[Frontiers in Bioscience, 4 d22-42, January 1, 1999]

PROTEIN DEPHOSPHORYLATION AND THE INTRACELLULAR CONTROL OF THE CELL NUMBER

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TABLE OF CONTENTS

1. ABSTRACT

Regulating the cell number is critically important for the development and maintenance of a multi-cellular organism. The cell number can be altered by inducing cell proliferation and/or programmed cell death (apoptosis). These two processes are linked by cell cycle-regulatory pathways, and protein phosphorylation and proteolytic degradation play key roles in both. Protein dephosphorylation has been a rather neglected aspect of cell cycle control. Recent advances in this field make it imperative to provide a view of the cell cycle from a phosphatase's vantage point. Although a number of protein phosphatases may be instrumental in cell cycle and apoptosis control, the emphasis here will be on the prototypical Ser/Thr-specific protein phosphatase PP1. Experiments will be considered in their historical context. The major goal of this review will be to re-evaluate the hypothesis that PP1 — and other protein phosphatases — may function as negative growth regulators. Currently available evidence suggests that PP1 activity is required for exit from mitosis, yet may also block cell cycle progression and facilitate apoptosis. Where appropriate, results highlighting the role of the other major phosphatase, PP2A, will also be discussed. This review will conclude with some unresolved issues including the question whether PP1 might be a suitable target for anti-cancer strategies.

2. INTRODUCTION

The idea that protein phosphatases might have something to do with cell proliferation can be traced back to the mid-to-late 1980s. The first clue was provided by the observation that microinjection of protein phosphatases 1 and 2A inhibits and microinjection of a phosphatase inhibitor induces maturation of starfish oocytes (1,2). The second line of evidence consisted of two independent studies showing that i) protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) are potently inhibited by okadaic acid (OA) (3,4), and that ii) this very compound is a powerful tumor promoter (5). These findings had two immediate consequences: First, the use of OA and related compounds (see Section 3.1.1) has contributed to the now prevailing concept that protein phosphorylation is a universal regulatory mechanism. Second, as it was tempting to speculate that the tumor promoting effect of okadaic acid was the result of phosphatase inhibition, it was quickly suggested that PP1 and PP2A might be tumor suppressors in their own right (6). These exciting discoveries occurred a short time after we learned that many oncogenes encode protein kinases, and, more specifically, that the enzyme responsible for triggering entry into mitosis, $p34^{cdc2/CDC28}$ (now re-named cyclin-dependent kinase 1 or Cdk1) shares homology with oncogenes (7), is a protein kinase itself (8), and represents the cytoplasmic mitosis-promoting factor (MPF) (9,10) that was postulated almost two decades earlier (11). Since the late 1980s, the work of many laboratories has helped us to build a molecular framework through which we can approach and ultimately understand fundamental processes such as development and differentiation, tissue homeostasis, aging and apoptosis, and tumorigenesis as well as tumor suppression. As tumors can arise from increased cell proliferation and/or decreased cell death, our much-improved understanding of many key pathways has

nurtured hopes to be able one day to design more "biological" therapies for human cancer.

While there is no shortage of excellent review articles on cell cycle regulation and related areas, the main goal of this article is to provide a view of the cell cycle from a phosphatase's vantage point. It is clear by now that several phosphatases have a role in cell cycle progression. In contrast to the Cdks, where we have arrived at some consensus about what these enzymes do, the phosphatase arena seems like a goldmine and a mine field at the same time: As always, there are open questions, but for nearly every role that was assigned to a protein phosphatase in growth control, there are studies which apparently support a different view. The aim of this review then, is to examine the evidence in its (historical) context, to reconcile, if possible, seemingly contradictory results, and thus help to diminish some of the confusion.

3. THE CONTEXT

Until recently, protein phosphatases have widely been considered as "cellular second banana" to the protein kinases (12). In a sense, this is well justified: If the default state of a protein is its unphosphorylated form, there is no need for protein phosphatase action unless a protein kinase attaches a phosphate to its substrate first. Likewise, there is no need for laboratories investigating phosphatases unless somebody, somewhere, has discovered the significance of a protein kinase-mediated reaction. Other, more trivial reasons include the widespread perception that it is more difficult to study phosphatases (probably true) and, secondly, that all it takes to alter the phosphorylation state of a protein is the forceful activation —or inhibition— of a protein kinase, or in other words, that the phosphatases could be counted upon to act as housekeeping enzymes (not true). Meanwhile, we have come to realize that i) although novel protein phosphatases continue to be identified, they are likely to be outnumbered by protein kinases by a wide margin, ii) protein phosphatases have a very high turnover rate that is often significantly higher than that of the opposing kinase, and iii) there is an ever increasing list of regulatory subunits, at least for PP1 and PP2A. Taken together, these findings have helped to gradually change the appreciation of these enzymes: It is now more commonly assumed that, to perform their biological functions in a meaningful way, protein phosphatases are —or even must be— tightly regulated, both in space and time (13-16).

3.1. Protein Phosphatase 1 as a Multifunctional Enzyme

For more than three decades after the discovery of protein phosphorylation in the area of glycogen metabolism (17,18), the study of protein dephosphorylation was pretty much confined to phosphorylase phosphatase (see this volume (19)). It is probably fair to say that the field of protein phosphatase research received a jumpstart thanks to the discovery of protein phosphatase inhibitors such as OA (3,4). Another, more recent finding that is likely to keep the excitement level high is that catalytic subunits of protein phosphatases appear to specifically bind to an increasing number of cellular proteins. Both aspects of phosphatase function are covered in detail elsewhere in this volume (19,20); here, I will discuss these rather novel features of the phosphatase family only insofar as they are relevant to the topic of this review.

3.1.1. Small Molecular Weight Inhibitors of Protein Phosphatases

As already mentioned, soon after OA was discovered to inhibit phosphatases and promote tumors in the mouse skin assay, it was proposed that protein phosphatases may be negative regulators of cell growth and/or proliferation (6). In the years that followed, hundreds of studies appeared that used OA and other phosphatase inhibitors to establish a role for protein phosphorylation in virtually every cellular process — or so it seems. In many of these studies, the reasoning was that OA inhibits phosphatases, therefore increases protein phosphorylation, which in turn is responsible for the net effect observed at the end of the experiment. In the area of cell growth control however, results obtained were more confusing rather than enlightening: Thus, in support of the above hypothesis, OA was found to transform cells (21,22), stimulate DNA synthesis (23) and induce Cdk1 as well as cyclin A and B expression (24), promote mitosis (25-27), and prevent apoptosis (28,29). In contrast, OA was also found to reverse the transformed phenotype (30) or inhibit *src*-mediated transformation (31), induce cell growth arrest, presumably by down-regulating Cdk1 expression (32), arrest mitosis (33,34), and induce apoptosis (35-37). Similar examples could be cited for other phosphatase inhibitors. How are we to make sense of this confusion without blaming the different cell types used in these studies? A re-examination of these results reveals that i) cells were treated with OA concentrations spanning several orders of magnitude, ii) the time of exposure to OA also varied from minutes to days, and iii) assays for phosphatase activity or the phosphorylation state of a (number of) protein(s) were rarely included. This makes it very difficult to evaluate the overall results seen in cells that have been exposed to OA for much longer than minutes (usually the time it takes to detect phosphatase inhibition). Long-term treatment of cells with low OA concentrations makes them resistant to OA: Remarkably, this is associated with a point mutation in PP2A (C269G) that increases its IC_{50} about fourfold (38). This suggests that the primary target of OA action may be PP2A. Of course, this does not rule out that OA affects molecular systems other than protein phosphatases. In fact, that it is necessary to exercise caution when interpreting results with phosphatase inhibitors was illustrated recently by showing that tautomycin-mediated phosphatase inhibition and induction of apoptosis can be attributed to distinct parts of the molecule (39). What this tells us is that phosphatase inhibitors may be utilized to assess the role of protein phosphorylation in a distinct molecular process (*e.g.* the activation or subcellular relocation of an enzyme), but they cannot typically aid us in evaluating the role of protein (de)phosphorylation in a complex biological process such as cell proliferation, unless the conclusions are supported by other independent evidence. Furthermore, because phosphatase inhibitors are not specific for any single phosphatase enzyme, they are rarely suited to delineate the

exact role(s) of a given protein phosphatase (for another account of the pros and cons of OA, see (40) this volume).

