ROLE OF pRB DEPHOSPHORYLATION IN CELL CYCLE REGULATION

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1. ABSTRACT

pRB, the tumor suppressor product of the retinoblastoma susceptibility gene, is regarded as one of the key regulators of the cell cycle. This protein exerts its growth suppressive effect through its ability to bind and interact with a variety of cellular proteins. In turn, pRB binding and interacting ability is governed by its phosphorylation state. In recent years, this negative growth regulatory protein has captured a great deal of attention from investigators around the world due to its ability to modulate the activity of transcription regulatory proteins, enzymes which modify chromatin, and other cellular proteins which contribute to its complex role in mammalian cells. Hypophosphorylated pRB binds and sequesters transcription factors, most notably those of the E2F/DP family, inhibiting the transcription of genes required to traverse the G1 to S phase boundary. This cell cycle inhibitory function is abrogated when pRB undergoes phosphorylation mediated by cyclin/cdk complexes following cell stimulation by mitogens. Removal of these phosphates appears to be carried out by a multimeric complex of protein phosphatase type 1 (PP1) and noncatalytic regulatory subunits at the completion of mitosis. This dephosphorylation returns pRB to its active, growth suppressive state. While the mechanism of pRB phosphorylation has and continues to be extensively studied, dephosphorylation of pRB has received disproportionately less attention. The goal of this review is to revisit the role of pRB dephosphorylation in regulating the cell cycle. Emphasis will be placed on understanding the function and regulation of pRB during the cell cycle as well as our ever-expanding notions of pRB-PP1 interaction and the mechanism of pRB dephosphorylation at mitotic exit.

2. INTRODUCTION

A major mechanism by which eukaryotic cells regulate protein function is to place phosphate groups on serine/threonine or tyrosine residues. The historical paradigm is that by placing such groups onto a protein, the function of the protein will be modified. As such, considerable effort has gone into the study of kinases. However, the steady state level of protein phosphorylation depends on the relative activities of both kinases and phosphatases. It has only recently become appreciated that phosphate removal by phosphatases can have effects just as dramatic as placing phosphates on. A prime example of this is the product of the retinoblastoma susceptibility gene, pRB. When this protein is phosphorylated, it is generally
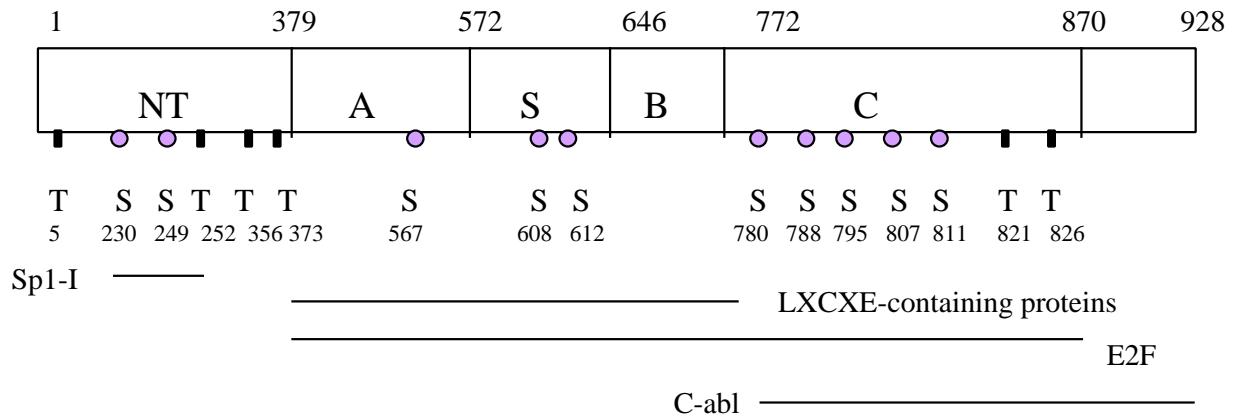
Figure 1. pRB structure, consensus cdk phosphorylation sites, and protein binding domains. Numbers at the top of the bar delineate amino acids comprising the amino terminus (NT), A-pocket (A), spacer region (S), B-pocket (B), C-pocket (C). Approximate locations for serine (S) and threonine (T) cdk phosphorylation sites, as well as amino acid position, are indicated on the underside of the bar. Solid lines denote regions of pRB involved in the binding of an inhibitor of Sp1 (Sp1-I), LXCXE-proteins, transcription factor E2F, and c-abl.

regarded as inactive with respect to growth suppression. Upon removal of phosphates, this protein becomes an active growth suppressor affecting the very engine which drives cell proliferation.

In this review, we will concentrate on the mechanism of pRB dephosphorylation as it relates to activating the growth suppressing property of pRB and cell cycle control. The purpose here is to highlight studies contributing towards the development of the current working models with regards to pRB dephosphorylation, PP1 activity, and cell cycle control.

3. THE pRB PROTEIN

3.1. Structure

In order to appreciate the functional significance of pRB dephosphorylation and cell cycle control, it is worthwhile to take a moment and discuss some of the structural features of this protein. Of critical importance here is the fact that pRB can be divided into a number of functional regions, with each region affecting different protein-binding capacities. Figure 1 summarizes these functional regions with respect to protein binding, as well as highlighting the various cyclin-dependent kinase (cdk) phosphorylation sites.

pRB is known to bind to over 30 cellular proteins ranging from transcriptional regulators, many of which are conduits to pRB's involvement in differentiation pathways (reviewed in1, 2), to enzymes involved in signal transduction and chromatin remodeling systems. Although far from a complete list, the functional relevance of many, but not all, of these interactions is known. For example, the N-terminal region of pRB, amino acids 1-378, has been shown to be important for Sp1 transactivation as it relieves repression by the inhibitor, Sp1-I (3). Amino acids 379-772 are considered the small pocket domain, and are further subdivided by a spacer region into the A- (amino acids 394-

571) and B- (amino acids 649-773) domains (4-6). The small pocket of pRB is necessary for binding proteins containing a conserved LXCXE amino acid motif. Most notable of these are the transforming proteins of the DNA tumor viruses including SV40 large T-antigen (7,8), adenovirus E1A (9-11), and human papillomavirus E7 (12,13). Other proteins that interact with pRB and may utilize this motif include the D-type cyclins (14-16), the transcription factor UBF (17), Elf-1 (18) and a phosphatase crucial for maintaining the integrity of pRB function, a type 1 serine/threonine protein phosphatase (PP1), which contains a slightly modified version of this motif (19). The large pocket domain of pRB is composed of the A/B region together with the C-pocket amino acids 773-870. This region of pRB is most noted for its ability to bind to a class of transcription factors known as E2F (20,21). It is through this interaction that pRB is believed to exert its most powerful growth suppressive effects. The C-pocket of pRB itself is important in other protein-binding functions such as those with mdm-2, the p53-regulatory protein (22), and the c-abl tyrosine kinase (23), although less is known about the biological effects of these latter two interactions. In summary, it is apparent that discrete regions of pRB are involved in different protein-protein interactions, resulting in different functional consequences.

