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PROTEIN PHOSPHATASE TYPE 1, THE PRODUCT OF THE RETINOBLASTOMA SUSCEPTIBILITY GENE, AND CELL CYCLE CONTROL

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1. ABSTRACT

Cell cycle regulation - three words which conjure in the minds of those conducting research in this area a myriad of proteins and biochemical pathways. In this examination, an overview of the mammalian cell cycle is presented with emphasis on the function of the negative growth regulatory protein, the product of the retinoblastoma susceptibility gene, pRb. Since the activity of this protein itself is regulated by phosphorylation on serine and threonine residues, more elaborate discussions on the enzymes involved in placing the phosphates on, and taking them off, are provided. The focus here is on the activity of the members of the type 1 class of serine/threonine phosphatases. More specifically, the role of PP1 in regulating cell cycle progression by dephosphorylating pRb during mitosis, thereby activating the growth suppressing function of pRb, is presented. Suggested avenues for further investigation regarding the functional significance, and ultimately the effect on cell cycle progression, of the complex between pRb and the type 1 phosphatases are also discussed.

2. INTRODUCTION

It is logical to assume that where protein kinases dare to tread, protein phosphatases will surely follow. While a single protein kinase is capable of phosphorylating serine, threonine and tyrosine residues, protein phosphatases, with few exceptions, are specific for either phosphoserine / phosphothreonine or phosphotyrosine. At the same time, since each kinase recognizes specific and distinct amino acid motifs, a protein acted upon by more than one kinase may be dephosphorylated by only one phosphatase. This may help to explain the discrepancy between the great number of kinases versus the relatively few number of phosphatases currently described. Further skewing this balance is the fact that phosphatases are found in higher order structures, complexed to noncatalytic subunits that modulate enzymatic activity and alter substrate specificity. Focus on the role of phosphatases in all areas of cellular processes from metabolism to RNA splicing to cell division necessitates periodic reviewing of this ever-expanding field. The intent of this review is to describe our current knowledge on the role of one member of the serine/threonine protein phosphatase family, protein phosphatase type 1 (PP1), with respect to indirect control of cell cycle progression by modulation of the activity of a crucial cell cycle-regulatory protein, pRb.

3. REGULATION OF THE MAMMALIAN CELL CYCLE

Before we discuss the details of PP1 activity towards pRb and this effect on cell cycle progression, an overview of mammalian cell cycle regulation is in order. In response to mitogenic stimuli, quiescent G_0 cells must sequentially activate the necessary protein complexes for entry into, and passage through, the four phases of the cell cycle: G_1, S, G_2, and M. In mammalian cells, a class of proteins known as cyclin dependent kinases (cdks) in concert with their cell cycle-oscillating partners, the cyclins, are the positive regulators of the G_1/S and G_2/M cell cycle transitions by sequentially phosphorylating

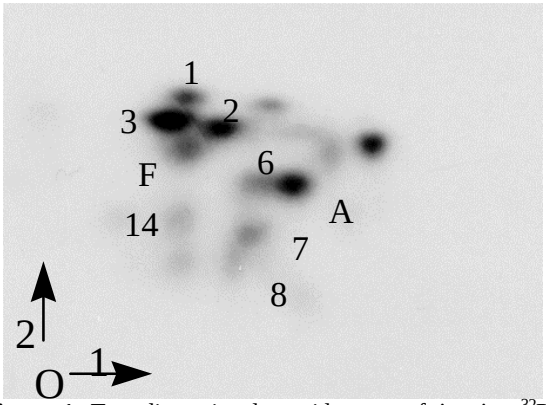


Figure 1. Two-dimensional peptide map of *in vivo* ^{32}P-labeled pRb. Immunopurified radiolabeled pRb was gel isolated and enzyme digested. The resulting peptides were then separated by electrophoresis in the first dimension and ascending chromatography in the second dimension, as described by Zarkowska and Mittnacht (39). Visualization of the separated peptides was achieved by autoradiography. Numbering scheme is as suggested by Zarkowska and Mittnacht (39).

substrate proteins activating entry into and progression through the cell cycle. Peaking in mid-G_1, the D-type cyclins, cyclin D_1, D_2, and/or D_3, associate with their catalytic partner cdk4 or cdk6. At the G_1/S transition, cyclin E forms an active kinase complex with cdk2 and both G_1 cyclin complexes are required for entry into S phase, as microinjection of anti-cyclin E or anti-cyclin D antibodies into cells during G_1 abrogates progression into S phase (1-3). Cyclin A expression peaks in S phase and complexes with cdk2, to operate in S and G_2, or with cdk1 (also called p34^{cdc2}) during M phase. Cyclin B is synthesized later in S phase and complexes with cdk1 to effect the G_2/M transition (4,5). Exit from mitosis requires the selective proteolysis of the M-phase cyclins A and B at anaphase by the multienzyme reactions governing the ubiquitin system together with the 26S proteasome (6-8).