These contradictory results notwithstanding, the hypothesis that phosphatases may be negative growth regulators continues to be feasible for two reasons: Like OA, other, chemically unrelated phosphatase inhibitors are tumor promoters: calyculin A (41), microcystin (42), and nodularin (43). More importantly, cell proliferation is driven by both Tyr- and Ser/Thr-specific protein kinases (see Section 3.2.2). Together, these findings led to the idea that the hyperphosphorylation often associated with malignant transformation may be, at least in part, due to phosphatase inhibition (reviewed in (44)).

3.1.2. Protein Phosphatase 1 Isozymes

PP1 was discovered 55 years ago as the first interconverting enzyme that inactivates phosphorylase *a* (45,46), although it took another two decades to realize that it is a phosphatase (47). During the 1970s much energy went into purifying phosphatases (see (19) this volume). In 1983, mammalian phosphatases were designated PP1, PP2A, PP2B, and PP2C according to their biochemical and enzymatic properties (48). Another 4 or 5 years later, the first protein phosphatases to be cloned were PP2A and PP1, both from rabbit skeletal muscle (49-51). Since then, progress in the cloning arena has revealed that protein phosphatases comprise three families: the Ser/Thr-specific families PPP (PP1/PP2A/PP2B) and PPM (PP2C-like), and the Tyr-specific or dual-specificity family PTP (52). Members of the PPP family are believed to represent the most conserved enzymes (53), which possibly testifies to their essential function in cellular regulation. Returning to mammalian PP1, three genes code for four distinct isoforms designated α, γ1, γ2, and δ (this has now been renamed 1β). Their primary structures are identical, except for a few N-terminal residues and the extreme C-terminal region (54). Initially, the general lack of isoform-specific antibodies made it difficult to assign specific roles to distinct PP1 isoforms. All isoforms are ubiquitously expressed except for PP1γ2, an alternative splicing product that is found only in testes (55). While PP1β is part of the glycogen particle (56), PP1α, along with PP1γ1, is over-expressed in hepatomas and other malignant tumors, suggesting to these authors that these two isozymes might contribute to tumorigenesis (54,57-61). The potential significance of these findings will be further discussed in Section 5.1.

3.1.3. Regulatory Subunits of PP1

The free catalytic subunit of PP1, a 37 kDa protein, has a broad substrate specificity overlapping with that of other phosphatases *in vitro*. In the cell, PP1 (as well as PP2A) is often complexed to regulatory subunits that modify their activity or target them to particular locales (reviewed in (14,62-64)). The first regulatory subunits were discovered by conventional protein purification and became known as inhibitor-1 and inhibitor-2 (65). Meanwhile, novel approaches including the yeast two-hybrid screen and affinity chromatography on immobilized microcystin (66) have identified a growing number of proteins that appear to specifically bind PP1 (19,20). Those that are particularly interesting in the context of this review will be introduced in Section 4.

In summary, the existence in most cells of three PP1 isozymes and close to twenty regulatory subunits means that there are — at least in theory — sixty or so distinct forms of PP1 alone. The situation with PP2A is similar if not more complicated by the fact that this enzyme mostly occurs in heterotrimeric form. As more PP1-binding proteins become known, this number is likely to go up. Even conceding that some cell types might not express all of the putative PP1 binding proteins, this still means that various PP1 heterodimers are probably involved in distinct cellular processes. Thus, only a fraction of all the PP1 molecules present in a cell can be expected to play a role in cell cycle regulation.

3.2. Cell Cycle Regulation — As we Know it

The cell number can be increased by generating exact copies of parental cells through cell division, and it can be decreased by programmed cell death. The first process requires entry into and/or progression through the cell cycle, whereas the latter requires exit from the cell cycle. These two fundamental processes were studied independently for many years, but nowadays, a discussion of the cell cycle would not make much sense without also considering apoptosis. This is because the pathways regulating the cell cycle and apoptosis are linked (reviewed in (67-70)). The synopsis of this field as presented below (Sections 3.2.1 and 3.2.2) is possible because of the tremendous progress during the last 15 years or so. It will probably contain several generalizations and lack important details but its main purpose is to set the stage for a discussion of the protein phosphatases in Section 4.

3.2.1. The Significance of G1 Regulation for the Organism

To divide or not to divide? The cell cycle is a series of orderly processes that proliferating cells go through in order to divide. Thus, growth in mass (G1) is followed by DNA replication (S-phase), synthesis of proteins required for mitosis (G2), mitosis and cell division (M-phase) and return to G1 (71,72). Early in life, cell cycle progression has to be coordinated with development and differentiation, whereas the overriding concern for an adult organism is to maintain tissue homeostasis, which includes organ integrity and an approximately constant mass (73).

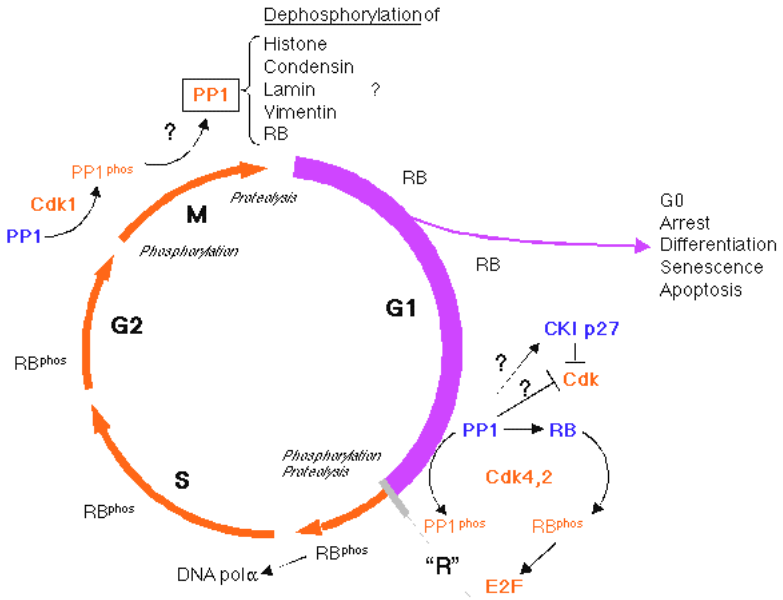

Figure 1. Three aspects of cell cycle regulation. 1) During the better part of G1 (orange) a cell is typically uncommitted and free to choose between several distinct fates. Passage through R is therefore especially important. 2) Reversible phosphorylation and proteolysis cooperate to drive cell cycle progression. 3) It takes PP1 to get into and remain in G1. At G2/M, PP1 undergoes inhibitory phosphorylation by Cdk1; later, PP1 is required to exit from mitosis = G1 entry which may be due to the necessary dephosphorylation of several proteins including RB. It is not known whether the mitotic requirement for PP1 activity involves just another population of PP1 molecules or reactivation by another phosphatase. Likewise, the exact roles of the different PP1 isozymes remain to be determined. After return to G1, PP1 would have several opportunities to activate RB and thereby maintain cells in G1: by directly dephosphorylating RB, inhibiting any of the Cdks or stabilizing CKIs such as $p27^{Kip1}$.