3.2. Phosphorylation

As stated above, a major mechanism by which eukaryotic cells regulate protein function is to modify the protein by phosphorylation. Indeed, prior to mitogenic stimulation, pRB exists in a hypophosphorylated, growth suppressive state. Upon cell stimulation by growth promoting signals, pRB becomes hyperphosphorylated and inactive with respect to growth suppression. This form of pRB is easily distinguishable on SDS-gels by its slower migration. Hyperphosphorylated pRB loses the ability to complex to many of its binding partners mentioned above, including the viral oncoproteins (24) and E2F (20,25,15). Free E2F is then available to transcribe genes required for

S-phase entry, and will be discussed later in this review. Initial phosphorylation of pRB is carried out by a complex of cyclin D, whose message is induced immediately following ras signaling via the Raf-1, MEK 1 and 2, and ERK pathway (26,27), and cdk4. In mid-G1 phase, cyclinE/cdk2 is active to further phosphorylate pRB. Cyclin A/cdk2 adds additional phosphates to pRB at the G1/S boundary. These cdks which phosphorylate pRB are temporally regulated, both by assembly with cell cycle-oscillating subunits, the cyclins, and by activating or inhibitory phosphorylation events (reviewed in 28). Two-dimensional phosphopeptide mapping of GST-pRB fusion proteins phosphorylated *in vitro* by baculovirus- expressed cyclin/cdk complexes revealed that no complex fully phosphorylates pRB (29). Rather, each complex has distinct, albeit overlapping, specificities for a subset of consensus sites. Thus, pRB becomes hyperphosphorylated sequentially, attaining full phosphorylation only in G2/M.

Inhibitors of pRB phosphorylation are, as one would predict, the cdk inhibitors. These inhibitors fall into two categories. The first are those which broadly inhibit cyclin/cdk complexes, and yet are generally believed to be required in stoichiometric concentrations for complex assembly (30, 31). There may be some exceptions to this general belief, however (32). Designated the KIP/CIP protein family, they include p21 (33, 34), p27 (35, 36) and p57 (37, 38). The second category are those specific inhibitors of cdk4, the so-called INK4 family, which is comprised of p15^{INK4b} (39), p16^{INK4a} (40, 41), p18^{INK4c} and p19^{INK4d} (42). Of all the above INK4 proteins, only p16 is recognized as a bona fide tumor suppressor protein. This gene is commonly found deleted, mutated (41, 43) or transcriptionally silenced by hypermethylation (44, 45) in many cancers, although its link to cancer and tumor suppression was first identified in familial melanomas (46, 47).

3.3. Relevance to Cancer

A functional pathway in growth control includes cyclinD/cdk4, p16 and pRB, in which any mutation in these members affects the cell cycle. The major function of cyclinD/cdk4 is to phosphorylate pRB, rendering it inactive as a growth suppressor. The role of p16 is to negatively regulate this cyclin/cdk activity. As one can imagine, any mutation that upregulates cyclinD/cdk4 activity or abolishes p16 activity will promote entry into the cell cycle, provided that functional pRB is present. Indeed, this scenario has been found to be true. Loss of p16 (48) or amplification of cyclin D or cdk4 (49) are functionally equivalent in pRB-containing cells, inducing hyperphosphorylation of pRB and cell cycle progression.

The corollary to this, of course, is that in cells lacking functional pRB, p16 loss (48, 50, 51) or overexpression of cyclinD/cdk4 (52) is of little consequence. The human pRB transcript includes 27 separate exons (53, 54), and a G-C rich promoter containing E2F-1, ATF and Sp1 binding sequences (55, 56), where deletions or point mutations in the DNA sequences corresponding to these regions resulted in inactive alleles (57, 58). Other naturally occurring pRB

mutations are a splicing mutation in a bladder carcinoma cell line which gives rise to a truncated pRB protein (59), a deletion of exons 21-27 in an osteosarcoma cell line (60), resulting in a truncated protein unable to properly translocate to the nucleus, an amino acid substitution at position 706 from a cysteine to phenylalanine in a small cell lung cancer (61, 62), a missense mutation at serine position 567 (63), and others originating from exon 21 mutations which all give rise to nonfunctional pRB which abrogates LXCXE binding (64, 65).

4. MEDIATION OF GROWTH SUPPRESSION

4.1.The pRB/E2F interaction

The growth suppressive effects of pRB function are best characterized by its role in repression of E2F-mediated transcriptional activation. E2F is a family of transcription factors originally identified as that required for adenovirus E1A transactivation of viral promoter E2 (66). E2F activity is comprised of one member of the E2F family, of which 6 are now known (68-70), plus one of the three members of the DP family (reviewed in 70). Many of the genes whose products are required for DNA replication or cell cycle progression contain E2F binding sites in their promoters (71). These include DNA polymerase alpha (72, 73) , thymidine kinase (74), dihydrofolate reductase (75), cyclin E (76, 77), cyclin A (78, 79), cdk1 (80), E2F-1 (81, 82), E2F-2 (83), and the pRB-family member p107 (84). The multifaceted mechanisms for E2F regulation are as yet not fully understood. What has been learned so far is that in addition to functional variations through heterodimer formation with distinct DP partners, E2F members are also regulated by their relative abundance during each phase of the cell cycle as well as by phosphorylation (85), cell cycle-dependent subcellular localization (86), and complex formation with pRB or related pocket-proteins p107 and p130. While pRB binds E2F 1-3, and possibly 4, (21, 28, 87, 88), which are capable of inducing S-phase entry in quiescent fibroblasts (89, 90), p107 and p130 binds to E2F-4 and 5 only (28,70, 87, 88).