In addition to the cell cycle-dependent expression of cyclins, cdk activity is regulated by phosphorylation and dephosphorylation events as well as interactions with specific inhibitors. For activation, cdk1, cdk2 and cdk4 are phosphorylated on a specific threonine residue by CAK (cdk-activating kinase), a complex of cdk7 (MO15) and its partner, cyclin H, itself activated by phosphorylation (9-11). A cdc25-related phosphatase then catalyzes the removal of inhibitory phosphates added to the cdks by the wee1/mik1 kinase complex (12). Cdc 25 is also regulated by phosphorylation in a positive feedback loop at G_1/S by cyclin E/cdk2 (13) and by cyclin B/cdk1 at G_2/M (14).

The specific inhibitors of mammalian cdks (cdkIs) fall into two categories: those that broadly inhibit the cyclin/cdk complexes and those that are restricted to binding cdks. The first class of cdkIs includes the p21 (p21^{cip1}/p21^{CDKN1}/CIP1/WAF1/SDI1/CAP20), the related p27 (p27^{kip1}/p27^{ICK1}) and p57 (p57^{kip2}) proteins (15,16). Although these proteins are potent inhibitors of the G_1 cyclin/cdk complexes when bound to them, it turns out that stoichiometrically low levels of p21 not only promotes but is required for complex assembly and enzymatic activity (15,17,18). The second family of cdk inhibitors is derived from the INK4 loci which give rise to p15 (INK4b), p16 (INK4a), p18 (INK4c) and p19 (INK4d) that specifically inhibit G_1 cdks, primarily cdk4 (15).

4. THE ROLE OF pRb IN CELL CYCLE CONTROL - AN OVERVIEW

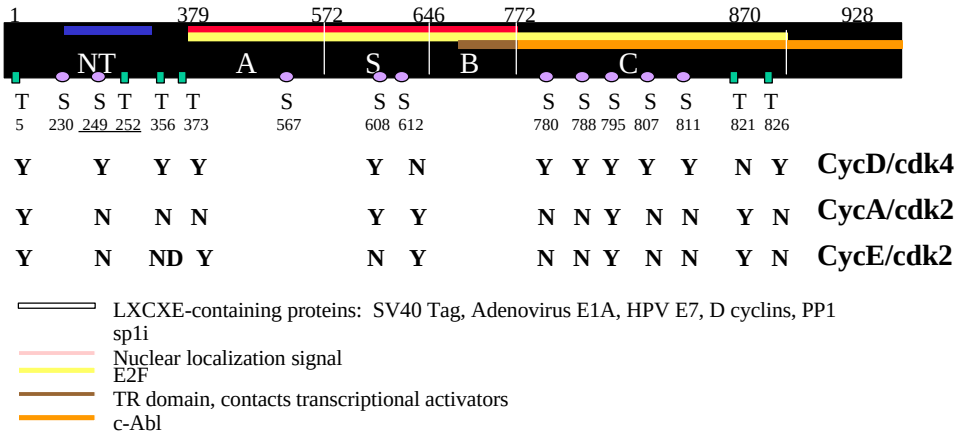
As illustrated above, reversible phosphorylation of key protein regulators plays a major role in the highly choreographed events that control cell cycle progression. The product of the *RB1* gene, pRb, a 110 kD nuclear phosphoprotein, is one such regulator. pRb undergoes cell cycle-dependent phosphorylation on serine and threonine residues. Two-dimensional peptide mapping of pRb reveals multiple phosphorylated sites (see figure 1). The hypophosphorylated form of pRb abounds in G_0 and G_1, at which time pRb exerts its growth suppressive effects by binding and sequestering the transcription factor E2F, repressing the transcription of genes required for DNA synthesis and cell cycle progression. At some time late in G_1, the growth-suppressive property of pRb becomes inactivated by phosphorylation. The regulation of pRb by phosphorylation is mediated by many of the cyclin-dependent kinases which play other key roles in cell cycle control. Hyperphosphorylated pRb releases E2F and cell cycle progression ensues until late in mitosis when pRb is dephosphorylated by a holoenzyme complex containing PP1 thereby restoring the growth suppressive function of pRb.

