} phosphorylation patterns of individual cdk sites in the wild type pRb and pRbm89 (Fig. 4B). The chymotryptic phosphopeptide containing the Ser-807/Ser-811 sites was previously mapped using the 807/811 mutant and the data confirmed with a synthetic peptide corresponding to the chymotryptic phosphopeptide containing Ser-807/Ser-811 (14). The chymotryptic phosphopeptides corresponding to Thr-821, Ser-795, and Thr-356 were mapped using the corresponding mutants \emph{in vitro and in vivo}. In order to quantitate the changes in phosphorylation at these four cdk sites that may have occurred in pRbm89, \emph{x}-ray film images of the two-dimensional patterns of wild type and mutant pRb were analyzed by scanning densitometry. As the phosphorylation status of Ser-795 showed little variation with growth conditions, it was used as the standard for comparison between samples. By normalizing the intensity of the peptide images of Ser-795 from the wild type and various pRb mutants, the phosphorylation status of the four cdk sites was compared. The data (Fig. 4B and Table I) revealed that phosphorylation at Ser-807/Ser-811 was absent in both the pRbmS788/S807/S811 mutant and in pRbm89, and was reduced in pRb extracted from TGFβ-treated, growth-arrested, MDAMB 231 cells. In contrast, negligible changes were observed for those peptides containing Thr-356 or Thr-821 in these mutants, or under TGFβ treatment conditions. As such, the data showed very clearly that the Ser-807/Ser-811 region in the m89 mutant, pRbm89, is inaccessible to kinases \emph{in vivo}. In contrast, the chymotryptic phosphopeptides corresponding to Thr-821, Ser-795, and Thr-356 (mapped using the corresponding mutants \emph{in vitro and in vivo}) were not affected by the m89 mutation. This \emph{in vitro} result was confirmed when wild type pRb and pRbm89 were phosphorylated \emph{in vitro} (data not shown).

That the mutation of m89 affects the phosphorylation of Ser-807/Ser-811 specifically is of great interest in view of the fact that the flanking Thr-821 and Ser-795 sites were not affected. Our previous data revealed that phosphorylation of Ser-807/Ser-811 were also specifically down-regulated in cells growth-arrested at the late G1 phase by TGFβ treatment (14). An example of this form of endogenous pRb extracted from TGFβ-treated MDAMB 231 cells is included here for illustration (Fig. 4B). We further showed that the Ser-807/Ser-811 sites became rapidly phosphorylated once TGFβ was removed and cells progressed into the S phase (14). These data suggest that pRb unphosphorylated at Ser-807/Ser-811 is in an active form capable of blocking cell cycle progression. It further suggests that pRb mutated at the m89 region should be refractory to inactivation by phosphorylation, since the critical Ser-807/Ser-811 sites are rendered inaccessible. As such, pRbm89 should be constitutively active. To test this hypothesis, we compared the growth suppression properties of the wild type and the mutant pRb.

Comparison of the Ability of Wild Type and Constitutively Active pRb Mutants to Block Cell Cycle Progression—To compare the effect of the mutation of the four cdk sites and the m89 region on the growth suppressor function of pRb, selected cdk site mutants and the m89 mutant pRbm89 were placed under the control of the CMVp in a plasmid together with CMVp-cyclin A and the fluorescent marker eGFP. The resulting plasmids, peGFP, peGFP/CyxA, peGFP/CycApRb, peGFP/CycA/pRbm89, peGFP/CycApRbmS807/S811, peGFP/CycApRbmT356/S807/S811, and peGFP/CycApRbmT356/S807/S811/T821, were transfected into the human diploid bladder carcinoma cell line, 5637. Cells were cultured for 56 h in the presence or absence of serum. The harvested cells were then fixed, counterrainted with propidium iodide, and FACs sorted into GFP-positive and -negative populations. Cell cycle analysis was performed on the GFP-positive population. In the peGFP-transfected, GFP-positive cells, the effect of serum deprivation could clearly be seen in the increase in cells in G0/G1, coupled by a marked decrease in the S phase population. When compared with serum-deprived GFP-positive cell culture, co-expression of cyclin A with eGFP resulted in a significant decrease in the G2/G0 population (*, p < 0.0005) while the S and G2/M populations showed significant increases (**, p < 0.0005; *** p < 0.02), even in the absence of serum (Fig. 5A).