In either case, what has become clear is that E2F is prevented from transcribing target genes through interaction with hypophosphorylated pRB. It is this isoform which can bind not only E2F, but other proteins which utilize the LXCXE motif. Upon cyclin/cdk phosphorylation, E2F is free to transactivate. However, from the above discussion, phosphorylation of pRB by the different cyclin/cdks is functionally distinct. Creation of phosphorylation-site specific pRB mutants (91), *in vitro* phosphorylation of GST-pRB constructs (29), and the use of pRB phosphorylation-site specific antibodies (92) have permitted investigators to analyze the protein-binding functions of pRB. Phosphorylation at each site will dissociate a subset of pRB binding activities, leaving others unaffected. For example, while pRB phosphorylation at serine 780 carried out by cyclinD/cdk4 disrupts E2F binding (93), phosphorylation at threonine 821/826 does not (91). Phosphorylation at either threonine 826 catalyzed by cdk4 or threonine 821 by cdk2-containing complexes, alone or in combination, *will* abrogate binding to other

LXCXE proteins, such as SV40 large T-antigen (29). Interestingly, only phosphorylation at one of these sites, threonine 821, by cyclinA/cdk2 will resolve pre-existing LXCXE-pRB complexes (29).

Other cyclinD/cdk4 phosphorylation sites such as serine 795 seem to play a major role in the growth arresting function of pRB, as microinjection of the serine-to-alanine mutant at position 795 into pRB-minus SAOS-2 osteosarcoma cells prevented a G1 exit, presumably due to the inability of cyclinD/cdk4 to phosphorylate this site (94). CyclinD/cdk4 also uniquely phosphorylates serine 807 and serine 811, disrupting c-abl binding (91). Since serine 780, a target of cyclinD/cdk4 alone, is among the first sites to be targeted for phosphorylation (95) , and has been shown to be sufficient for disruption of E2F/pRB complexes, it is tempting to speculate that afferent signals converge on cyclin D, activating cdk4, and immediate release of the anti-proliferative pRB/E2F interaction ensues. This simple scenario does not appear to be the case, however, despite the extensive data that measured pRB-E2F binding following phosphorylation. Recent work now shows that complete phosphorylation of pRB by the sequential activities of cyclinD/cdk4 and cyclinE/cdk2 is required to relieve E2F inactivation and allow transcription from E2F-responsive promoters (96-98). How can the apparent discrepancy between these data be resolved ? It appears that different types of experiments yield data which may need to be interpreted by taking into account various protein levels. It has been suggested that cyclin/cdk levels which exceed physiologic concentrations may yield artifactual data (96).

The scenario is further complicated by the notion that pRB acts as a transcriptional repressor through interactions with proteins other than E2F. Most importantly, pRB binds the LXCXE-containing histone deacetylase, HDAC1, recruiting its chromatin-remodeling activity to repress transcription at some genes (99, 100). HDAC1 and E2F-1 compete in Sp1 binding, resulting in transcriptional repression or activation, respectively (101). In early G1, when E2F levels are low, HDAC1 will repress transcription, but initial phosphorylation of pRB by cyclinD/cdk4 releases HDAC1 (98). This may facilitate an intramolecular interaction between the C-terminal region of pRB and the A/B pocket domain. Such an interaction appears to be required for cyclinE/cdk2 access to serine 567, relieving E2F transcriptional repression (98). The subsequent rise in E2F levels, in an autoregulatory manner, promotes Sp1 transcription, as well as transactivation of other E2F responsive genes required for S-phase entry. This model presents an attractive way of viewing how the sequential activation of cycD/cdk4 and cycE/cdk2 allows the differential regulation of genes repressed by pRB interactions (98).

4.2. Other roles for pRB in cell growth

A less studied role of pRB is its function of repressing transcription by RNA polymerase I and polymerase III. As opposed to its polymerase II-repressing activity through E2F sequestration, the interactions that pRB makes with UBF (17) prevents the synthesis of large

ribosomal RNA by polymerase I. During interaction with TFIIIB (102, 103), pRB represses the transcription of 5S rRNA and tRNA, both known to exist at elevated levels in pRB-minus and tumor cells (104-108). Although pRB cannot repress basal levels of transcription, pRB may have indirect effects on cell growth by limiting the production of rRNA and tRNA available for protein synthesis via these interactions.

5. DEPHOSPHORYLATION OF PRB

5.1. Introduction

Now that we have re-capped what is generally known and accepted concerning pRB structure, modification, and function, we will now turn our attention to pRB dephosphorylation and cell cycle control. Reactivation of pRB's growth suppressing function is achieved at exit from mitosis by dephosphorylation events. pRB dephosphorylation has received considerably less attention due perhaps to the significance of pRB inactivation by phosphorylation during oncogenesis. A formal link between tumor development/cell cycle deregulation and the failure of pRB reactivation has yet to be established. However, it appears that the phosphatase implicated in pRB dephosphorylation is indispensable for mitotic exit. PP1 mutations in diverse organisms (109-111) exhibit defects in mitotic structures and display varying degrees of lethality. Other PP1-neutralizing experiments all revealed the absolute requirement for PP1 activity at mitotic exit (112-114). The mitotic cyclinB/cdk1 substrates are all potential targets of PP1, but it was only recently that pRB was identified as a crucial cell cycle regulatory substrate of PP1. To underscore the importance of the mitotic timing of pRB dephosphorylation and the importance of PP1 in this cell cycle stage-dependent process, we have been able to demonstrate that preventing cells from entering metaphase/anaphase does not affect pRB dephosphorylation normally scheduled for this time during mitosis (115). These data strengthen the argument that pRB dephosphorylation is crucial for mitotic exit even if additional downstream biochemical events are impeded, and further highlights the need to characterize the molecular mechanism by which PP1 activity towards pRB is regulated.