5. THE STRUCTURE AND FUNCTION OF pRb

Of the critical substrates for the G_1 cdks, pRb has been hypothesized to be the main target of cyclin D/cdk4. Evidence for this includes the observations that microinjection of anti-cyclin D_1 antibodies into fibroblasts have no inhibitory effect on cell cycle progression in pRb$^-$ tumor lines and homozygous pRb$^{-/-}$ mouse embryo fibroblasts (1); and overexpression (19) or microinjection (20) of p16, which should cause cell cycle (S phase) arrest, fails to do so in these pRb-deficient fibroblasts. In addition, the minimal region of pRb required to bind cyclins D_2 and D_3 *in vitro* were those residues conferring pRb's growth suppressive activity (21).

pRb is the product of the retinoblastoma susceptibility gene, *RB1*. Its identification, cloning and characterization ten years ago was a result of the discovery that patients with the retinoblastoma disease state had deletions or mutations in both copies of chromosome 13q14. Disruption of *RB1* was later shown to be associated with other common tumors, sparking interest in pRb as a tumor suppressor protein. As such, the transforming ability of the viral oncoproteins SV40 large T-antigen, adenovirus E1A, and human papillomavirus E7 protein, depend on their ability to associate with pRb and inactivate its growth suppressive function. Indeed, mutants of these proteins that lose pRb-binding capabilities fail to transform cells (22-27).

Table 1. pRb phosphorylation sites, functional domains and binding proteins



The phosphorylation state of pRb changes with the cell cycle, and, as stated above, is catalyzed by the activity of cyclin/cdk complexes. Until some time in G_1, close to, and perhaps with, the restriction point transition when cells become committed to division refractory to mitogenic stimuli, pRb is hypophosphorylated. Initial phosphorylation of pRb is most likely carried out by cyclin D/cdk4 or cdk6-containing complexes and thereafter maintained by the other cyclin/cdks: cyclin E/cdk2 and cyclin A/cdk2 or cdk1 (28). Hyperphosphorylated pRb no longer binds the E2F-DP heterodimeric transcription factor which it formerly bound in its hypophosphorylated state. Free E2F-DP activates transcription from a number of different genes required for DNA replication, such as DHFR, thymidine kinase, DNA pol-α, or growth-promoting genes such as c-myc, cyclin A and cyclin E (29). The viral oncoproteins mentioned above induce the G_1/S transition by binding only hypophosphorylated pRb, releasing E2F-DP, thus promoting proliferation (30-33).

The regions of pRb involved in protein-protein interactions can be mapped to distinct sites. To date, over thirty cellular proteins have been reported which bind to pRb. The 928 amino acid pRb protein is composed of a number of different structural and functional motifs. The A/B pocket (aa 379-572 and 646-772), separated by a spacer region (aa 573-645), binds proteins containing a conserved LXCXE (X= any amino acid). LXCXE-containing proteins include the oncoproteins of the DNA tumor viruses and the D-type cyclins. A variant of this motif, LXSXE, which is equally able to bind pRb (34), is found in PP1. It is this phosphatase which has been shown to act on pRb at the conclusion of M phase (35). The C-pocket of pRb encompasses carboxy-terminal residues 772-870 and binds both c-Abl tyrosine kinase and the oncoprotein mdm-2. The large pocket domain of pRb is composed of both the A/B and C pocket regions, residues 379-870, and is the binding site for the E2F family of transcription factors. There are sixteen different ser/thr-pro motifs in pRb, serving as cdk consensus sites, and eleven of these potential sites have been shown to be phosphorylated *in vivo* (36-39). Each site inhibits different protein binding functions, as determined in experiments using pRb mutants lacking specific cdk phosphorylation sites (40).

In more recent work, tryptic phosphopeptide maps of cdk-mediated hyperphosphorylated pRb have been generated. These experiments demonstrated that no single cyclin/cdk can reproduce the fully hyperphosphorylated form of *in vivo*-labeled pRb, rather, each cdk phosphorylates a subset of the potential consensus sites (39). Functional analysis has revealed that phosphorylation of thr 821 by cdk2-containing complexes or thr 826 by cyclin D_1/cdk4 can abolish large T-antigen binding; phosphorylation on ser 807 and ser 811 by cyclin D_1/cdk4 disrupts the C pocket (39); and phosphorylation of ser 608 and ser 612 or ser 780 disrupts E2F binding to pRb, though this requires the action of either cyclin D_1/cdk4 or cyclin A/cdk2 (37,41). This information is summarized in table 1.

6. BIOLOGICAL SIGNIFICANCE: CELL CYCLE REGULATORS AND CANCER

Tumorigenesis is a result of the deregulation of both cell cycle-inhibitory and growth-stimulatory signals. Therefore, knowledge of the control of cell cycle progression has profound implications for the treatment of cancer. A critical point in the G_1 phase of the cell cycle, called the restriction point, is the commitment to enter the division cycle. In G_1, the sum of growth stimulatory and growth inhibitory signals determines whether cells enter the division cycle or whether they remain quiescent. The genetics of cancer involves both growth-promoting oncogenes as well as tumor suppressor genes such as pRb that act as brakes to constrain cell proliferation. What should become apparent in this section is that through the various layers of control, the functions of these genes are interconnected.