The effect of co-expression of cyclin A and pRb (wild type or mutants) on cell cycle progression is shown in Fig. 5B. Previous studies have demonstrated that pRb, ectopically co-expressed with cyclin A, is fully phosphorylated and inactivated (28). Consistent with these previous findings, our results revealed that there is no significant difference in the size of the cycling cell population in cells expressing transfected cyclin A, with (Fig. 5B, lane 4) or without (Fig. 5B, lane 3) co-expressed wild type pRb. However, when pRb was mutated at either the m89 region or at the cdk sites Ser-807/Ser-811, the size of the cycling cell population was significantly decreased and to the same extent (Fig. 5B, lanes 5 and 6).

As the four cdk sites regulate the conformation of pRb independently, we tested the effect of mutating additional cdk sites on the progression of the cell cycle. The data showed that the addition of a mutation at Thr-356 to mutations at Ser-807/Ser-811...
(figure), or when Thr-821 is also unphosphorylated, in combination with Ser-807/Ser-811, in pRB. The fully phosphorylated version of pRB, in which all four cdk sites (Thr-356, Ser-807, Ser-811, and Thr-821) are mutated (lane 1), was compared with unphosphorylated wild type (lane 2), the phosphorylated form of the m89 mutant pRbm89 (lane 3), and to the fully phosphorylated forms of the mutant Ser-807/Ser-811 (lane 4) and wild type pRb (lane 5). Panel B, two-dimensional phosphopeptide patterns were obtained by chymotryptic digestion of wild type and mutant pRB proteins extracted and immunoprecipitated from transfected Saos2 (AT) (wild type, pRbm89, and pRbmS788S807S811) cells and TGFβ-treated MDAMB 231 cells, metabolically labeled with [32P]H3PO4. For each panel, the phosphopeptides corresponding to those contributed by cdk sites Thr-356, Ser-788, Ser-795, Ser-807/Ser-811, and Thr-821, determined by chromatographic mapping of cdk site mutants, are circled and labeled. For the combination cdk site mutant Ser-788/Ser-807/Ser-811 from Saos2pRbm788/807/811C2, mutation at the Ser-788 site had no effect on either the one- or two-dimensional phosphorylation patterns of pRbmS807/S811 (data not shown), and therefore, this mutant is used as an example of the pRbmS807/S811 pattern. For quantitation of phosphorylation at the four critical sites due to mutation or TGFβ treatment, see Table I.
TABLE I
Phosphorylation site status

| Peptide     | Wild type | pRbmT356 | pRbmT356/S807/S811 | Thr-821 | TGFβ | pRbmT356/S807/S811 | Wild type |
|-------------|-----------|----------|--------------------|---------|------|--------------------|-----------|
| Ser-795     | 1.0       | 1.0      | 1.0                | 1.0     |      | 0.6                | 0.8       |
| Ser-807/Ser-811 | 0.9     | 0.9      | 0.1                | 0.1     | 0.4  | 0.2                | 0.8       |
| Thr-356     | 1.0       | 1.0      | 1.0                | 0.9     | 0.9  | 1.0                | 0.8       |
| Thr-821     | 0.8       | 0.8      | 0.6                | 0.6     | 0.6  | 0.8                | 0.8       |

811 caused a relatively small decrease in the number of cycling cells when compared with the Ser-807/Ser-811 mutation alone (Fig. 5B, lane 7). The most dramatic decrease in the cycling population occurred in the cells transfected with pEGFP/CycA/pRbmT356/S807/S811/T821 expressing pRb mutated at all four sites (Fig. 5B, lane 8). Although co-expressed with cyclin A, this form of pRb reduced the cycling population of the transfected cells to a level (Fig. 5B, lane 8) even lower than that observed in serum-deprived pEGFP (no cyclin A)-transfected cells (Fig. 5B, lane 2).

DISCUSSION

The Phosphorylation Status of Four cdk Sites Determines the Conformation of pRb—The data presented here describe the identification of four cdk sites, the phosphorylation status of which determines the conformation of pRb. Several conclusions can be drawn from these data, summarized in Fig. 3B. First, the phosphorylation status of each of the four cdk sites (Thr-356, Ser-807/Ser-811, Thr-821) can independently control the conformation of pRb. Second, the conformational changes appear to affect different domains of pRb, such that mutation of more than one cdk site leads to further conformational changes resolvable by SDS-PAGE. Third, the conformation of pRb appears to be determined mainly by these four sites, as the mutation of other cdk sites has little effect. Finally, the five forms of pRb extracted from randomly growing cell cultures can be attributed to different conformations created by the differential phosphorylation of these four sites. The observed greater intensity of form 4 on SDS-PAGE can be explained simply by the observation that the conformation brought about by an unphosphorylated Thr-356 and/or Thr-821 cannot be resolved by one-dimensional SDS-PAGE. One of these four sites, Thr-356, was previously reported to be inconsequential to the determination of the conformation of pRb (12). The data presented here demonstrated a drastic effect of the mutation of this site, alone or in combination with the others, on the conformation of pRb. Thus, the conformation of pRb is regulated by cdk sites both at the N terminus and the C terminus.

