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Oxidative Stress Induces Protein Phosphatase 2A-dependent Dephosphorylation of the Pocket Proteins pRb, p107, and p130*

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Oxidative stress induces cell death and growth arrest. In this study, the regulation and the functional role of the retinoblastoma family proteins pRb, p107, and p130 in the cellular response to oxidative stress were investigated. Treatment of endothelial cells with H_2O_2 induced rapid hypophosphorylation of the retinoblastoma family proteins. This event did not require p53 or $p21^{Waf1/Cip1/Sdi1}$ and was not associated with cyclin/cyclin-dependent kinase down-modulation. Four lines of evidence indicate that H_2O_2-induced hypophosphorylation of pRb, p107, and p130 was because of the activity of protein phosphatase 2A (PP2A). First, cell treatment with two phosphatase inhibitors, okadaic acid and calyculin A, prevented the hypophosphorylation of the retinoblastoma family proteins, at concentrations that specifically inhibit PP2A. Second, SV40 small t, which binds and inhibits PP2A, when overexpressed prevented H_2O_2-induced dephosphorylation of the retinoblastoma family proteins, whereas a SV40 small t mutant unable to bind PP2A was totally inert. Third, PP2A core enzyme physically interacted with pRb and p107, both in H_2O_2-treated and untreated cells. Fourth, a PP2A phosphatase activity was co-immunoprecipitated with pRb, and the activity of pRb-associated PP2A was positively modulated by cell treatment with H_2O_2. Because DNA damaging agents inhibit DNA synthesis in a pRb-dependent manner, it was determined whether the PP2A-mediated dephosphorylation of the retinoblastoma family proteins played a role in this S-phase response. Indeed, it was found that inhibition of PP2A by SV40 small t overexpression prevented DNA synthesis inhibition induced by H_2O_2.

Formation of reactive oxygen species (ROS)[1] is an unavoidable consequence of aerobic metabolism. Accumulation of ROS, which include hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radicals, can inflict damage to DNA, proteins, and fatty acids. It is generally believed that oxidative stress caused by ROS plays a causal role in aging as well as in numerous pathologies, including tissue ischemia and reperfusion, cancer, diabetic vasculopathy, atherosclerosis, Alzheimer disease, and pulmonary fibrosis (1–3).

To cope with the burden of oxidative damage, a series of enzymatic and non-enzymatic antioxidant defenses have evolved that neutralize ROS (2). In addition, cells also deploy "adaptive responses" aimed at minimizing the damages caused by ROS. Mammalian cells respond to oxidative stress with an increase in expression of antioxidant enzymes and activation of protective genes.

To defend against DNA oxidation induced by ROS, proliferating cells arrest their cell cycle, preventing the mutated DNA from being replicated and transferred to daughter cells. This delay in cell growth also allows the cells to repair the damaged DNA and to set up an adaptive response to counteract further oxidative damage. According to whether or not damaged DNA is repaired, cells either resume cell growth or enter a status of permanent growth arrest. Thus, growth arrest is a crucial component of the cellular response to oxidative stress. Moreover, ROS can also induce cell death, either by apoptosis or by necrosis, according to the level of oxidative stress experienced by the cell and its genotype (3).

Formation of ROS is not always associated with cell damage but is also induced during many physiologic cellular processes, including the regulation of signal transduction, gene expression, and proliferation (4). For instance, activated growth factor receptors increase intracellular ROS (5). Moreover, fibroblasts expressing a constitutively active isoform of Ras produce large amounts of ROS, and the mitogenic activity of cells expressing Ras is inhibited by treatment with antioxidants (6).

Therefore, depending on the concentration, the molecular species, and the subcellular localization, ROS exert two physiopathological effects, damage to various cell components and activation of specific signaling pathways.