6.1. Cyclins/cdk

Of all the cyclins in mammalian cells, only the D-type cyclins, their associated cdks and inhibitors have been implicated in cancer. In cell culture systems, overexpression of cyclin D_1 accelerates progression

through G_1 (2). Overexpression of cyclin E in fibroblast cultures also shortened G_1 phase (3), and cyclin E is abnormally expressed in a large number of breast cancers, although there is no evidence of amplification of the cyclin E gene (42). Cyclin D_1, located on chromosome 11q13, undergoes chromosomal translocation in 95% of B cell lymphomas where genes naturally undergo Ig rearrangement in the process of lymphocyte differentiation. The rearrangement that occurs in mantle cell lymphomas (a name that includes a collection of intermediately differentiated lymphomas), namely the t(11;14)(q13;q32) translocation, puts the cyclin D_1 gene immediately downstream of a breakpoint (43,44). Activation of cyclin D_1 in B cells that do not normally express this protein impairs the ability of the cell to exit the cell cycle and prevents differentiation into Ig-secreting plasma cells. The cyclin D_1 gene is amplified in an average of 13% of breast cancers, 43% of head and neck squamous cell carcinomas, 35% of esophageal and 12% of bladder cancers (43). Cdk4, located on chromosome 12q13, lays within an amplicon observed in 18% of human sarcomas and 10% of gliomas, and cdk4 is overexpressed in these cancers as a result (43).

6.2. Cdk Inhibitors

As mediators of cell cycle arrest, the cdk inhibitors are prime candidates for acting as tumor suppressors. Although no tumor specific alterations have been observed in p21 and p27, the p16 gene is mutated or deleted in a high frequency of primary tumors. p16 was first identified as a tumor suppressor gene in relation to its high frequency of mutation in familial melanoma (45). Many of the deletions that affect p16 on chromosome 9p21 include p15, though there are examples of p16 deletions that do not encompass it. p16 mutations include insertions, frameshift mutations, missense mutations, splicing defects (46), chromosomal translocations and silencing by hypermethylation (47,48).

6.3. The pRb Pathway

Cyclin D_1 and p16 are functionally interconnected in the control of the pRb-mediated regulation of cell proliferation, by acting as the positive and negative regulators, respectively, of cdk4. As described above, a major function of cyclin D-associated kinases is to phosphorylate pRb, releasing the brake on cell cycle progression. Mutation in any of these genes in human cancer is likely to have consequences that promote entry into the cell cycle. Loss of p16 and amplification of cyclin D_1 or cdk4 will both induce the hyperphosphorylation of pRb, release of E2F, loss of growth suppression, and cells will proliferate uncontrollably. Supporting this model are the facts that cyclin D_1 is dispensable in pRb^- cells (1) and p16 requires functional pRb to inhibit growth (19,20). Therefore, tumors that have lost or mutated forms of pRb do not require the alterations of these upstream genes, and visa versa, as tumors that do overexpress cyclin D_1, or have deleted or mutated p16, are also pRb^+ (33). Thus, loss of p16, overexpression of cyclin D_1, and inactivation of pRb all favor cell proliferation, and are components of a common pathway to tumorigenesis. As such, each is a potential target for therapeutic intervention. Because of this, much effort is being expended in our laboratory, as well as others, to elucidate the details of the pRb protein-protein interactions and the specific phosphorylation sites targeted by PP1 that would restore its anti-proliferative effects. This brings us to the involvement of PP1 and its indirect effects on the control of cell proliferation.

7. THE DEPHOSPHORYLATION OF pRB

Serine/threonine phosphatases are classified according to their biochemical properties rather than phylogenic relationship. Classification depends on specificity for the α or β subunit of phosphorylase kinase, a key enzyme in the glycogen metabolic pathway. The type 1 protein phosphatases include the α, $\gamma 1$, $\gamma 2$, and δ isoforms which dephosphorylate the β subunit of phosphorylase kinase. The type 2 protein phosphatases are specific for the α subunit of phosphorylase kinase and are further subdivided according to their requirement for divalent metal ions (49).

Before PP1 activity was ever implicated in the regulation of pRb, it was known to have a role in the regulation of mitosis and cell division. PP1 mutations in *Drosophila* (50), yeast (51) and fungi (52) displayed varied mitotic defects and different degrees of lethality. Mitotic blocks were observed upon microinjection of PP1-neutralizing antibodies (53) and PP1 inhibitors such as okadaic acid (54,55). In addition to this, the distribution of PP1 changes with the cell cycle, accumulating at the nucleus to associate with chromatin during G_2 and M phase (53). Potential targets for PP1 in the nucleus are the mitotic cyclin B/cdk1 substrates that include histone H1, lamins, microtubule-associated proteins and perhaps other proteins that have yet to be identified. These data further underscore the importance of PP1 for exit from mitosis.