Thus, the ability to inhibit, rather than to promote, cell cycle progression becomes more important during an animal's and our own lifetime. During which phase(s) does a cell decide between the alternative fates of proliferation, temporary arrest or quiescence, differentiation, apoptosis or senescence? The study of cultured cells has defined a checkpoint (R-point) in late G1: Before this point, cell cycle progression depends on extracellular growth factors, whereas passage through the R-point commits the cell to DNA replication and the ensuing cell division, even in the absence of growth factors (74,75). It thus appears that a cell is free to choose between the above alternatives only in G1. This would make the transition from G1- to S-phase the most important step in cell cycle regulation (figure 1). It may not be surprising then that a malfunctioning of proteins involved in G1/S regulation is a leading cause of malignant transformation, as it allows cells to escape from checkpoint control (76-80). Cancer has even been termed a disease of the cell cycle (81), and a molecular framework now exists that may be exploited in the quest for more rational cancer therapies. The following paragraphs will briefly describe the molecular pathways that are relevant for the cell cycle transitions in which PP1 is involved.

3.2.2. The Role of Post-translational Modification in Cell Cycle Control

Protein kinases drive cell cycle progression. Following the discovery that, in budding and fission yeast, entry into mitosis depends on two protein homologs, *Cdc28p* and *cdc2p* (reviewed in (82)), which are being recognized as the prototypical cyclin-dependent protein kinase Cdk1, it turned out first that vertebrates encode a Cdk of similar structure and function (83). As their name suggests, activation of these enzymes requires binding between the Cdk and a cyclin regulatory subunit; unlike the cyclins, Cdk catalytic subunits appear to be present in constant amounts throughout the cell cycle. Whereas in yeast, one Cdk can "choose" between different cyclins, higher eukaryotes express at least ten cyclins and nine Cdks, giving rise to the now well-known concept that cell cycle progression is driven by a family of Cdks (84,85). Following the association between a Cdk and a cyclin, the Cdk catalytic subunit undergoes inhibitory phosphorylation at Thr^{14}/Tyr^{15}, activating phosphorylation at Thr^{161} and activating dephosphorylation at Thr^{14}/Tyr^{15} (86). Cdk/cyclins can be inhibited by several cyclin-dependent kinase inhibitors (CKIs) (87-90) or by proteolytic destruction of the cyclin component (91,92). Beyond activating the kinases, cyclins appear to target Cdk activity to particular substrates (93). Therefore, different Cdk/cyclin complexes promote distinct phases of the cell cycle. As for G1/S regulation, it is thought that Cdks 4 and 6 in complex with D-type cyclins promote G1 progression, Cdk2/cyclin E commits a cell to enter S-phase, and Cdk2/cyclin A initiates and maintains S-phase: Cyclins D, E, and A are all rate-limiting for S-phase entry and have independently been identified as proto-oncogenes ((81,94) and references therein). However, with one notable exception, these Cdks have yet to be assigned to *in vivo* substrates.

Tumor suppressors are targets for inhibitory phosphorylation by Cdks. The only G1 Cdk target in mammalian cells whose role is universally recognized is the prototype of tumor suppressors, the retinoblastoma protein RB (81,94). RB becomes phosphorylated at multiple serines and threonines as cells approach late G1. It is now assumed that, in normal healthy cells, RB blocks cell cycle progression in G1, and phosphorylation of RB is required for S-phase entry (reviewed in (95-97)). The kinases that inactivate RB are, in this order, Cdk4, 6/cyclin D1, 2 in mid-to-late G1, most likely Cdk2/cyclin E in late G1, and Cdk2/cyclin A during S-phase (97). Whereas these Cdks readily phosphorylate RB *in vitro*, the details and consequences of these reactions *in vivo* remain to be elucidated (see Section 4.2). The importance of cyclin D in RB phosphorylation is highlighted by the observation that Cdk4, 6/cyclin D1 is dispensable in cells lacking RB (98-100). In contrast, cyclin E is required for S-phase entry even in RB^{-} cells (101). Furthermore, forced expression of both cyclins D1 and E leads to a greater acceleration of S-phase entry than either cyclin alone (102). These results suggest that Cdk2/cyclin E targets other proteins besides RB. Among the primary targets of RB activity are the E2F transcription factors which, upon phosphorylation of RB, dissociate from it and activate the transcription of S-phase genes including cyclin E (103). Another widespread view —once RB has been phosphorylated, it is more or less inert (104)— has been challenged by recent reports suggesting that RB^{phos} inhibits apoptosis (29) and stimulates DNA polymerase α (105). Other tumor suppressor proteins that are modified by Cdk-mediated phosphorylation include p53 (106), WT1 (107), APC (108,109), BRCA1 (110,111), and the RB family members p107 (112) and p130 (113,114). Unless phosphorylation marks them for cell cycle-dependent proteolysis —and so far, there is no evidence for this— a phosphatase is responsible for their cell cycle-dependent reactivation.

Tumor suppressors inhibit Cdks. Cdk inhibitors (CKIs), which were discovered about 5 years ago (87,88), are now represented by several major types: $p16^{Ink4}$, $p21^{Cip1}$, and $p27^{Kip1}$. Some of them share critical features with RB: Loss-of-function is found in a variety of human tumors (reviewed in (79,80,115)) and associated with accelerated tumor development in knockout mice (reviewed in (116)). As could be expected, over-expression of all types of CKIs invariably causes cell cycle arrest (99,117,118) and/or apoptosis (119-121). Therefore, CKIs are now being considered true tumor suppressors. p21 provides a link between the growth-suppressing role of p53 and the cell cycle machinery: p53 induction in response to irradiation or DNA-damage induces p21 which in turn inhibits G1-specific Cdks (122). In addition, p21 is induced by TGFβ, thus bypassing p53 (123). The p16 family of CKIs, which are also rapidly induced by TGFβ (124), specifically inhibit cyclin D kinase complexes, thereby blocking the initial RB phosphorylation (99). In essence, CKIs may function through setting a threshold that Cdks must overcome to trigger cell cycle entry or certain transitions (90).

Cell proliferation and cell death requires proteolytic degradation. The discovery of the first cyclin in 1983 also included a novel regulatory mechanism: This cyclin is synthesized and proteolytically destroyed in a cell cycle-dependent manner (125). We know now that proteolysis plays a pivotal role in both cell proliferation and cell death (figure 1). Thus, in addition to mitotic cyclins, proteolysis destroys other cyclins during other phases of the cell cycle, and in addition to cyclins, proteolysis destroys other cell cycle regulators, some during normal cell cycle progression (reviewed in (126)), and others at the beginning of and during programmed cell death (reviewed in (127)). A particularly illuminating example for a presumably general strategy is the Cdk inhibitor $p27^{Kip1}$, as it provides a link between ubiquitin-mediated proteolysis (128) and cell cycle-dependent phosphorylation, especially by Cdk2/cyclin E (129-131). In fact, the phosphorylation of $p27^{Kip1}$ triggers its proteolytic degradation (131,132). Thus, $p27^{Kip1}$ joins the ranks of several other cell cycle regulators (G1 cyclins (133) and CKIs (134,135) in yeast, human *cdc25p* (136)) that undergo both types of post-translational modification. Studies in yeast have suggested that ubiquitin-mediated proteolysis is required for progression into S-phase (137). While phosphorylation can have all sorts of effects on a protein's activity, the one obvious goal of ubiquitin-mediated proteolysis is to destroy a protein. In the cell cycle, the true significance of proteolysis might be to inactivate a protein reliably, when its normal function would prevent initiation of the next phase, or when it is not required or even detrimental for extended periods of time; in other words, proteolysis might ensure irreversible cell cycle transitions. Perhaps not surprisingly, phosphorylation and proteolysis have been found to play a key role in apoptosis as well, possibly providing the molecular tools for exercising checkpoint controls over cell death pathways (138) (for examples, see Section 4). Late in apoptosis, the degradation of many structural proteins probably serves the purpose to eliminate the dying cell without a trace.