Functional Significance of the Four cdk Sites and the Conformation of pRb at the Molecular Level—pRb is a binding target for many proteins. Different domains are required for binding to different proteins. A number of proteins, such as MCM7 and Ap-48 (29, 30) bind to the N terminus of pRb, whereas RBP-1, RBP-2 (31), PU.1 (32), cyclin D (33, 34), E2F-1 (35–37), histone deacetylase (38), and others bind to the pocket region, while c-Abl binds exclusively to the c-terminus (11). The fact that some of these binding proteins, such as c-Abl and E2F-1 (11) and HPV E7 and E2F-1 (39), can bind to pRb independently and simultaneously further confirms that each of these proteins recognizes a non-overlapping domain of pRb. Our data, which suggest that each of the four cdk sites can independently regulate the conformation of a particular domain, suggest that the phosphorylation status of these sites may affect the binding of pRb to a particular binding protein.

Indeed, previous data suggest that certain proteins always recognize particular forms of pRb. The phosphorylation status of Thr-821 and Thr-826 affects the interaction of pRb with the LXXE motif, whereas that of Ser-807/Ser-811 affects pRb interaction with c-Abl. How the phosphorylation status of Thr-356 may affect the interaction between pRb and pRb-binding proteins that bind to its N terminus remains to be determined.

In addition, it is entirely possible that there are other functionally important conformational changes in pRb brought about by the phosphorylation of other cdk sites which are too subtle to be detected by one-dimensional SDS-PAGE analysis. Indeed, it was recently reported that, although the phosphorylation status of Ser-795 does not noticeably affect the conformation of pRb, it is nonetheless functionally important (27). On the other hand, our data on the influence of the mutation at the cdk sites on cell cycle progression does support the contention of an important role of the phosphorylation status-related conformational change in the proper functioning of pRb.

Functional Significance of the Critical cdk Sites and the Conformation of pRb at the Cellular Level—There are three lines of evidence that support an important role for the four cdk sites in proper pRb function. First of all, the active form of pRb from TGFβ-growth-arrested cells contains unphosphorylated Ser-807/Ser-811 sites. TGFβ treatment results in down-regulation of cdk activity and up-regulation of cdk inhibitors p27 and p15 (40–44), and the phosphorylation of most cdk sites in pRb is down-regulated. The Ser-608 site, for example, is inefficiently phosphorylated under this condition (26). Interestingly, five pRb cdk sites, at Thr-5, Ser-252, Ser-373, Ser-788, and Ser-795, are also potential kinase sites for mitogen-activated protein kinase. Recent studies suggest that some mitogen-activated protein kinase-related kinases are activated when TGFβ is used as an inhibitor of epithelial cell growth (45). The inability of TGFβ treatment to down-regulate the phosphorylation of these sites could be due to this overlap of kinase specificity. The phosphorylation of Ser-807/Ser-811, on the other hand, appears to be most severely affected, as shown by the complete absence of detectable phosphopeptides when the amount of protein used for the analysis was adjusted such that phosphopeptides for other sites are detected at high level. As there are a number of cdk sites that are substrates for cyclin D1/cdk4 and cdk6, the more severe inhibition of phosphorylation at Ser-807/Ser-811 perhaps suggests these two sites are less accessible to the lowered level of cyclin D/cdk4/6. Our previous data showed that the progression of the cells into S is accompanied by the heavy phosphorylation of these two sites (14).