Unlike a pathway, a cycle by definition returns to the starting position. For G1 to ensue, all the properties of G1, including the presence of functionally active pRB, need to be reestablished. Accomplishing this could entail either the cells' removal of the inactivating phosphates on pRB, or degrading all of the inactive pRB present and synthesis of new molecules. pRB is known to have a long half-life of at least 12 hours (116, 117), and rapid destruction of all the pRB at mitotic exit is not observed. Pulse-chase radiolabeling experiments have shown that pRB molecules from the preceding cycle are carried over into the next G1 phase (118, 119). Thus, dephosphorylation is the method by which a cell reactivates pRB, a process favored energetically over synthesis of new molecules.

5.2. The pRB phosphatase

Beginning in anaphase, pRB is dephosphorylated by the enzymatic activity of PP1 (120). Belonging to the

larger serine/threonine phosphatase family, PP1 was first discovered as an enzyme which dephosphorylates the beta-subunit of phosphorylase kinase (PK). The type 2 serine/threonine phosphatases are specific for the a subunit of PK, and are further distinguished by their requirement for divalent metal cations for their enzymatic activity (121, 122). The identification of PP1 as the pRB phosphatase was accomplished through differential inhibition of other cellular serine/threonine phosphatases by okadaic acid (120). This compound selectively inhibits the other major cellular phosphatase, PP2A, at lower concentrations than those required to inhibit PP1 (123-125).

Serine/threonine phosphatases are rarely found as free catalytic subunits within the cell. Rather, they are associated with various regulatory subunits which function to direct substrate specificity, subcellular localization, or catalytic activity. PP1 is found associated in multimeric structures with a variety of interacting subunits. Through various methods of purification and identification including affinity chromatography, gel filtration, ion-exchange chromatography, and database searches, many of these subunits are now known. These include two heat stable cytoplasmic inhibitors, I-1 and I-2 (126), a nuclear inhibitor of PP1, NIPP-1, (127), a phosphatase 1 nuclear targeting subunit, PNUTS (128), glycogen targeting G subunits in liver (129) and muscle (130), myosin binding M-subunits (131, 132), and a p53 binding subunit designated p53BP2 (133). While each of these higher order structures serves to regulate PP1 activity, PP1 regulation is made more complex by its cell cycle-specific inhibitory phosphorylation (134, 135), and the fact that 4 different isoforms of this protein, designated PP1-alpha, PP1-delta, PP1-gamma1, and PP1-gamma2, exist in mammalian cells (see below).

A report by Berndt et al. (136) suggests that PP1-alpha has the potential to arrest cell growth in G1 unless it is inactivated by phosphorylation on threonine at position 320. This phosphorylation is presumably carried out by cdks (134). To summarize their results, recombinant protein was electroporated into G1-synchronized pRB-positive and pRB-minus cells. It was found that PP1-alpha levels became elevated 6- to 16-fold and remained stable for at least 48 hr. In pRB-positive cells, mutant PP1-alpha T320A (a threonine-to-alanine change at position 320, thus eliminating a cdk phosphorylation site), but not wild-type PP1-alpha, caused cell cycle arrest in late G1 and was associated with hypophosphorylation of pRB. In pRB-minus cells, neither phosphatase elicited a change in cell cycle progression, suggesting that PP1-alpha requires functional pRB to induce growth arrest. These data are also in agreement with our earlier suggestion that PP1 can regulate the cell cycle indirectly by modulating the activity of the growth suppressive function of pRB (137). At about this same time, a paper was published by Kwon et al. (138), reporting on the mitotic phosphorylation of PP1-alpha at threonine 320. This raises the possibility that PP1-alpha activity may be regulated at two points during the cell cycle by phosphorylation on threonine 320; from late G1 through to early S, and from G2 through to early M-phase. Taken together with our reports on the cell cycle stage-specific

dephosphorylation of pRB (118, 119), and the published reports on the phosphorylation state of pRB during the cell cycle (139-141), the following scenario may be proposed. Phosphorylation of PP1 and pRB mediated by cdk's, beginning during mid G1, functionally inactivates the ability of PP1 to dephosphorylate pRB. This ensures that the growth suppressive property of pRB is inactivated by keeping this protein hyperphosphorylated. Having the block to growth progression lifted by these phosphorylations, the cell then progresses through S, G2, and early M. During these times, cdk activity continues to maintain pRB in a hyperphosphorylated form by direct phosphorylation as well as inhibition of PP1-mediated pRB dephosphorylation. During mid-M-phase, cdk-mediated phosphorylation of PP1 and pRB ceases, thus allowing PP1 to become active towards pRB. This phosphatase activity triggers the growth suppressive ability of pRB for the ensuing G1 by dephosphorylation. In fact, a new report by Liu et al. (142) supports just such a scenario.

As mentioned above, there are four different isoforms of PP1 in mammalian cells that differ only in their carboxy-terminal sequences. Three isoforms, alpha, gamma1, and delta, are encoded by three separate genes (143-145) while the gamma2 isoform is a splice variant of the gamma1 gene (146). The existence of these various isoforms allows an added level of substrate specificity, though many of the PP1-catalyzed reactions are known to be carried out by all three isoforms with varying degree of efficiency. In identifying and characterizing the PP1 holoenzyme which dephosphorylates pRB during mitotic exit, our laboratory has discovered that although each isoform complex may act on pRB, it was the delta isoform which appears to do so most efficiently (137). We also found three PP1-associated proteins, having apparent molecular weights of 111-, 125- and 180-kDa. While the 125- and 180-kDa proteins have yet to be identified, the111-kDa protein we believe to be the recently identified PNUTS subunit (128). PNUTS appeared earlier to inhibit PP1 activity towards a substrate classically used to measure PP1 phosphatase activity, phosphorylase a. Recent in vitro experiments performed by us using GST-PNUTS fusion protein revealed a similar inhibitory activity towards PP1-mediated pRB dephosphorylation. The significance of finding this apparent inhibitory protein in fractions of mitotic mammalian cell lysate containing active PP1-mediated pRB dephosphorylating ability remains to be determined.