The identification of PP1 as the enzyme that catalyzes the dephosphorylation of pRb at mitotic exit has implicated this phosphatase as possessing a crucial role in growth control. PP1 is found associated with pRb during this time and into the G_1 phase of the next cycle (56). This temporal association between PP1 and pRb appears to have functional significance in that it coincides with the reactivation of pRb-mediated growth suppression (57). Thus, by activating a demonstrated negative regulator of cell cycle progression, PP1 can be considered to play an indirect role in the regulation of the cell cycle. Interestingly, in the coimmunoprecipitation and yeast two-hybrid screens that originally identified the association between these two proteins, it was hypophosphorylated pRb that was bound to the PP1α catalytic subunit, and its splice variant, PP1α2 (56). Work from our laboratory recently corroborated this observation for all three PP1 isoforms (58) and with the α and δ isoforms in affinity chromatography experiments (S.Tamrakar, and J.W. Ludlow, unpublished observations), though the reason for the preferential interaction with the hypophosphorylated form of pRb is not clear. When perceiving this interaction in terms of a general enzyme-substrate relationship, one would predict that *hyper*phosphorylated rather than *hypo*phosphorylated pRb would bind PP1 more readily. On

the other hand, the probability of capturing a phosphorylated substrate in complex with its phosphatase enzyme is dependent on the myriad and most likely complex concentration-dependent enzyme kinetics governing the given reaction. The possibility also exists that the associated pRb may have been dephosphorylated while in complex with PP1. Addressing these possibilities necessitates a closer look into the mechanics of interaction between PP1 and pRb.

Is the relationship between PP1 and pRb more than merely an enzyme-substrate association? Is pRb affecting the activity of PP1 much like the other known PP1 -associated proteins ? Considering the multiple regulatory subunits that direct phosphatase activities, could other proteins be involved? Preliminary data from our own studies using affinity chromatography has revealed both hypophosphorylated and, to a lesser degree, hyperphosphorylated forms of pRb interacting with PP1 (S.Tamrakar, S.Mittnacht, and J.W. Ludlow, manuscript in preparation). Work currently in progress has begun to address the question of whether PP1 activity can be modulated by its association with hypophosphorylated and/or hyperphosphorylated pRb.

Another venue currently being explored in our laboratory is the potentially different roles that the various PP1 isoforms play with respect to pRb-directed activity. Previous work using immunocomplexed mitotic PP1 isoforms to dephosphorylate metabolically labeled pRb showed that although the α, γ1 αnd δ isoforms all effectively dephosphorylated pRb, the PP1-δ isoform contained the greatest pRb-directed activity (59). As we have earlier hypothesized, this may be due to its association, unique among the PP1 isoforms, with a 110 kD interacting protein (59). The generation of pRb phosphopeptide maps, following PP1-isoform specifiic dephosphorylation, should lead to valuable information about any specificities that these enzymes have for a subset of phosphorylation sites. Such analysis will most certainly contribute towards elucidating the manner in which pRb is so efficiently reinstated to its growth suppressive form.

8. PP1 REGULATION

The enzymatic activity of PP1 may be regulated by at least two methods: by phosphorylation at a specific carboxy-terminal threonine that is the only region that differs among PP1 isoforms, and by association with noncatalytic, cellular proteins that inhibit, modulate or target PP1 to various subcellular structures and substrates. The cyclin B/cdk1-mediated phosphorylation of PP1 is covered in greater detail elsewhere in this issue. However, for our purposes here, it is noteworthy that this phosphorylation inactivates PP1 activity during the onset and middle of M-phase (60, 61). Dissipating with the destruction of cyclin B, this phosphorylation times the pRb-directed activity of PP1 to mitotic exit (62-64). PP1 phosphorylation in phases other than mitosis may also be important for cell cycle regulation, as mutant PP1 in which the phosphorylatable threonine is replaced by alanine arrests cells in G1, presumably because of its inability to be phosphorylated (65).

The PP1 catalytic subunit is rarely found alone in nature. Rather, higher order structures of PP1 are formed by oligomerization with associated, regulatory subunits. The biological advantage of this is, of course, for ensuring multiple pathways for the inhibition, regulation or direction of PP1 activity in response to signal-transducing stimuli. By the existence of varied regulatory subunits which can each be modified, PP1 activity can be quickly and decisively regulated. This form of regulation furthers the disparity between the relatively small number of phosphatases needed to reverse the actions of a multitude of kinases.

A fine example of this form of regulation is illustrated by the regulation of two thermostable proteins commonly found complexed to cytoplasmic PP1 catalytic subunit. Inhibitors 1 and 2 both render PP1 activity latent until further modifications. Inhibition by I-1 and its brain-specific isoform, DARPP-32, is dependent on its prior phosphorylation by cAMP-PK in response to hormonal activation, while the inhibition by I-2 is *reversed* upon phosphorylation by GSK3. Association of PP1 with I-1 and I-2 is thought to control many cellular processes through the temporal inhibition of PP1 (66).