4. THE CELL CYCLE FROM A PHOSPHATASE'S POINT OF VIEW

The potential significance of dephosphorylation. From the above discussion it should be clear that Cdk activity (and therefore, cell cycle progression) is primarily regulated on four levels: *de novo* synthesis of cyclins as well as the CKIs, the assembly or disassembly of Cdk/cyclin/CKI complexes, the proteolytic destruction of cyclins or CKIs (proteins whose continued presence may prevent entry into the next phase of the cell cycle), and, of course, phosphorylation and dephosphorylation. However, with the notable exception of the dual-specificity phosphatase *cdc25p*, protein phosphatases are hardly ever mentioned in even the most recent reviews. Clearly, the phosphorylation of key proteins is required or even sufficient (139) to allow transition from G0/G1 to S and thus, promote cell proliferation. Given this premise, phosphatases might be expected to be the most natural inhibitors of cell proliferation (*cf.* ref. (6)), either by activating growth inhibitors or by inhibiting growth stimulators.

Timing is everything. It follows from the above that identification of the protein phosphatases that oppose Cdk action and understanding their regulation is necessary to fully appreciate cell cycle control. Phosphatase regulation may be required or at least beneficial for two reasons: Although changing kinase activity alone would result in altered phosphorylation state of a protein, changing kinase and phosphatase activity in opposite direction would be much more cost-efficient. This reciprocal regulation would ensure maximal phosphorylation or dephosphorylation of a given substrate at a given time and is well-documented for the enzymes regulating glycogen metabolism (140). Considering those proteins that are phosphorylated and then degraded, one might argue that a phosphatase cannot re-activate them. However, a phosphatase may interfere with the phosphorylation event triggering proteolysis and thus stabilize a protein. Second, to ensure an orderly cell cycle, phosphorylation and dephosphorylation occur at the appropriate, yet distinct times (figure 1). Inasmuch as phosphorylation at inappropriate times accelerates the cell cycle (refs. quoted above and (139)), dephosphorylation at inappropriate times may be expected to inhibit cell cycle progression, an idea that we have tested experimentally for PP1α (see Section 4.2).

4.1. Mitotic Entry and Exit

Protein phosphatases enter the realm of molecular cell biology. Despite the excitement created by the discovery of okadaic acid and its *in vitro* mechanism of action (which provided indirect clues about protein phosphatases in cell growth control), the first convincing evidence establishing a role for PP1 came from an unexpected corner. In 1989, shortly after the cDNA encoding mammalian PP1 was cloned (50,51), a series of studies based on the analysis of fungal or yeast mutants revealed that in *Aspergillus nidulans* and *S. pombe*, proteins that are over 80% identical to mammalian PP1, are required for the separation of daughter nuclei, completion of anaphase or chromosome segregation (141-143). The same approach was used the following year to demonstrate that PP2A from *S. pombe* inhibits entry into mitosis (144). As in fungi and fission yeast, PP1 was found to be essential for mitosis in *Drosophila* (145). Supporting the notion that cell cycle-regulatory mechanisms are conserved across distant species was the finding that PP1α can rescue the *Aspergillus* mutant *bim*G (146). These data were confirmed biochemically in vertebrate cells: Microinjection of neutralizing PP1 antibodies into early mitotic cells arrests them in metaphase (147). The same study also showed that PP1 translocates from the cytoplasm to the nucleus during G2 (147). One crucial mitotic function of PP1 may be to bring about the necessary inhibition of Cdk1 by dephosphorylating Thr^{161} in Cdk1 (148). Another important role of PP1 may be to dephosphorylate the nuclear Cdk1 substrate lamin (149), thereby permitting the repolymerization that is necessary to rebuild the nuclear envelope at the onset of cytokinesis (150). An important substrate for PP1, at least in mammals, is the RB tumor suppressor protein that is dephosphorylated by a type-1 phosphatase activity during mitosis (151). Whether this reaction is essential for mitotic exit is unclear at this point. However, this seems unlikely, because PP1 is also essential for mitotic completion in organisms (see above) which — as far as we know— do not express a homolog of mammalian RB. Because of the significance of RB for G1 regulation, this topic will be further discussed in Section 4.2.

PP2A's inhibitory effect on mitotic entry may be explained by its regulation of the mitosis-specific form of Cdk1 in mammalian cells (25,152), *S. pombe* (144) and *Xenopus* (153). PP2A most likely inactivates *cdc25p* (154-156), which removes the inhibitory phosphate groups at Thr^{14}/Tyr^{15} in Cdk1 (157,158), and activates the dual-specificity kinase *wee1/myt1*, which phosphorylates Thr^{14}/Tyr^{15} (159,160). The inhibition of mitotic entry by PP2A cannot be absolute, because otherwise cells would never be able to proliferate. In *S. cerevisiae*, the activity of PP2A towards the Cdk1-regulating enzymes appears to be reduced by the PP2A-regulatory subunit *Cdc55* (a homolog of the mammalian B subunit) (161). In mammalian cells, the potentially oncogenic homeobox gene product of *hox11* primarily inhibits PP2A, thereby eliminating a G2/M checkpoint (162). PP2A's role in mitotic progression is more complicated, however. In addition to preventing Cdk1 activation, PP2A may act downstream of Cdk1, *i.e.* reverse the phosphorylations catalyzed by Cdk1. Evidence for this scenario also comes from at least two angles: Loss of the B subunit in *Drosophila* results in abnormal anaphase (163), and PP2A dephosphorylates physiological Cdk substrates *in vitro* (164).

In summary, early evidence from a variety of organisms suggests that i) PP2A inhibits entry into mitosis, and ii) PP1 is necessary to complete mitosis. So far then, the experimental evidence is inconclusive with regards to the tumor suppressor hypothesis. As we have seen in Section 3.2, it might make more sense for a typical tumor suppressor to interfere with cell proliferation in G1, *i.e.* before cells commit to replicate its DNA and divide.

4.2. The G1/S Transition

The enormous interest in the regulation of G1 progression was spurred by the discovery that a gene product known to play a critical role in human cancer is involved in cell cycle control. The first experimental evidence for this concept came from the observation that the prototypical tumor suppressor protein, RB, is phosphorylated in a cell cycle-dependent manner (165-168) by Cdks (see Section 3.2.2). This original finding has inspired many elegant and informative experiments in the last 9 years, and thus, RB is the best-studied protein in G1 progression. RB is now being thought of as the key player in regulating passage through the R point, and the accumulated evidence, suggesting that one or more members of the RB-pathway ($p16^{Ink4}$, Cdk4/cyclin D, RB itself) is mutated in virtually every human cancer (122), justifies the tremendous efforts invested in this area. Nonetheless, the molecular interactions of the distinctly phosphorylated forms of RB as well as the significance of the up to 16 potential Cdk phosphorylation sites still remain controversial and will be further discussed below.

RB is dephosphorylated by PP1. Despite these undisputed challenges, it was generally accepted very quickly that phosphorylation of RB abrogates its inhibitory effect on cell proliferation (169). The distinct phosphorylation pattern of RB (active, unphosphorylated RB during G1, and hyper-phosphorylated RB during late G1 through M) implied that one or more phosphatases might function to activate a tumor suppressor. This hypothesis was in fact confirmed by several different approaches. Equally important was to ask the question which phosphatase(s) act upon RB. Several laboratories including this one have identified PP1 as being partially or wholly responsible for the activation of RB. Thus, mitotic cell extracts treated with inhibitor-2 fail to modify the phosphate content of RB, suggesting that the mitotic dephosphorylation of RB is due to PP1 (151), while microinjection of PP1 into cells synchronized in G1 accumulates (presumably hypophosphorylated) RB in the nucleus and inhibition of DNA synthesis (170). In a yeast two-hybrid screen, RB specifically associates with a form of PP1α (171). Finally, inhibition of PP1 in quiescent cells significantly increases RB phosphorylation (172). Of course, none of these results can rule out that other phosphatases participate in RB dephosphorylation as well, but, as RB maintains cells in G1, we hypothesized that PP1 might be required to keep RB in a dephosphorylated, fully active state during G1 and thus play an important role in G1/S regulation (172).