A second line of evidence that supports an important role for the four cdk sites in proper pRb function comes from the observation that cell cycle progression is more effectively blocked by ectopically expressed cdk site pRb mutants than by wild type pRb. Although the finding that serum deprivation can block cell cycle progression in pRb-negative cells (Fig. 5B, lane 2) suggests that active pRb is not the only critical factor for transducing a growth arrest signal, our data indicate that expression of cyclin A in these cells is quite effective in reversing the serum deprivation effect. In addition, this effect of cyclin A can be most successfully counteracted by pRb in which the Ser-807/Ser-811 cdk sites were mutated. A similar effect of the expression of cyclin A on 807/811 was noted previously, in Saco2 cells (11). Of the four cdk sites, Thr-356 and Ser-807/Ser-811 are substrates for cyclin D1/cdk4/cdk6 but not for cyclinA/cdk2 or cyclin E/cdk2, whereas Thr-821 can be phosphorylated in vitro by either cyclin A/cdk2 or cyclin E/cdk2, but not by cyclin D/cdk4/6 (46). This perhaps explains the observation that pRbmT356S807S811T821,
FIG. 5. Cell cycle analysis of GFP-positive human bladder carcinoma 5637 cells expressing wild type or mutant pRb/eGFP fusion proteins and cyclin A. The percentage of cells at various cell cycle phases were determined for cells transfected with various peGFP plasmids. Data represent the mean ± standard deviation for three separate FACS analyses. Statistical significance was calculated using Student's t test. Panel A, comparison of G0/G1, S, and G2/M phase profiles from populations of peGFP-transfected cells maintained in medium plus or minus serum, and cells transfected with a plasmid co-expressing peGFP and cyclin A (peGFP/CycA), also under serum-deprived conditions. For peGFP/CycA-transfected GFP-positive cells, the decrease in the G0/G1 population (*, p < 0.0005) and the increase in the S and G2/M populations were significant (**, p < 0.0005; *** , p < 0.02). Panel B, cell cycle analysis of multiple cdk site and m89 region pRb mutant-transfected cells. All cell lines were analyzed following transfection and maintenance in medium without serum with the exception of sample 1, which controls for the effect of eGFP expression alone, and represents a random population in the presence of serum. Results are expressed as the number of cells in S phase following transfection and treatment, and are considered representative of the cycling portion of the population, as opposed to cells which may have become blocked at the G0/G1 or G2/M phases. N0 significant decrease in the number of cycling cells was observed by co-expressing wild type pRb with cyclin A (compare bars 3 and 4). However, mutation at the m89 region (• • ) or at Ser-807/Ser-811 (• • • ) did cause a significant drop in S phase cells when compared with levels observed in the wild type transfected cells (••, p < 0.01; •••, p < 0.01). Addition of the Thr-356 mutation to the Ser-807/Ser-811 mutation caused a significant difference in the cycling population when compared with wild type pRb (*, p < 0.005), and this difference was more significant than the difference between Ser-807/Ser-811 alone and wild type (*, p < 0.005; compare with •••, p < 0.01). Addition of a mutation at Thr-821 caused a significant decrease in the number of cycling cells when compared with either the triple mutant, containing Thr-356/Ser-807/Ser-811 only (*, p < 0.005) or with wild type (*, p < 0.0005). This mutant was also capable of decreasing the level of cycling cells to a level significantly below that observed in serum-deprived cells with eGFP-transfected cells in the absence of serum (•, < 0.005).
in which all four sites were resistant to phosphorylation, was more efficient than pRbmT356S807S811T821 at blocking progression of the cell cycle in cells overexpressing cyclin A. The fact that pRbmT356S807S811T821 was the most efficient at blocking progression of the cell cycle, more so than serum deprivation alone (Fig. 5B), perhaps reflects residual phosphorylation of the cdks sites even in medium containing 0.1% serum. Finally, pRb mutated at the m89 region, which affects the phosphorylation status of cdks sites Ser-807/Ser-811, resulted in a block in cell cycle progression equivalent to that produced by pRb mutated at Ser-807/Ser-811. Taken together, these data support the claim that the Ser-807/Ser-811 cdks sites are critical in determining the conformation and function of pRb.

The Significance of the m89 Motif—Data presented here reveals that mutation at m89 renders the Ser-807/Ser-811 sites inaccessible to cdks and that this conformation is maintained at all phases of the cell cycle. It is interesting to note that phosphorylation of Ser-807/Ser-811, but not Thr-821 or Thr-356, is much more severely inhibited in cells growth-arrested in anaphase and that this conformation is maintained in a cell cycle progression of the cell cycle, more so than serum deprivation alone under certain growth conditions.

How the m89 region regulates the accessibility of Ser-807/Ser-811 is far from clear, but the fact that the conformation is maintained at all phases of the cell cycle suggests that m89 is engaged in an intramolecular interaction. The mutation appears to affect, selectively, the conformation of the immediate upstream region where cdks site 807/811 is located. The phosphorylation status of these two sites in turn affects the further upstream region where c-Abl binds. It is of great interest that we have recently extended these studies and discovered that the m89 region is part of an antigenically conserved domain, p5, between pRb and the tumor suppressor protein p53. Moreover, mutation of the p5 region in p53 also induces a conformational change in the upstream cdks site Ser-315, such that it becomes inaccessible for cdks phosphorylation (47). The phosphorylation status of this serine, in turn, controls the ability of p53 to bind to its target DNA sequence (48). It is possible that the presence of this regulatory domain in both tumor suppressor proteins may afford a coordinated regulation of their activities under certain growth conditions.

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