Other proteins serve to direct and modulate PP1 activity rather than inhibit it. The well-known role of PP1 activity in glycogen metabolism is highly regulated by its interaction with the muscle (G_M) and liver (G_L) subunits that are highly sensitive to the actions of glucagon, adrenaline and insulin in the regulation of PP1 activity in glycogen particles (67-69). Other regulatory subunits include the myofibril binding M110 protein, which directs PP1 activity towards myosin light chain (70), a ribosomal inhibitor of PP1 (71), a p53 binding protein, p53BP2 (72), and a nuclear inhibitor of PP1, NIPP-1, which, like I-2, is itself regulated by phosphorylation (73).

Recently, our work identified several PP1-associated proteins that could be found complexed with mitotic PP1 during its pRb-directed activity (59). One of the more prominent associated proteins had an apparent molecular weight of 110kDa. Formerly termed R_{111} (73), this subunit has recently been isolated and characterized as a PP1 nuclear targeting subunit, aptly named PNUTS (74). In its activity towards pRb, we envision a scenario in which the mitotic PP1 160 kD holoenzyme that we have identified is a complex of the targeting function of the 110 kD PNUTS plus the 37 kD catalytic subunit of PP1, or, more precisely, PP1-δ. From the enzyme affinity assays employed to detect interacting proteins, it was evident that only the PP1-δ isoform was complexed to the 110 kD subunit, and from corresponding phosphatase assays, it appeared that it was this isoform that most efficiently dephosphorylated pRb. Thus, a model of the control of PP1 activity towards pRb at mitotic exit and into the ensuing G_1 phase may include the specific association of the subunit PNUTS to the δ isoform of mitotic PP1. In so doing, PP1 may be targeted to the nucleus to its cell cycle-

suppressive substrate, pRb, perhaps conferring differential substrate specificity and heightened enzymatic activity during a crucial window of opportunity in the cell's return to interphase. Further studies are required to establish the cell cycle profile of the PNUTS/PP1 association, and to address the puzzling phenomenon of its isoform-specific binding. In addition, while the above described scenario suggests that PNUTS somehow acts as an activator of PP1 towards pRb, can this model be compatible with the current *in vitro* data which says that PNUTS binding to PP1 inhibits phosphatase activity, at least towards the substrate phosphorylase *a* (74) ?

9. CELL CYCLE REGULATION DURING HYPOXIA –SYNERGY BETWEEN PHOSPHATASE AND KINASE ACTIVITY

Studying cell cycle regulation under abnormal circumstances can provide a wealth of information on the inner workings of cell cycle regulation during normal situations. Towards this end, we have developed a model system whereby cells are cultured under hypoxic conditions and this effect on cell proliferation is then studied. Indeed, overall cell proliferation is regulated in response to changes in the extracellular environment. Low oxygen availability, or hypoxia, causes an inhibition of the respiratory chain thereby lowering ATP synthesis by the cell (75). In accordance with the need by the cell to decrease energy usage under low oxygen conditions, hypoxia inhibits cell proliferation as well as general transcription and translation in multiple cell types (76-78). Published work from our own laboratory and others is consistent with the hypothesis that the arrest of cellular proliferation by hypoxia involves disrupting the synthesis and degradation of cyclins, as well as the post-translational modification of pRb (79-81).

We previously reported that hypoxia-induced growth arrest results in accumulation of hypophosphorylated pRb in both transformed and non-transformed cells (80, 81). We have recently completed a study to determine the mechanism of hypoxia-induced pRb hypophosphorylation (82). To establish if hypoxia induces specific activation of PP1 leading to the conversion of pRb from a hyper- to hypo-phosphorylated form, pRb-directed phosphatase assays were performed using lysates from cells exposed to hypoxia for up to 48 hours . The summary data from four independent experiments (0-48 hr time course) indicate that there is a 30-50% increase in pRb-directed phosphatase activity which peaks at 18 hours of hypoxia relative to aerobic controls. These data are consistent with conversion of hyper- to hypo-phosphorylated pRb *in vivo* as detected by immunoblotting (80, 81). pRb-directed phosphatase activity remains elevated by 20% relative to aerobic controls after 24 hours and returns to aerobic levels by 48 hr of hypoxia. For comparison, cells arrested in G_1 by serum starvation, which demonstrate very little pRb-directed phosphatase activity by this assay (57), exhibit approximately 50% less PP1-mediated pRb dephosphorylation than cells arrested in G_1 by hypoxia.