Which of the many forms of PP1 dephosphorylates RB? More recently, Nelson *et al.* presented evidence that the mitotic dephosphorylation of RB in monkey kidney cells is catalyzed by a heterodimeric complex consisting of PP1 and a 110 kDa regulatory subunit (p110) which seems to increase the RB phosphatase activity of PP1 (173,174). All three isoforms of PP1 associate with p110 and have RB phosphatase activity (173). In contrast, in mitotic HeLa cell extracts, RB appears to associate with PP1β only (175). Yet, another variation of this issue was described earlier this year, when a novel nuclear PP1α targeting subunit, named PNUTS, was identified in a yeast two-hybrid screen (176). PNUTS, curiously enough, has an apparent molecular mass of 110 kDa. However, it is doubtful whether p110 and PNUTS are identical, as PNUTS appears to inhibit PP1 activity and prevent PP1 from binding to RB (176). The possible involvement of another subunit directing PP1 towards RB notwithstanding, PP1 itself has two potential RB-binding sites (LXSXE). Whether these RB-recognition sites are significant remains to be investigated (for another review of the RB-PP1 interaction, see (177) this volume).

It may take more than one phosphatase to activate RB. Although cell cycle-dependent RB phosphorylation has been known since 1989, we have yet to come to an understanding of the significance of the up to 16 potential Cdk phosphorylation sites in RB (178-182). Thus, the role of individual kinases and RB phosphorylation sites remains controversial. A decisive role was suggested for Cdk4/cyclin D1, based on the observation that this kinase is the only one that phosphorylates Ser^{795}, which is sufficient to inactivate the G1-arrest function of RB (180), whereas other workers showed that Ser^{795} is effectively phosphorylated by Cdk4/cyclin D1, Cdk2/cyclin E, and Cdk2/cyclin A (182), and that Cdk4/cyclin D1 generates a form of RB that is still active (183). Also, phosphorylation of $Thr^{821/826}$ disables binding of RB to LXCXE-containing proteins (179,182), phosphorylation of $Ser^{807/811}$ disables binding of RB to c-*abl* (179), and phosphorylation of Ser^{780} abolishes the association with E2F (181). In contrast to these experiments, which were based on *in vitro* incubations or over-expressing Cdk/cyclins, the specific inhibition of Cdk/cyclin complexes led to the conclusion that both Cdk4/cyclin D and Cdk2/cyclin E are required to inactivate RB (184).

At any rate, it is now possible to ask whether PP1α directly dephosphorylates RB, and if so, whether PP1α prefers a subset of sites. Data from this lab suggest that the answer to both questions is yes, making it worthwhile to ask which sites in RB are dephosphorylated *in vivo*. The ongoing characterization of RB phosphorylation sites and the responsible kinases will hopefully assist to solve this problem. It is generally assumed that the active, fast-migrating form of RB protein present in G0-early G1 is converted into a slow-migrating form upon inactivating phosphorylation at multiple sites in late G1. The contribution of individual residues to this conformational change has now been determined: Fully phosphorylated RB mutants lacking Thr^{356}, $Ser^{807/811}$, or Thr^{821}, either alone or in combination, migrate to a position that corresponds to the hypophosphorylated form of RB and show enhanced growth-suppressing activity (185). The availability of these recombinant RB wild-type and mutant forms has allowed us to identify the phosphoamino acid residues that are dephosphorylated by PP1α. Following Cdk-mediated phosphorylation of RB and addition of purified PP1α, it became clear that PP1α preferred to dephosphorylate certain sites, most notably Thr^{356}, as well as $Ser^{807/811}$ (Driscoll, B., C. Liu, and N. Berndt, manuscript in preparation). These sites were found to be associated with G1 arrest earlier (178). Despite the caution that is necessary when interpreting this *in vitro* experiment, two things are remarkable about these results: First, PP1α possibly dephosphorylates sites that are predicted to convert inactive to (more) active RB; secondly, complete dephosphorylation of RB may require another phosphatase acting upon RB before or after PP1α.

The PP1 catalytic subunit is regulated by phosphorylation. The finding that PP1 apparently activates RB raised the question whether PP1 itself is modulated during the cell cycle. It turns out that in synchronized MG63 cells, cytoplasmic PP1 activity is maximal in G0/G1 and dramatically reduced in the remaining phases of the

Table 1. Effects of PP1α on cell cycle progression in RB^+ and RB^- cells (196)

Enzyme	MG63 cells	HL60 cells	Saos2 cells
	$RB^+/p53^+$	$RB^+/p53^-$	$RB^-/p53^-$
		Effect on G1 progression	
PP1α	G1 delay	G1 delay	None
PP1αT320A	G1 arrest	G1 arrest	None
		Effect on cell death	
PP1α	None	None	Increase
PP1αT320A	Increase	Increase	Increase

cell cycle; nuclear, or chromatin-associated PP1 activity peaks in G0/G1 and in mitosis (186). As the PP1 amounts do not change, we asked whether post-translational modification could account for the activity changes. This hypothesis was further supported by the fact that eukaryotic PP1 isoforms (except in *S. cerevisiae*) bear a motif (TPPR) in the C-terminal region that corresponds to a preferred Cdk phosphorylation site. Our experiments showed that, following incubation with Cdk1/cyclin A and ATP, purified PP1 is readily phosphorylated and inactivated in a time-dependent manner, and the site of phosphorylation is the suspected residue, Thr^{320}, in PP1α (186). An important question was whether this phosphorylation event takes place *in vivo*. This problem was approached from two different angles: First, we generated a recombinant PP1α where Thr^{320} was replaced by alanine. As this mutant, PP1αT320A, is fully active, we predicted that it functions as a constitutively active PP1 in cells expressing wild-type PP1α. This enzyme would therefore be a valuable "tool" to explore the biological function of Cdk-mediated phosphorylation of PP1α (see below). Second, we used specific antibodies kindly provided to us by Emma Villa-Moruzzi and Angus C. Nairn to probe directly for cell cycle-dependent phosphorylation of PP1α. As has been suggested before for human cells (187,188), these experiments confirmed that (up to 20% of) PP1α is phosphorylated at the onset of mitosis; in addition, a smaller fraction (perhaps 5-10%) of cellular PP1α was also phosphorylated in cells released from a late G1 or G1/S arrest (Liu, C. and N. Berndt, manuscript in preparation). Attempts are now underway to identify the kinases that inhibit PP1.