The retinoblastoma family of growth-inhibitory proteins is an integral part of the mechanisms that regulate cell growth in normal conditions as well as after exposure to genotoxic stimuli. It consists of three members: the retinoblastoma protein (pRb) and the related p107 and p130 (also known as $p130^{pRb2}$) (7, 8). Collectively, these proteins are called "pocket" proteins because they have a domain, named the pocket, that can bind

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[1] The abbreviations used are: ROS, reactive oxygen species; pRb, retinoblastoma protein; CDK, cyclin-dependent kinase; PP1, PP2A, and PP2B, protein phosphatase type 1, 2A, and 2B; PP2A-C, PP2A-A, and PP2A-B, PP2A catalytic, structural, and regulatory subunits; p21, $p21^{Waf1/Cip1/Sdi1}$; HPV16-E6, oncoprotein E6 of human papilloma virus 16; HUVEC, human umbilical vein endothelial cells; NAC, *N*-acetyl-L-cysteine; BCNU, 1,3-bis(2 chloroethyl)-1-nitrosourea; OA, okadaic acid; CL-A, calyculin A; BrdUrd, bromodeoxyuridine; wt, wild type; red/ox, reduction/oxidation.

and regulate the activity of several cellular proteins. The family of E2F transcription factors is one major target of the pocket proteins. E2F plays an important part in cell cycle-regulated gene expression because it modulates, either positively or negatively, the transcription of a number of genes that are required for DNA synthesis (9). The ability of the pocket proteins to bind proteins such as E2F is abolished through cell cycle-regulated serine/threonine phosphorylation by cyclin-dependent kinases (CDKs). In the G_1 stage of the cell cycle, pRb is hypophosphorylated. At the G_1 to S-phase transition, it receives additional phosphates, and phosphorylation increases even further as cells progress through S and G_2. Not only pRb, but also p107 and p130, can be phosphorylated and thereby inactivated in a similar fashion (7, 8).

Inhibitors of CDKs, like $p21^{Waf1/Cip1/Sdi1}$ (hereafter named p21), provide another level of regulation (10, 11). High levels of p21 lead to the inhibition of CDKs and, in turn, to the accumulation of the pocket proteins in their under-phosphorylated form. Certain genotoxic stimuli elicit the activation of p53, which, in turn, transactivates the transcription of the *p21* gene. Alternatively, oxidative stress induces p21 protein levels, triggering the stabilization of p21 mRNA in a p53-independent manner (12).

Little is known about how phosphates are removed from the serine/threonine residues of the pocket proteins. During the cell cycle, pRb is dephosphorylated by protein phosphatase 1 (PP1) at mitotic exit and a physical binding between select forms of phosphorylated as well as hypophosphorylated pRb can be found with the isotype α of PP1 (13). Another major cellular phosphatase that could potentially contribute to dephosphorylation of pocket proteins is protein phosphatase 2A (PP2A) (14, 15). PP2A consists of a catalytic subunit (PP2A-C) and a structural subunit (PP2A-A). These two proteins form a core dimer to which a multitude of regulatory PP2A-B subunits associate. PP2A-B subunits regulate phosphatase activity, substrate specificity, and localization to cellular compartments (14, 15). Recently, Voorhoeve *et al.* (16) described the cloning of a PP2A component that specifically associates with p107, leading to the subsequent dephosphorylation of p107. Moreover, p107 is rapidly dephosphorylated in response to UV, and this phenomenon appears to be mediated through activation of a PP2A-related phosphatase (17). Finally, elevated cAMP in NRK-52E cells causes a marked reduction of pRb phosphorylation, and this dephosphorylation is blocked by the PP1 and PP2A inhibitor okadaic acid (OA) at concentrations selective for PP2A (18).

Understanding the cellular responses to oxidative stress will provide useful insights into the mechanisms of aging, tissue damage because of ischemia, and transformation as well as into the pathogenesis of a variety of aging-related diseases. In the present study, the regulation and functional role of pocket proteins in the cellular response to oxidative stress was investigated. The results obtained showed that following oxidative stress pocket proteins undergo PP2A-dependent dephosphorylation. PP2A core enzyme physically interacts with pRb and p107, and a PP2A phosphatase activity that is co-immunoprecipitated with pRb is positively modulated by cell treatment with H_2O_2. This event may be part of an intra-S-phase response that leads to a reduction of the rate of DNA synthesis.

MATERIALS AND METHODS

Cells and Drug Treatment—Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2-95% air. Human umbilical vein endothelial cells (HUVEC) (Clonetics) were maintained in EGM-2 (Bio-Whittaker). Cells were used between passage 4 and 6. H_2O_2 (30% w/w solution, Sigma) was administered to the cells as a solution in phosphate-buffered saline. *N*-acetyl-L-cysteine (NAC, Sigma) and 1,3-bis(2 chloroethyl)-1-nitrosourea (BCNU, Sigma) were dissolved in water, whereas OA (Sigma) and calyculin A (CL-A, Sigma) were dissolved in 10% Me_2SO. Aphidicolin was resuspended in Me_2SO. For S-phase synchronization, cells were incubated with 2 μg/ml aphidicolin for 14 h, washed twice with phosphate-buffered saline, and incubated for one additional hour in pre-warmed medium.