To test whether the hypoxia-induced increase in the pRb-directed phosphatase activity is due to an overall elevation of PP1 activity in the cell, we measured phosphatase activity using phosphorylase *a* as a substrate. Phosphorylase *a* is phosphorylated on a single serine residue (83) and used as the standard substrate for monitoring enzymatic dephosphorylation by PP1 and the type 2A serine/threonine protein phosphatase (PP2A). However, in CV-1P cell lysate greater than 90% of the phosphatase activity is attributed to PP1 (D. Nelson and J.W. Ludlow, unpublished observations). The results obtained showed that total phosphatase activity after 18 and 24 hours of hypoxia remained unchanged relative to aerobic controls. The levels of PP1 isoforms in CV-1P cell lysates remained relatively constant during hypoxia, suggesting that the observed increase in pRb-directed phosphatase activity was not due to an increase in PP1 catalytic subunit abundance. This suggests that PP1 associated proteins, perhaps the PNUTS protein which we have putatively identified in a complex with mitotic cell fractions containing pRb-directed PP1 activity (59), may play as yet undefined roles in modulating this increase in pRb-directed activity during hypoxia.

Accompanying this induction of phosphatase activity is inhibition of both cdk4 and cdk2 activity, decrease in cyclin D, CDK4 and cyclin E abundance, and a dramatic increase in p27 protein abundance. We can also demonstrate that p27 associates with CDK2 complex under hypoxic and not aerobic conditions in this system, thus mediating inhibition of CDK2 activity. Taken together, it appears that not only is there an increased activity to remove phosphates from pRB (i.e.: increased pRb-directed PP1 activity), but there is also a decreased activity to put phosphates on (i.e.: decreased cyclin/cdk activities) during hypoxia. We conclude from these data 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, and have summarized these findings in a model presented in figure 2. While supporting the notion that PP1 plays an indirect role in modulating the cell cycle by activating the growth suppressive function of pRb, having a system whereby PP1-specific activity towards pRb can be manipulated also provides an opportunity to more fully investigate the functional interaction between these proteins.

Clearly, the above discussion 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 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 phosphatase activity with regard to other biochemical aspects of cell cycle regulation.

10. PERSPECTIVE

Considered together, pRb is a cell cycle regulatory protein whose function is modulated by cell cycle regulatory protein whose function is modulated by

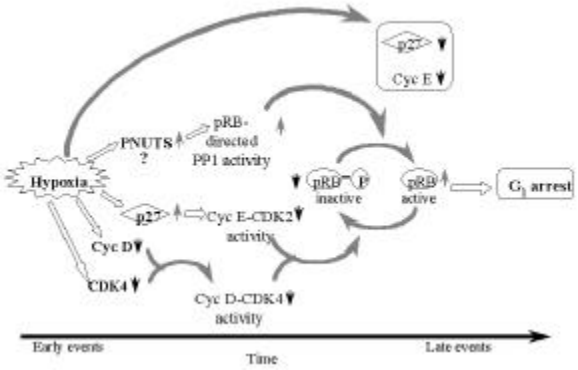


Figure 2. Model of the molecular mechanisms involved in hypoxia induced cell cycle arrest in CV-1P cells. Cellular events in response to hypoxia are depicted along the time course from left to right. Early upon cell exposure to hypoxia, the abundance of CDK inhibitor p27 is elevated and, as a consequence, more p27 is available to bind cyclin-CDK2 complexes, resulting in the observed decrease in CDK2 kinase activity. Simultaneous with inhibition of the kinase activity is an increase in pRB-directed phosphatase activity. The concerted action of CDK2 inhibition and increased pRB dephosphorylation leads to the net accumulation of pRB in its active, hypophosphorylated form. This results in G_1 growth arrest through repression of early S phase gene transcription and consequent inhibition of DNA synthesis. In addition, DNA synthesis may also be directly affected through inhibition of cyclin A-associated kinase activity. Long term hypoxia results in a decrease in both cyclin A and cyclin E abundance. Consequently, there are fewer active cyclin-CDK2 complexes available and lower concentrations of p27 are needed for their inhibition, which may account for the lower levels of p27 observed. If hypoxia is too severe and oxygen levels are too low for the particular cell to maintain its basic functions, it will undergo apoptosis; if the basic functions can be sustained, the cell will arrest until conditions become more favorable. Question marks designate hypothetical pathways.

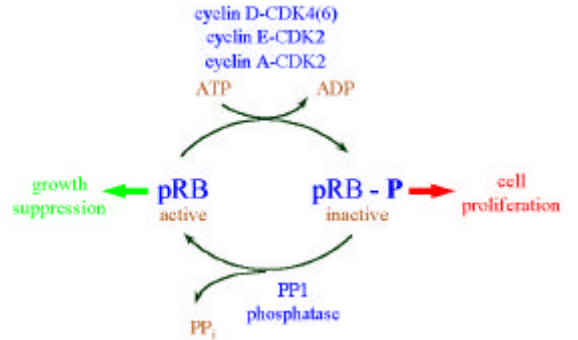


Figure 3. Summary of pRb phosphorylation/ dephosphorylation reaction. Growth suppressive, hypophosphorylated pRb present during G_1 is functionally inactivated by phosphorylation mediated by cyclin/cdk complexes, thus removing the G_1 block to S-phase entry and subsequent proliferation steps. During mitosis, PP1 activates the growth suppressive function of pRb by removing phosphates, thus ensuring that pRb is functional during the subsequent G_1 phase of the cell cycle.

cell cycle dependent serine/threonine phosphorylation. A model which summarizes key features in the pRb phosphorylation/dephosphorylation reaction is presented in figure 3. As a reminder, pRb enters mitosis hyperphosphorylated, yet it is hypophosphorylated pRb that is maintained through mitosis and the subsequent G_0/G_1 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. 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 pRb and PP1 in cell cycle regulation.