Failure to inactivate PP1α arrests cell proliferation. Another example for the notion that very similar mechanisms regulate the cell cycle across species is the finding that one of the PP1 isoforms in fission yeast, *dis2*, is also phosphorylated by Cdk1 at Thr^{316} (which corresponds to the mammalian Thr^{320}) during early mitosis (189). Interestingly, overexpression of *dis2*T316A causes G2/M arrest (189). To investigate the role of PP1 in the mammalian cell cycle, we compared the effects of wild-type PP1α and PP1αT320A on G1 progression, RB phosphorylation state, proliferation rates, and cell viability in MG63 ($RB^+/p53^+$) osteosarcoma cells. Avoiding the infamous problems of over-expressing active PP1 in mammalian cells, we purified PP1α expressed in *E. coli* (190) and introduced the recombinant protein into cells by electrotransfer. These experiments showed that both phosphatases lengthen the G1 phase when they are introduced into cells in early G1. When transferred into cells in late G1, wild-type PP1α is unable to interfere with RB phosphorylation and prevent cells from entering S-phase. In contrast, mutant PP1α appears to prevent RB phosphorylation entirely and blocks S phase entry. Consistent with these data, a moderate (8-fold) excess of wild-type PP1α decreases the proliferation rate, whereas a 6-fold excess of mutant PP1α causes a complete growth arrest that lasts for about 3 days, at which time the recombinant protein is getting degraded. To our surprise, in Saos2 ($RB^-/p53^-$) osteosarcoma cells, neither PP1 affects the G1/S transition or the proliferation rate (table 1). This was unexpected, as there are other proteins besides RB whose phosphorylation is thought to enable cells to replicate DNA (*e.g.* DNA polymerase α (191,192), DNA topoisomerase II (193)) or ensure undisturbed cell cycle progression (*e.g.* transcription factor E2F (194,195)), not to mention the other tumor suppressors introduced in section 3.2.2. Any of these proteins would be a potential substrate for PP1 (table 2). In HL60 ($RB^+/p53^-$) leukemia cells, the effects of both wild-type and mutant are similar to those in MG63 cells, suggesting that PP1α bypasses p53-regulated pathways (196). 24 h after loading asynchronous RB^+ cells with enzyme, it becomes apparent that PP1αT320A, but not PP1α, causes cells to die more frequently (30 % trypan blue-positive cells *vs.* < 5 % in controls). Yet another surprise was that, in RB^- cells, the apparent induction of cell death is seen in response to both wild-type and mutant PP1α (196). Before these data will be further evaluated in context in Section 4.4, it seems necessary to introduce current concepts about phosphatases and apoptosis.

4.3. Apoptosis—Another Way of Exiting the Cell Cycle

Certain proteins link the cell cycle to cell death. Apoptosis, in providing an elegant means of removing unwanted cells, is thought to be necessary for the development and homeostasis of the multicellular organism. It is beyond the scope of this article to provide an in-depth review of the field, however, as already mentioned, we have recently come to realize that certain cell cycle-regulatory proteins provide a link between cell cycle control and apoptosis (reviewed in (67-70)). At the heart of the apoptotic program appears to be a series of proteolytic reactions, catalyzed by the caspase family or proteases (127,205). Proteins undergoing proteolytic cleavage include structural proteins, signaling proteins, enzymes involved in

Table 2. Potential substrates for Ser/Thr-specific protein phosphatases that are important in cell cycle control or apoptosis

Protein	*Result of phosphorylation*	Phase	PK-PP	Refs.
Proteins where the phosphatase is known				
RB	Inhibition of G1 arrest function	Late G_1-M	Cdks - PP1	See text
cdc25p	Activation—Mitosis	G_2/M	Polo - PP2A	See text
Lamin	Depolymerization of nuclear envelope	M	Cdk1 - PP1	See text
Growth or tumor suppressors				
APC	Loss of function?	M	Cdk1	See text
BRCA1	Dissociation from centrosome?	M	Cdk1	See text
$p21^{Cip1}$	?		Cdk2/cycA	1
p53	S15—decreased inhibition by mdm2		Cdk	2
$p27^{Kip1}$	Recruitment by ubiquitin	G_1	Cdk2/cycE	See text
p107	Inhibition of G1 arrest function?	Mid G_1	Cdk	See text
p130	Inhibition of G1 arrest function?	Early-mid G_1	Cdk	See text
WT1	Inhibition of G1 arrest function?	G_1	Cdks	See text
MyoD	Degradation—no p21 induction	G_1	Cdk	3
Cell cycle-activating proteins				
Cdk-Thr^{161}	Activation—Cell cycle progression	All phases	CAK	See text
Condensin	Activation— Chromosome condensation	M	Cdk1	4
E2F	Inhibition of transcription	S-M	Cdks	See text
DNA pol α primase	Activation—DNA replication		Cdk2/cycA,E	See text
DNA topoisomerase	Activation		PKC	See text
Cell death-activating proteins				
Bad	Inhibition—Survival	n.a.	PKB	5
Caspase-9	Inhibition—Survival	n.a.	PKB	6

References: [1] $p21^{Cip1}$ is the latest addition to the CKIs that undergo proteasome-dependent degradation; whether it is the mere association with a Cdk or its phosphorylation that triggers proteolysis is not yet clear (197). [2] p53 is phosphorylated at multiple sites but the best-understood phosphorylation event so far is that occurring at Ser15 which decreases p53's interaction with its negative regulator mdm2 (106). [3] The unphosphorylated transcription factor MyoD induces p21 thereby causing cell cycle arrest that precedes the differentiation of myogenic cells; upon phosphorylation it becomes proteolytically degraded (198-200). [4] The novel protein condensin which is involved in chromosome condensation necessary for faithful distribution of genetic material becomes activated by Cdk1 (201). Both [5] Bad (202,203) and [6] caspase-9 (204) are inhibited by protein kinase B, thereby inhibiting apoptosis and/or promoting survival.

DNA/RNA modification and others. In contrast to cell cycle-dependent proteolysis, where proteins appear to be just destroyed, apoptotic proteolysis provides a way to either destroy (inactivate) or activate a protein. At any rate, the ultimate goal of apoptosis appears to be removal of a dying cell without collateral damage to neighboring cells. Just as cancer cells have a tendency to proliferate out of control, they are frequently unable to die. Typically, this is due to activation of oncoproteins that block cell death (*e.g.* Bcl-2, (206)), and/or the loss of tumor suppressors such as p53 and the CKIs that can stimulate apoptosis (*cf.* Section 3.2.2).

RB, phosphatases, proteases, and cell death. Paradoxically, the RB protein has been called an inhibitor of apoptosis, a role that is not easily reconcilable with its supposed function as a tumor suppressor. There is good evidence for RB-mediated protection from apoptosis though, most notably the increased cell death observed in neuronal tissues of $RB^{-/-}$ mice (207). Furthermore, in tumor cells that have lost RB function, over-expression of wild-type RB inhibits apoptosis (208-211). Consistent with this model is the recent discovery that RB undergoes caspase-dependent cleavage during apoptosis (212-216). However, these studies did not rigorously distinguish between the distinct phosphorylation states of RB. Thus, it is not clear that differentially phosphorylated forms of RB have the same effect on apoptosis, or are equally good substrates for caspases. In other words, it is not clear when in the cell cycle RB inhibits apoptosis. Neither is it clear that loss of RB is equivalent with phosphorylated (putatively inactive) RB. That it might be necessary to clarify these issues before definitive statements can be made about RB's role in apoptosis is illustrated by several other recent studies that —by the way— have introduced phosphatases into the equation. For example, exposure of hepatoma cells to TGFβ induces RB dephosphorylation

and apoptosis (217). Short-term inhibition of phosphatases with OA, calyculin A, and cantharidin prevents DNA fragmentation, PARP cleavage and dephosphorylation of RB by several apoptosis-inducing agents, and induction of apoptosis is associated with S-phase and loss of RB phosphorylation (29). Thirdly, treatment of HL60 cells with the anti-cancer drug cytosine arabinoside (araC) induces RB phosphatase activity, G1 arrest and apoptosis (218). The last group of researchers also found that RB becomes dephosphorylated first and then degraded (212,218). All of these results suggest that a phosphatase acts on RB first before the apoptotic program is executed. As such these data are reminiscent of our own data described above and are consistent with a model in which one or more forms of RB^{phos}, rather than RB *per se*, inhibits apoptosis. Note that loss of RB has been shown to be associated with inappropriate entry into S-phase both in mouse fibroblasts (219) and mice (207) Given that a cell replicating its DNA is bound to divide, it is conceivable that mechanisms exist to protect a cell in S-phase from apoptosis. If RB prevents S-phase entry, and if RB^{phos} stimulates DNA polymerase α (105), the hypothesis that RB^{phos} may play such a role is worthy of further testing. As in the above studies the phosphatases involved were not identified, we have initiated a study to characterize the role of PP1 in araC-induced apoptosis. The evidence so far suggests that PP1α and β is activated before DNA fragmentation, PARP cleavage and propidium iodine uptake is detectable (Wang, R.-H. and N. Berndt, unpublished). Further investigations using elevated phosphatase and phosphatase inhibition in both araC-responsive and araC-resistant cells will clarify whether PP1 is required for launching cell death.