5.3. Sequential pRB dephosphorylation
As discussed above, pRB is phosphorylated at multiple sites and growing evidence suggests that different patterns of phosphorylation on pRB impart various biological and biochemical properties to pRB. Chiefly, phosphorylation modulates the ability of pRB to bind different proteins. The pattern of sequential phosphorylation of pRB is only beginning to be elucidated, and cell-type specific differences are sure to exist. As a prerequisite to addressing the possibility that pRB is sequentially and temporally dephosphorylated, we have undertaken the task of analyzing the site-specific phosphorylation of pRB during different stages of the cell
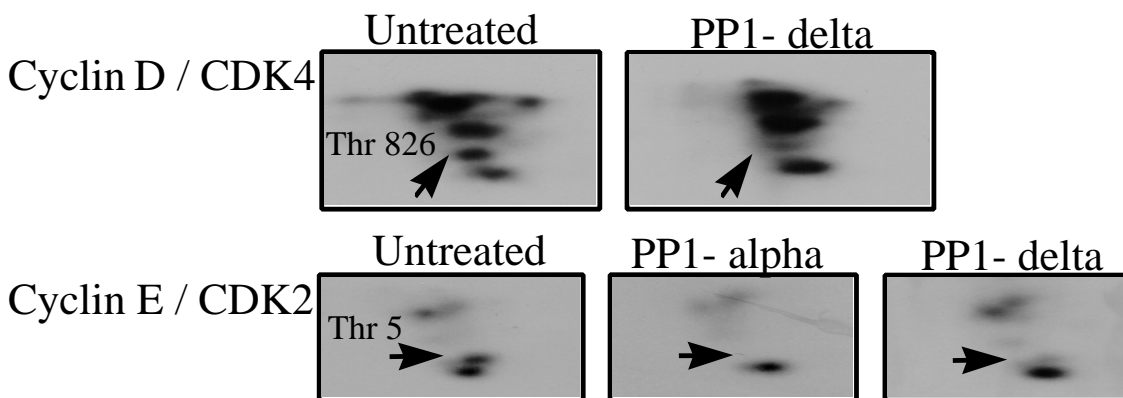
Figure 2. Phosphopeptide map comparison of cyclin D/CDK4 phosphorylated GST-pRB (top 2 panels), untreated or treated with PP1-delta, reveals a preference for Thr 826 dephosphorylation. Phosphopeptide map comparison of cyclin E/CDK2 phosphorylated GST-pRB (bottom 3 panels), untreated or treated with PP1-alpha or PP1-delta, reveals a preference for Thr 5 dephosphorylation.

cycle. Using CV1-P cells, we have found that while levels of phosphorylation at some sites rise as cells approach the G2/M boundary, other sites show a biphasic nature to their phosphorylation pattern. These studies further support the notion that differential

5.4. Phosphorylation of pRB may result in differential regulation of downstream effector pathways

We reasoned that if there is site-specific cyclin/cdk specificity for placing phosphates onto pRB, there may very well be site-specific PP1 specificity for removing phosphates from pRB. To address this possibility, we have recently completed a timecourse of pRB dephosphorylation by western blotting in which mitotic-block-then-released cells were lysed, the soluble proteins separated by SDS-PAGE, transferred to nitrocellulose, and probed with recently developed antibodies generated towards pRB site-specific phosphorylated residues. If cells were indeed undergoing sequential pRB dephosphorylation, then the pattern of signal disappearance on these western blots would differ. Indeed, it appears that this is the case. Our preliminary results suggest that threonine 252, threonine 356, and threonine 821 dephosphorylation begins almost immediately upon M-phase entrance, and appears to be complete by the subsequent G1 phase. Threonine 826 appears to be completely dephosphorylated within the first 10 minutes of mitosis, while serine 249 and serine 608 dephosphorylation does not begin until 30 minutes into M-phase. In contrast, serine 780, serine 795, and serine 807 residues may become increasingly phosphorylated during mitosis. While a more extensive report on these observations will be published in the near future (E. Rubin, S. Mittnacht, and J.W. Ludlow, *manuscript in preparation*), these results further support our notion of temporal regulation for site-specific pRB dephosphorylation while strengthening our previously published data in which we concluded that PP1 does not remove all of the phosphates from pRB (137).

Since we have found differences among the PP1 isoforms that carry out the enzymatic removal of these pRB phosphates, namely, that the delta isoform had greater pRB-directed activity, we wondered whether each isoform exhibited specificity for a subset of phosphorylation sites, much like the cyclin/cdk complexes. To address this, we currently have available in our laboratory the same bacterial expression system for full-length GST-pRB and the baculovirus-expression system for cyclin D/cdk4, cyclin E/cdk2, and cyclin A/cdk2 which Zarkowska and Mittnacht (29) used to map the cyclin/cdk -specific phosphorylation sites of pRB. In preliminary studies carried out with Dr. Sibylle Mittnacht (see Figure 2 below), we have demonstrated site-specific threonine 826 and threonine 5 dephosphorylation by the delta- and alpha-isoforms of PP1. These experiments were performed by mixing *in vitro* phosphorylated GST-pRB with bacterially-expressed GST- PP1-alpha and GST-PP1-delta affinity purified by glutathione-Sepharose chromatography.

This result is extremely exciting for the following reason. As described above, one of the clustered pairs of pRB phosphorylation sites, threonine 821 and threonine 826, has previously been implicated in regulating the association of pRB with proteins containing an LXCXE motif. It has since been shown that phosphorylation on these sites disables pRB binding to LXCXE-containing proteins (91), thereby downregulating the growth suppressive ability of pRB. It is thus logical to assume that dephosphorylation of one or more of these sites during mitosis will restore the growth suppressive ability of pRB observed during G1 by permitting pRB to complex with cellular proteins. In support of this notion, as shown in Figure 2 and the timecourse of dephosphorylation discussed above, threonine 826 appears to be a specific site dephosphorylated by PP1, and both threonine 821 and threonine 826 appear to be dephosphorylated early during mitosis. As a reminder, it is also during this time that PP1 and pRB can be found complexed with each other (19,

147), and PP1-directed activity towards pRB is the greatest (19, 147).