Retroviral Infection—Phoenix-ampho cells (American Type Culture Collection) were cultivated and transfected with pBABE-puro (19) or pBABE-puro E6 as described by Pear *et al.* (20). The medium containing the emerging retrovirus was harvested 36–48 h after transfection. To assay for infectious virus, HUVEC were infected and selected in puromycin-containing medium (0.5 μg/ml, Sigma). pBABE-puro E6 was generated from pGST-E6 with standard techniques (21).

Adenoviral Infection—SV40 small t wt- and C103S-encoding adenoviruses (22) are a kind gift from K. Rundell (Northwestern University, Chicago, IL.). Adenovirus infection was carried out as previously described (23). HUVEC were infected with 100 plaque-forming units/cell and cultured for an additional 18 h before further treatment.

Western Blotting—Cells were lysed in 2× Laemmli buffer and boiled for 5 min. Proteins were separated in SDS-polyacrylamide gels and transferred to nitrocellulose by standard procedures. The following antibodies, all from Santa Cruz Biotechnology unless differently specified, were used to detect the proteins of interest: anti-pRb (C15 or G3–245, BD PharMingen), anti-p107 (C-18), anti-p130 (C-20), anti-p53 (Ab-6, Oncogene Research Products), anti-p21 (C-19), anti-cyclin A (BF683), anti-cyclin E (C-19), anti-cyclin D1 (A-12), anti-cyclin D2 (C-17), anti-cyclin D3 (C-16), anti-CDK4 (C-22), anti-CDK2 (C-22), anti-CDK6 (C21), anti-CDK1 (C17), anti-E2F1 (C20), anti-p16 (H-156), anti-p18 (N-20), anti-p27 (C-19), anti-$p57^{Kip2}$ (C-20), anti-PP2A-C (610555, BD Transduction Laboratories), anti-PP2A-A (C-20), anti-α-tubulin (Ab-1, Oncogene Research Products), anti-phospho-pRb antibodies (pT356, 44–578; pT821, 44–582; pT826, 44–576, BIOSOURCE International).

Flow Cytometry—HUVEC were incubated for 10 min with 20 μM bromodeoxyuridine (BrdUrd, Sigma) and then fixed with 70% ethanol. Cell cycle analysis was performed by combined BrdUrd and propidium iodide staining using a Becton Dickinson flow cytometer (24). Cell Quest software was used to determine the percentage of BrdUrd-positive cells.

Immunoprecipitation—Immunoprecipitations were performed as previously described (25). Cells were suspended in lysis buffer containing 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 0.1% Tween 20, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 50 mM NaF and protease inhibitors (complete EDTA-free protease inhibitor mixture tablets, Roche Applied Science), followed by 30 min of incubation on ice and clearing by centrifugation at 14,000 rpm for 10 min. After pre-reclearing with A/G-agarose for 15 min, supernatants were immunoprecipitated for 2–3 h at 4 °C with protein A/G-agarose and 1 μg of relevant antibody. Immunoprecipitated proteins on beads were washed three times with wash buffer containing 60 mM Na_2HPO_4, 17 mM NaH_2PO_4, and 35 mM NaCl (pH 7.4), resuspended in 2× Laemmli buffer, separated by SDS-PAGE, and then immunoblotted with relevant antibodies.

Kinase Assay—Kinase assays were performed as previously described (25). Immunoprecipitations were performed as described in the previous section using 5 μg of the following antibodies: anti-cyclin D1 (M-20), anti-cyclin D2 (C-17), anti-CDK4 (C-22), anti-CDK2 (M2) (all from Santa Cruz Biotechnology). The following substrates were used: Histone H1 (Roche Applied Science) was used with anti-CDK2 immunoprecipitates and Rb-769 (pRb 769–921 domain, Santa Cruz Biotechnology) was used with anti-cyclin D1, anti-cyclin D2, and anti-CDK4 immunoprecipitates. Phosphorylated proteins were visualized by exposure to BioMax film (Kodak) and quantified using GS710 calibrated densitometer imaging (Bio-Rad) and Quantity one 4.1.1 software (Bio-Rad).