Although proteolytic cleavage seems to be instrumental in apoptosis, so far no single protein has been identified whose degradation is absolutely required for cells to die. At this time, it is more likely that apoptosis requires the cleavage of a large number of proteins (220). If this turns out to be true, protein phosphatases and kinases can be expected to participate in apoptosis in a variety of ways, all of which may ultimately modulate proteolytic activity or convert proteins into better or worse caspase substrates. Dephosphorylation of a variety of proteins is emerging as a positive regulator or apoptosis. Undoubtedly, this model will be confirmed or perhaps modified by future discoveries of other apoptotic phosphorylation events. The phosphatases identified so far are PP1 (see above) and PP2A (221-223), both of which appear to act downstream of caspases. However, the first caspase to be inhibited by protein kinase B-mediated phosphorylation has just been identified (204). As phosphorylation seemingly regulates everything, others may soon follow. Thus, a phosphatase would also be predicted to activate apoptosis by directly regulating an enzyme involved in the execution of apoptosis.

4.4. A Model for the Cell Cycle Regulation and Function of PP1

PP1 — Positive or negative regulator of the cell cycle, or both? There is compelling evidence that PP1 is required for completion of mitosis in many eukaryotic organisms. This can be seen as promoting cell cycle progression. On the other hand, constitutively active PP1 mutants, *i.e.* PP1 molecules that are resistant to inhibition by Cdk-mediated phosphorylation are able to block cell cycle progression in both *S. pombe* (189) and human cells (196). The interesting distinction is that such a PP1 blocks mitotic initiation or progression in fission yeast (189), but initiation of S-phase in human cells (196). Thus, it seems that inhibitory phosphorylation of PP1 may be required to permit progression into the next phase of the cell cycle. In *S.pombe*, this inactivation is transient, as PP1 becomes dephosphorylated at the onset of anaphase (224). Given that in fission yeast, cell growth (and growth control) is primarily associated with G2, whereas mammals are thought to make the important decisions about growth and proliferation in G0/G1 (see Section 3.2.1), a mechanism implicating PP1 in the control of entry into S-phase may be more useful. Interestingly, over-expression of mouse PP1 catalytic subunits also causes mitotic growth arrest and cell death in *S.pombe* (225). The role of PP1 in mammalian M-phase entry is uncertain. While our experiments did not provide evidence that PP1α induces G2/M arrest (*cf.* figure 3 in ref. (196)), we did not directly address this issue as that study focused on the G1/S transition. It is conceivable though that sustained activity of PP1α causes apoptosis in G2. This hypothesis will need to be tested. Considering this body of work, mammalian PP1 is both a positive and a potentially negative regulator of the cell cycle at the same time. However, PP1 and other proteins which are necessary for mitotic exit, or in other words, G1 entry, cannot be considered as positive regulators of cell growth or proliferation, as they function <u>after</u> the last important decision to divide has already been made and <u>before</u> the next growth period. In fact, proteins promoting mitotic entry or exit at the right time, may even be advantageous as they would contribute to the integrity and genomic stability of the cell.

Regulation of PP1 activity. Most regulatory subunits of PP1 are modified by phosphorylation and thus direct and modulate its activity towards various substrates. The discovery that PP1 catalytic subunits are phosphorylated in a cell cycle-dependent manner in both yeast and human cells suggests that, the significance of regulatory subunits notwithstanding, they are insufficient to fully regulate PP1. It is, however, possible that two or more mechanisms cooperate in order to modulate the cell cycle functions of PP1. Particularly noteworthy is inhibitor-2, which fluctuates in a cell cycle-dependent manner (226) and translocates to the nucleus at the G1/S transition, thereby providing another means to inhibit nuclear PP1 (227).

Downstream targets of PP1. The apparently unphosphorylated RB that is prevalent in response to elevated levels of PP1αT320A can be explained by several mechanisms, alone or in combination: PP1α may directly dephosphorylate RB, or it may interfere with the pathways that activate the RB kinases Cdk4, 6 and/or Cdk2 thereby

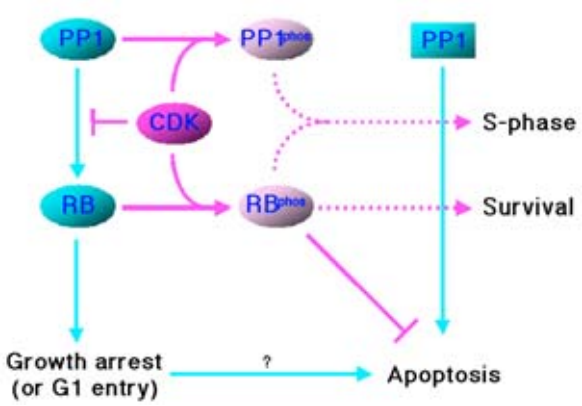

Figure 2. Proposed role for PP1 in the G1 phase of the cell cycle. This model is based on the observation that phosphorylation-resistant PP1 causes RB-dependent G1 arrest (196). During G0/G1, PP1 may be required to keep RB in an active form (172), which is also consistent with the observation that PP1 binds to hypophosphorylated RB (177). In late G1, PP1 is —like its target RB— inhibited by Cdk-catalyzed phosphorylation in order to commit cells to S-phase entry. RB^{phos} appears to be consistent with survival or may even inhibit apoptosis. PP1 may be able to induce apoptosis via RB-independent pathways without prior cell cycle arrest. Solid green arrows show reactions that promote cell proliferation, dashed green arrows show reactions that permit cell proliferation. Red arrows show reactions that block cell proliferation.

preventing RB phosphorylation. The first model is not necessarily in conflict with our observation that PP1α prefers certain sites on RB, as those sites appear to be critical ones for RB's growth-suppressing activity. Other candidate substrates for PP1 are the critical threonines (Thr^{161} in Cdk1) in all Cdks and the inhibitor $p27^{Kip1}$ (figure 1). Further experiments will be needed to determine the exact position of PP1 in RB-regulatory pathways. In addition, it remains to be seen what the other isoforms of PP1 are capable of doing in the cell cycle. Recently it was shown that mammalian PP1 isozymes locate to markedly different cellular compartment/structures in the cell cycle. While all isozymes can be found in the cytoplasm during interphase, the nuclear localization varies considerably as cells proceed from interphase to mitosis: PP1α is associated with the nuclear matrix and then centrosomes, PP1β with chromatin and then chromosomes, and PP1γ1 with the nucleolus and then mitotic spindles (228). This study will certainly aid in future experiments aimed at determining PP1 substrates.