Why this difference in apparent PP1 activity towards the various pRB phosphorylation sites? Can it be that the pRB phosphatase(s) are less active at some sites than others due to differential regulation induced by their multimeric assembly with different subunits that direct or restrict their substrate specificity ? This may be the case given the known functions of some PP1-associated proteins (148, 149). However, there has yet to be reported a PP1-associated protein which targets the enzyme to pRB. Perhaps the 125- and/or the 180-kDa PP1-associated proteins which we have observed may function in this capacity (137). An alternative explanation may be that these difference lie in the substrate specificity of the catalytic subunit of each PP1 isoform. *In vitro* experiments, such as shown in Figure 2 above, may support this idea since the bacterially-expressed PP1-alpha and PP1-delta are presumed devoid of associated proteins found in mammalian cells. There is also the possibility that kinetics play a role, and that the phosphatase isoforms will each act on every site, but that the reaction rates at each site differ among the isoforms. Another scenario that one can envision is based on the growing evidence of the need for cdk4-specific site phosphorylation of pRB prior to other cyclin/cdk phosphorylations (96). The rationale here is that access to subsequent sites is dependent upon previous phosphorylations, resulting in crucial conformational changes required for enzyme access. Relating this notion to PP1 catalytic activity towards pRB, perhaps dephosphorylation at the immediate-early sites at M-phase exit is required before the phosphatase can gain access to other sites, due to the same issue of required conformational change.

We are currently conducting experiments addressing some of these possibilities. Using a variety of techniques, it seems thus far that the PP1 isoforms exhibit specificity towards some pRB sites. Indeed, these differences may not be not restrictively inherent in the catalytic subunits since the specificity of recombinant fusion constructs of PP1-alpha and PP1-delta do not always mirror that of their respective endogenous isoforms immunoprecipitated from cells (E. Rubin and J.W. Ludlow, unpublished observations).

5.5. Dephosphorylation during S- and G2-phase
Contrary to the widely accepted observations presented above, there have been a few studies which demonstrate hypophosphorylation of pRB during S and G2. We have found that hypoxic stress can cause pRB to change from overtly phosphorylated to hypophosphorylated in S-phase CV-1P cells (150). Flow cytometric DNA histogram analysis and [^{3}H]-thymidine incorporation assays demonstrated that hypoxia-inhibited cell cycle progression and cell division. These data suggest that hypoxic stress blocks the progression of these cells through the phases of the cell cycle and further supports the notion that maintaining pRB in a hyperphosphorylated state is crucial for S-phase progression. We have recently completed a study to determine the mechanism by which hypoxia

induces pRB hypophosphorylation (151). Similar to cell cycle arrest induced by serum starvation, we show here that hypoxia-induced arrest of CV-1P cells is accompanied by a decrease in pRB-directed cdk4 and cdk2 activities, lower cyclin D and E protein levels, and by an increase in p27 protein abundance. In contrast to cell cycle arrest induced by serum starvation, hypoxia increases PP1-mediated pRB dephosphorylation. These data reveal that synergy between decreased pRB-directed cyclin/cdk activity and increased pRB-directed phosphatase activity contribute towards inducing and maintaining pRB in its hypophosphorylated, growth suppressive state during hypoxia. A similar mechanism appears to be involved in hypoxia-induced hypophosphorylation of pRB in human ovarian cancer cells (152). Clearly, this work helps to further illustrate the "cooperation" between enzymes whose functions are in direct opposition with respect to phosphorylation of proteins. As presented, there is a sense of these enzymes working together to ensure that, when proliferation conditions are not optimal, a key regulator of cell cycle progression (pRB) is activated to stop proliferation by maintaining it in a hypophosphorylated form. Additionally, this hypoxia system may be more extensively used to study the synergy between kinase and PP1 phosphatase activity with regard to other biochemical aspects of cell cycle regulation.

Along this line of thought regarding synergy between kinases and phosphatases, Yan and Mumby (153) have just reported that PP1 *and* PP2A, or PP2A-like phosphatases, play distinct roles in regulating pRB function. While the role of PP1 still includes direct dephosphorylation of pRB, PP2A appears to affect pRB phosphorylation indirectly by modulating the ability of G1 cdks to place phosphates onto this protein.

Studies by others have also demonstrated hypophosphorylation of pRB during S- and G2-phase (154, 155). This can be observed when treating human leukemia cells with growth arresting chemicals or when such cells are induced to undergo differentiation by retinoic acid treatment. In the general context of these experiments, such untimely dephosphorylation of pRB still does not contradict the fundamental role of the hypophosphorylated form of this protein, which is to function as a negative growth regulator.

5.6. Intracellular activators of pRB dephosphorylation

While the aforementioned hypoxic chemical-treatment studies did not address the intracellular signals by which pRB dephosphorylation may be modulated, progress in this area has been made by studying the effect of sphingosine of pRB dephosphorylation (156). Here, it was shown that soon after treatment of hematopoietic cells with D-erythro-sphingosine, pRB went from overtly phosphorylated to hypophosphorylated. This apparent dephosphorylation preceded cell growth inhibition resulting in a G0/G1 arrest. Other lipids, amphiphiles, long chain amino bases, and structural sphingosine analogs did not have this effect. Activation of second messenger systems involving protein kinase C, cAMP-dependent kinases, and calcium ionophores

also failed to induce pRB hypophosphorylation. Sphingosine-induced hypophosphorylation of pRB could be alleviated by inhibiting serine/threonine protein phosphatases, thus establishing for the first time an intracellular activator of pRB dephosphorylation. Subsequent studies have shown that pRB is a downstream target for a ceramide-dependent pathway of growth arrest (157). The addition of C6-ceramide, a lipid mediator, to MOLT-4 cell cultures results in a concentration- and time-dependent dephosphorylation of pRB. Linking pRB to growth arrest was demonstrated by poor growth suppression in ceramide-treated cells lacking functional pRB. In addition, protection from ceramide-induced growth suppression was demonstrated in cells which separately express SV40 large T-antigen and adenovirus E1A protein, both of which bind and thereby functionally inactivate the growth suppressive property of pRB (7, 11).

Most recently, the observed changes in ceramide levels during cell cycle progression suggest that ceramide synthesized *de novo* may function as an endogenous modulator of pRB protein and cell cycle progression (158). This study revealed that when cells are released from a G2/M nocodazole block, just prior to pRB dephosphorylation normally occurring during this time, endogenous ceramide levels transiently increase. Fumonisin B1, an inhibitor of ceramide synthase, was found to inhibit this transient increase as well as pRB dephosphorylation. Additional support for ceramide-induced PP1-mediated pRB dephosphorylation comes from studies using phosphatidic acid (159). These data support the notion that phosphatidic acid can act as a specific regulator of PP1 and counteract the effects of ceramide that are mediated by PP1, such as pRB dephosphorylation.