Avenues for further investigation may include the functional significance of PP1 binding to pRb with regard to the transcriptional regulation mediated by both of these proteins. For example, PP1 has been shown to modulate the transcriptional and DNA binding activities of retinoic acid receptors (84). Recently, it was shown that the glucose-mediated increase in binding of the Sp1 transcription factor to DNA is due to dephosphorylation of the existing Sp1 in the nucleus (85). Based on a series of elegant experiments, the conclusion drawn from this work is that the enzyme involved in Sp1 dephosphorylation is PP1, thus implicating this phosphatase in promoting Sp1 transcriptional activation. pRb also has demonstrated an ability to regulate Sp1 transactivation (86-88). Sp1-mediated transcription can also be stimulated by pRb (86-88), suggesting that pRb may regulate transcription in part by virtue of its ability to functionally interact with Sp1, although a direct physical association between these two cellular proteins has yet to be demonstrated. However, there is evidence to suggest that Sp1 trans-activation domains are bound by cellular proteins that may negatively regulate their activity (86). From these observations, one hypothesis put forth is that pRb may bind to and remove these factors thereby liberating Sp1 from negative regulation or by facilitating the interaction of Sp1 with the components of the basal transcription complex.

Taken together, it appears that pRb can stimulate Sp1-mediated transcription perhaps by complexing with and thereby removing an inhibitor to Sp1 binding, and dephosphorylation of Sp1 by PP1 can also stimulate Sp1-mediated transcription. An indirect role for the activity of PP1 in pRb-stimulated Sp1-mediated transcription can be inferred since it is only the hypophosphorylated form of pRb which is active in transcriptional regulation, by virtue of its ability to complex with transcription factors and accessory proteins. One question which begs to be

addressed is whether a direct role for PP1-stimulated Sp1-mediated transcription involves pRb. Specifically, does pRb, as a PP1-associated protein, modulate the enzymatic activity of this phosphatase towards Sp1? If so, support of the idea that a complex between PP1 and pRb has *in vivo* significance as a transcriptional regulator will be obtained.

Continuing with this line of thought, overexpression of the pRb-specific phosphatase and/or its regulatory subunits in mammalian cells will be important to address the physiological significance of this biochemical reaction. Overexpression of the PP1 catalytic subunit may be difficult to effectively achieve, however. Recent work on PP2A has provided evidence that an autoregulatory mechanism exists in which forced overexpression of exogenous PP2A causes an underexpression of the endogenous enzyme. This results in a constant level of PP2A expression and, presumably, activity (89). Taking this cue, we have developed an inducible-PP1 expression system, and can demonstrate similar results thus supporting an autoregulatory mechanism for PP1 expression also (J.E. Reeder, M.P. Sowden, E.M. Messing, E. Villa-Moruzzi, and J. W. Ludlow, unpublished observations). Nonetheless, one of the goals in our laboratory is to determine the functional significance of M-phase specific pRb dephosphorylation in growth suppression. Perhaps increased activity of the phosphatase towards pRb (by modulating PP1-associated regulatory subunits) will result in the cell being deficient in the control of cell growth attributed to the wild-type pRb. Alternatively, inhibiting pRb dephosphorylation may prevent the cell from going through an orderly mitosis, potentially contributing to development of a transformed phenotype. Support for this notion comes from the observation that mutations in chromosome 11q13, the chromosomal location of the human gene for the PP1α catalytic subunit, have been linked with the development of some cancers (90).

Thus, to conclude this review, a major mechanism by which eukaryotic cells regulate protein function is to place phosphate groups on serine/threonine or tyrosine residues. While the steady state level of protein phosphorylation depends on the relative activities of both kinases and phosphatases, until very recently, much greater effort has gone into the study of the former than the latter. Protein phosphatase type 1 (PP1) is one of the major cellular serine/threonine protein phosphatases. Studies employing many eukaryotic systems all point to a crucial role for PP1 activity in cell cycle progression, and an absolute requirement of this activity for mitotic exit. While the evidence thus far supports a view whereby PP1 activity plays an indirect role in cell cycle regulation by activating the growth suppressive property of pRb, this is not to say that a more direct role for this enzyme does not exist. With the current investigative scrutiny being given to this group of phosphatases, evidence for such a direct role may be provided very soon.

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