PP1 at the crossroads of cell cycle arrest and cell death. Furthermore in human cells, the ability of PP1α to induce late G1 arrest not only depends on the failure to be phosphorylated at Thr^{320}, but also on the presence of functional RB protein. The continued presence of —presumably dephosphorylated—RB is apparently consistent with or even required for the cell death that follows G1 arrest (196). In $RB^{-/-}$ cells, cell death occurs independent of PP1α's ability to serve as a substrate for Cdks and without detectable cell cycle arrest. Therefore, we believe that PP1α may facilitate apoptosis by acting on proteins other than RB, and that different populations of PP1α may be regulating apoptosis and the cell cycle, with only the latter being subject to Cdk phosphorylation. In agreement with other reports (29,212,217,218), these data would suggest that RB^{phos} protects cells from apoptosis or is consistent with survival.

In summary, the regulation and function(s) of PP1 in the G1-phase of the cell cycle are portrayed in figure 2. The key features of this model, taking into account data from this and other laboratories, are that PP1 promotes G1 entry and thereby contributes to cellular integrity and may help to maintain cells in G1 unless the conditions for commitment to DNA replication are met.

5. PERSPECTIVES

5.1. Malignant Transformation, Tumorigenesis and Tumor Progression

At the conclusion of this review, a re-evaluation of the tumor suppressor hypothesis seems in order. Conventional wisdom has taught us that to qualify as a tumor suppressor, a protein has to inhibit cell proliferation and/or permit cell death, it has to inhibit oncogenic transformation and/or tumorigenicity, and it should have undergone loss-of-function mutations in malignant tumors. PP1α meets at least one out of the three criteria in that its activity is able to trigger exit from the cell cycle. It remains to be determined whether PP1 can inhibit oncogenic transformation. As already mentioned, the basis for transformation is often associated with escape from G1 control. For example, *ras*, a frequently amplified human oncogene, targets cyclin D1, thereby causing premature activation of Cdk4, 6 and RB phosphorylation (229,230). As PP1α interferes with this pathway, it is worth testing whether PP1 can suppress cellular transformation by *ras* and other oncogenes that disrupt cell cycle regulation. Provided the experimental outcome supports the hypothesis, it may then be worthwhile to investigate whether PP1 also can reduce tumorigenicity *in vivo*. Similar predictions could be made for PP2A: In addition to being a potential inhibitor of mitosis, PP2A may induce ceramide-mediated apoptosis (221), and inhibit nuclear telomerase (231), an enzyme that is thought to play a pivotal role on the way to malignancy (232). Upstream regulators of PP2A include SV40 small-t antigen (233) which appears to induce cell proliferation by inhibiting PP2A (234), oncogenic protein tyrosine kinases which inactivate PP2A *in vitro* (235) and *in vivo* (236), and a novel inhibitor of PP2A that is associated with certain leukemias (237), suggesting that inhibition of PP2A may be critical to permit oncogenic transformation.

However, there is only scant evidence as to whether phosphatases play a role in human tumorigenesis. No loss-of-function mutations for either PP1 or PP2A have been reported so far. On the contrary, PP1 isozymes are over-expressed in a variety of malignancies (54,57-61). In the

light of PP1's apparent function as a cell cycle inhibitor (170,189,196,225), these findings, however, do not support the claim that PP1 contributes to tumorigenesis. This conclusion would have to be based on a demonstration that loss of PP1 reduces susceptibility to tumors. What can we expect from a phosphatase knockout mouse? In conjunction with PP1's and PP2A's requirement for multiple cellular processes, the failure to detect phosphatase mutations strongly suggests that phosphatase gene disruption turns out embryonically lethal. In fact, this was shown for the first time during the writing of this article: Mouse embryos lacking the catalytic subunit PP2Aα show several defects related to differentiation causing them to die around day 5-6 (238).

Arresting cell cycle progression has recently become a much-debated way to combat cancer; another suitable if not better strategy would be to restore the response to apoptosis-inducing signals in malignant tumors. In principle, both of these aims can possibly be achieved by down-regulating oncogenes or replacing tumor suppressor genes, by gene therapy or by targeting cell cycle control pathways with specific drugs. Looking further ahead, it may be useful to ask whether phosphatases such as PP1 are suitable targets to restore cell cycle control in tumor cells. Such a strategy could perhaps aim at activating the PP1 system, inhibiting PP1 inhibitors or preventing the inhibition of PP1. Current evidence suggests that in most if not all tumors the PP1 system is intact. It may also prove helpful to identify other proteins that similarly suppress proliferation but are essential for survival. Targeting such proteins might be more feasible than seeking to overcome the well-known difficulties associated with gene replacement therapy. Activation of such proteins would conceivably have no adverse effect on non-proliferating cells. Developing drugs designed to interact with PP1 will certainly be assisted by its known crystal structure (239,240) and the soon-to-follow structure-function analyses.

5.2. Other Phosphatases and Other Cellular Processes Related to Growth Control

PP1 and other phosphatases remain a fascinating target for future research, especially in the area of cell cycle control. A virtual unknown is the question, to what extent are phosphatases important in processes that have an impact on the cell cycle (size control, cell adhesion) or are regulated by it (differentiation). With the significance of protein phosphorylation established in all of these processes, the roles of protein phosphatases are asking to be discovered. As I hope this review has made clear, many unresolved issues remain until we understand the phosphatases' role as well as their fellow players, the cyclin-dependent kinases. It has been generally assumed that protein phosphorylation promotes, and protein dephosphorylation inhibits cell growth and proliferation. So far, the behavior of PP1 and other phosphatases appears to fit this model. For instance, MAP kinase phosphatase 1 has been shown to block S-phase entry (241) and induce apoptosis by dephosphorylating Bcl-2 (242). Likewise, two phosphatase 2C-like enzymes, Wip1 and FIN13, have been reported to inhibit cell proliferation upon over-expression (243,244). However, a note of caution may be appropriate here. There are examples demonstrating that no rule is without exceptions. The novel protein phosphatase 5, a member of the PPP family, does not fit the general pattern: Inhibition of PP5 expression by antisense oligonucleotides causes late G1 arrest (245). Furthermore, one should remember that the tyrosine phosphatase *cdc25p* is oncogenic (246), oncogenic E2F can apparently inhibit proliferation and induce apoptosis (247,248), and even protein kinases can be tumor suppressors (249,250). As the cellular functions of the PP1 enzyme system become unraveled, it may help to have an open mind for the occasional surprise.

6. SUMMARY

PP1 and other protein phosphatases that reverse the action of Cdks are emerging as important cell cycle regulatory enzymes. PP1 undergoes inhibitory phosphorylation by Cdk1 at the beginning of mitosis, perhaps to maximize the phosphorylation of Cdk substrates; PP1 is then required for completion of mitosis, but the critical substrate(s) remain to be identified. Beginning in mitosis, PP1 also reactivates the tumor suppressor RB which is most important in controlling the G1/S transition. To be able to enter S-phase, cells must inactivate RB by Cdk-mediated phosphorylation. PP1 can interfere with this pathway thereby causing G1 arrest, unless itself is phosphorylated and inhibited by Cdks. The mechanism by which PP1 activates RB or prevents the phosphorylation of RB remains to be investigated. PP1 activity also seems to play a positive role in initiating or executing apoptosis. Together, these features are reminiscent of other, better-characterized tumor suppressors. To evaluate whether this also makes PP1 a suitable target for anti-cancer strategies is one of the challenges lying ahead.

7. ACKNOWLEDGMENTS

I wish to thank past (Mariam Dohadwala) and present members (Cathy Liu, Rui-Hong Wang) of my laboratory for their invaluable contributions to this work and Barbara Driscoll as well as Yuen-Kai Fung for making available results before publication. Work in this laboratory discussed in this review was supported in part by grants to the author from the Tobacco-Related Disease Research Program of the State of California (3KT-0193) and the National Institutes of Health (R01-CA54167).

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Keywords: Protein phosphatase 1, protein phosphatase 2A, cell cycle, apoptosis, cyclin-dependent kinase, tumor suppressor, CDK inhibitor, RB protein

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Received 11/16/98, Accepted 12/15/98