6. THE PP1-pRB INTERACTION

6.1. Complex formation

Although PP1 is the enzyme that acts upon hyperphosphorylated pRB, these two proteins, as enzyme and substrate, have been difficult to detect as a complex. In fact, both co-immunoprecipitation and yeast two hybrid system assays demonstrated the interaction only with the hypophosphorylated form of pRB (19, 147), and the level of this association fluctuated during the cell cycle. Indeed, pRB can be found maximally bound to PP1 at mitotic exit and during early G1 (19, 147). PP1 affinity chromatography carried out in our laboratory has demonstrated for the first time complex formation between PP1 and the hyperphosphorylated pRB substrate (92), although it still holds true that more hypophosphorylated pRB can be found complexed with PP1 than this hyperphosphorylated form. Interestingly, we found that PP1 association with overtly phosphorylated pRB does not appear to take place when certain specific sites of pRB are modified in this manner. Using pRB phosphorylation-site specific antibodies, we can showed that phospho-serine 608-, -serine 612-, -serine 807-, and -serine 780-modified pRB can associate with GST-PP1-alpha, whereas pRB phosphorylation at threonine 373, serine 249, serine 811, threonine 821, and threonine 826 are not detected in the complex (92). Since the pRB used in these experiments

was derived from mitotic cell lysates, phosphorylation of the sites which seem to prevent PP1 binding may be necessary to maintain the integrity of an ordered G2/M progression and exit. Thus, by inhibiting binding to PP1, untimely dephosphorylation of pRB may be prevented.

While not rigorously addressed here, the simplest explanation for this observation is that phosphorylation at one or more of these sites impedes pRB binding to PP1. If indeed true that phosphorylation of these sites prevents pRB binding to PP1, what is the mechanism behind this observation? Perhaps an as yet undefined PP1-associated regulatory protein facilitates binding between PP1 and phosphorylated pRB. Such regulatory proteins have been described for this enzyme, and appear to function in targeting the substrate for catalysis (137, 128). In preliminary studies carried out in our laboratory, differences have been found to exist in the ability of bacterial recombinant PP1 and mammalian cell-isolated PP1 to dephosphorylate certain pRB sites. This may suggest that PP1-associated proteins, which are assumed not to be present in recombinant PP1 isolated from bacteria, are involved in this apparent selectivity. Alternatively, the apparent selectivity for different forms of phosphorylated pRB binding may lie in different affinities for the various PP1 isoforms. We are currently investigating these possibilities.

The formation of PP1-pRB complexes *in vitro* does not depend on the enzyme being catalytically active. PP1-alpha inhibited by okadaic acid or microcystin, two compounds which are routinely used to inhibit *in vivo* and *in vitro* PP1 activity, is still quite capable of binding both hypo- and hyper-phosphorylated forms of pRB. This capability is retained regardless of whether the source of enzyme is from bacterial or mammalian cells (92). In fact, the forms of pRB that bind appear to be identical to those found binding to catalytically active phosphatase. Recently, Liu *et al*. (142) have found another form of inactive PP1, phosphorylated on a threonine residues at position 320, which can coprecipitate with pRB during different periods of the cell cycle. Taken together with the fact that hypophosphorylated pRB also binds quite effectively to PP1, these data may suggest that non-catalytic interactions between PP1 and pRB are also possible. Similar to other pRB-binding proteins whose functions are altered upon their association with pRB, PP1 activity may be modulated by pRB in the complex, much like a PP1-associated regulatory subunit. To address this possibility, we have initiated experiments designed to test the catalytic activity of PP1 when bound to hypophosphorylated pRB. Although incomplete, the data suggest that binding of a small portion of the pRB protein to PP1 is sufficient to downregulate catalysis towards phosphorylase *a*, a routinely used, standard PP1 substrate, as well as hyperphosphorylated pRB (S. Tamrakar and J.W. Ludlow, unpublished observations).

6.2. The role of LXSXE in the PP1-pRB interaction

LXCXE is a conserved pRB binding motif present in several cellular and viral oncoproteins, as discussed above. The importance of this sequence in pRB

binding interactions is demonstrated by the fact that LXCXE peptides are able to compete off proteins harboring this motif, such as SV40 large T-antigen (160), Elf-1 (18), or UBF (17), from pRB. Proteins bearing this amino acid motif bind to the A/B pocket domain of pRB when threonine 821 or threonine 826 is unphosphorylated (91). The mutant form of the peptide, LXCXK , was unable to compete with the binding protein in the same experiment (160). Replacement of cysteine with serine to generate LXSXE was shown to have little if any effect on pRB binding to the adenovirus E1A protein (161). As may be expected, PP1 also contains two such conserved pRB binding motifs; one towards the amino terminus and the other more towards the middle of the molecule. While it is currently unknown if these motifs are involved in pRB binding to PP1, peptide competition assays as described above should help to address this issue. It would not be surprising if one or both of these LXSXE motifs are important for this interaction. Indeed, pRB binding to PP1 may be prevented by phosphorylation of threonine 821 or threonine 826, as suggested by the absence of these residues being found phosphorylated in the pRB binding to PP1 (92). This would be consistent with the failure of other known pRB binding proteins to form a complex when these residues are modified by phosphorylation (91).

7. DEPHOSPHORYLATION OF OTHER POCKET PROTEIN FAMILY MEMBERS

Although not the focus of this review, it is worth briefly discussing what is known about phosphorylation and the function of the other members of the pocket protein family, p107 and pRB2/p130, in cell cycle regulation. Like pRB, p107 and p130 both act as transcriptional repressors, which is best described by their association with and repression of E2F family of proteins. As mentioned in section 4.1 above, each of the three members binds to its own set of E2Fs and imparts growth suppressive effects. p107 and p130 phosphorylation may not be as crucial in regulating the activities of these proteins as it is for pRB. Indeed, cell cycle-dependent modulation of p107 and p130 protein abundance may be a more significant factor (87, 162-166). Nonetheless, their activity may also modulated by phosphorylation on multiple sites by the shared or unique sets of cyclin cdk complexes (87, 163, 164, 167-169). While pRB phosphorylating cyclinD/cdk4 complex can also phosphorylate p107 and p130, interaction of these two latter two proteins with cyclin A/cdk2 and cyclin E/cdk2 may be more than a kinase-substrate interaction, perhaps serving as regulators of kinase activity or as bridges to other proteins (170). With regard to the significance of p107 and p130 phosphorylation on their function, our current understanding is not as extensive as it is for pRB and would clearly benefit from additional analyses.

Voorhoeve *et al.* (171) were the first to report the possible involvement of PP2A in the dephosphorylation of p107. They observed a correlation between UV dosages required for G1 arrest of NIH3T3 cells and p107 dephosphorylation. This UV-mediated dephosphorylation could be alleviated by treating the cells with the serine/threonine phosphatase inhibitors calyculin A and okadaic acid. In addition, they found that alteration of the PP2A holoenzyme complex by overexpression of a PP2A-specific associated protein interfered with this dephosphorylation, and that p107 could be dephosphorylated with PP2A *in vitro*. This same group then went on to report on the functional interaction between a novel PP2A regulatory subunit, designated PR59, and the p107 protein (172). Coprecipitation studies revealed that PR59 complexes with PP2A, and also associates *in vivo* with p107, but not pRB. When these authors elevated expression of PR59, they observed dephosphorylation of p107 (but not pRB) and accumulation of cells in G1. These data may suggest that that PR59 targets PP2A to p107, facilitating p107 dephosphorylation and subsequent activation of its growth suppressive function. Taken together, this is an important and significant beginning towards making a reliable comparison between p107 and pRB dephosphorylation in regulating cell cycle progression.

8. PERSPECTIVES

Considered together, pRB is a cell cycle regulatory protein whose function is modulated by cell cycle dependent serine/threonine phosphorylation. pRB enters mitosis hyperphosphorylated yet hypophosphorylated pRB is maintained through mitosis and the subsequent G0/G1 phase. The activity of PP1, a serine/threonine protein phosphatase, appears to be involved in M-phase progression, a time during which PP1 and pRB can be found complexed together. It may be speculated that PP1 association with pRB prevents the untimely phosphorylation of pRB, thus indirectly contributing towards the temporal progression of the cell cycle. One might therefore predict that PP1 mutations which prevent binding to pRB may promote pRB phosphorylation and ultimately contribute towards cellular transformation. Along this line of thought, it is interesting to note that mutations in chromosome 11q13, the location of the human PP1-alpha gene, have been linked with the development of certain cancers (173). How PP1 is displaced from pRB and its relationship to pRB phosphorylation during late G1- and S-phase, is not clear at this time.

Of key importance to further defining the functional relationship between PP1 and pRB with regard to cell proliferation is understanding the regulation of PP1 activity. One of our most recent endeavors with respect to this area has resulted in the development of a PP1-inducible expression system for eukaryotic cells (Reeder JE, Sowden MP, Messing EM, Villa-Moruzzi E, and Ludlow JW., *unpublished data*). The motive behind development of this PP1-inducible expression system is to facilitate investigation into the *in vivo* effect of PP1 activity on pRB and growth regulation. Our prediction was that inducibly expressing PP1-alpha would result in an increase in PP1-alpha protein content and enzymatic activity within the cell, leading to pRB dephosphorylation and changes in cell cycle progression. This prediction was based on multiple examples in which induction of pRB kinases indeed results

in increased protein content, activity, pRB phosphorylation changes, and changes in cell cycle progression. As such, a stable human cell line has been derived in which expression of PP1-alpha was regulated by doxycycline using the tetracycline induction system. A fusion protein of the catalytic subunit of PP1-alpha with a 6-histidinin and hemagglutinin epitope (6His-HA-PP1-alpha) appears within 4 hours following addition of doxycycline to the culture medium. This induced protein localized to the nucleus, and associated *in vitro* and *in vivo* with PNUTS, a PP1-nuclear targeting subunit. Like endogenous PP1-alpha, immunoprecipitated 6His-HA- PP-alpha was active toward phosphorylase a and pRB. For intact cells, 6His-HA-PP1-alpha induction did not appear to alter pRB phosphorylation. While the PP1-specific activity from lysates prepared from induced cells was comparable to that of uninduced controls, a decrease in endogenous PP1-alpha levels following 6His-HA-PP1-alpha induction was observed. This suggests the existence of a previously undescribed autoregulatory mechanism for the control of PP1-alpha expression and activity.

While not totally unexpected, these data provide a mixed contribution towards addressing the *in vivo* effect of PP1 activity on pRB and cell cycle regulation. Just as is the case for PP2A (174), it seems clear that efficient overall increases in PP1 protein abundance may not be achieved due to a putative negative feedback or autoregulatory mechanism. However, induced expression of exogenous PP1 which behaves identically to endogenous PP1 while remaining physically distinguishable can expedite the use of mutants to address such functional questions. For example, the use of a constitutively active mutant of PP1 has already been shown to result in pRB-dependent G1 arrest in human cancer cells (136). Placing this mutant into the inducible expression system described here has the potential to down-regulate expression of wild-type endogenous PP1. Doing so in a timed, controlled manner will surely facilitate *in vivo* studies addressing PP1 function and activation of pRB by dephosphorylation during discrete phases of the cell cycle.

In summary, with such an apparent change during mitosis in the pRB phosphorylation state, together with the critical timing of PP1 activity for M-phase progression, defining the functional relationship between these two cellular proteins affords a unique opportunity for understanding the role of PP1 and pRB in cell cycle regulation. Of critical importance to this understanding is knowledge concerning the regulation of PP1 activity, specifically towards pRB. A multidisciplinary strategy employing biochemical, immunological, and molecular biology approaches affords us the best opportunity to address this regulation. In addition, isolation and identification of additional PP1-associated proteins may help define the mechanism responsible for targeting pRB for dephosphorylation by PP1 during a very discreet window of mitosis. With the current attention being given to tumor suppressor proteins and their modifying enzymes, answers to these questions may not be far off.

9. ACKNOWLEDGEMENTS

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