In Vitro Dephosphorylation Assay—Whole cell extracts were prepared from HUVEC after treatment with 800 μM H_2O_2 or solvent alone for 15 min. Cell extracts were then incubated for 4 h at 30 °C in phosphatase buffer (50 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM EDTA, 0.9 mg/ml bovine serum albumin, 0.09% β-mercaptoethanol) in the presence or absence of phosphatase inhibitors (1 mM sodium orthovanadate and 50 mM NaF), followed by Western blotting as previously described. Endogenous pRb was detected by Western blotting using a specific pRb antibody (C15, Santa Cruz Biotechnology).

Immunoprecipitation-PP2A Assay—HUVEC were suspended in lysis buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10% glycerol, 1.5 mM $MgCl_2$, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and protease inhibitors (complete

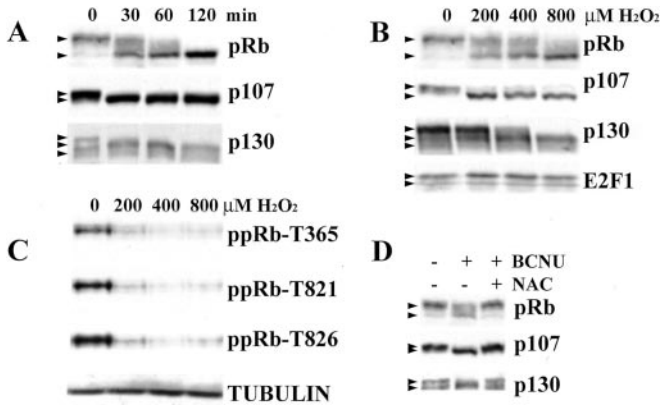

FIG. 1. **Oxidative stress induces pocket protein hypophosphorylation.** *A*, pocket proteins accumulate in their hypophosphorylated form following H_2O_2 treatment. HUVEC were incubated with 800 μM H_2O_2 for the indicated periods of time followed by immunoblots with antibodies to pRb, p107, and p130. *Arrowheads* indicate different phosphorylation forms of each protein. *B*, pocket protein hypophosphorylation following H_2O_2 treatment is dose-dependent. HUVEC were incubated for 1 h with the indicated concentrations of H_2O_2, followed by immunoblots with antibodies to pRb, p107, p130, and E2F1. *C*, hypophosphorylation of specific pRb sites upon H_2O_2 treatment. HUVEC were incubated for 1 h with the indicated concentrations of H_2O_2, followed by immunoblots with the following antibodies: anti-T356-pRb, recognizing pRb phosphorylated at threonine 356, anti-T821-pRb, recognizing pRb phosphorylated at threonine 821, anti-T826-pRb, recognizing pRb phosphorylated at threonine 826, and anti-tubulin, for gel loading control. *D*, BCNU, an inhibitor of glutathione reductase, induces pocket protein hypophosphorylation. HUVEC were either preincubated with 10 mM NAC (+) or sham-treated (−) for 30 min, followed by addition of 0.5 mM BCNU for 2 h. pRb, p107, and p130 were detected by immunoblotting.

EDTA-free protease inhibitor mixture tablets, Roche Applied Science). Immunoprecipitation was performed as described above, using 1 μg of the following antibodies: anti-Rb C15 (Santa Cruz Biotechnology), anti-PP2A-C (Upstate Biotechnology), and anti-rabbit IgG (Cappel). After two washings with lysis buffer, beads were washed twice with PP2A phosphatase buffer with no Ca^{2+} and Mg^{2+} that favors PP2A over PP2B and PP2C phosphatase activities (50 mM imidazole, pH 7.2, 0.2 mM EGTA, 0.02% mercaptoethanol, 0.1 mg/ml bovine serum albumin). Phosphatase activity was assayed using the serine/threonine phosphatase assay kit (Promega) according to the manufacturer's instructions (26, 27).[2] Substrate used, RRA(pT)VA, is a suitable substrate for PP2A but a very poor substrate for PP1.[2] PP2A activity was defined as the activity that was inhibited by incubation of the immunoprecipitates with 5 nM OA for 2 h.

Statistical Analysis—Variables were analyzed by Student's *t* test. A value of $p \leq 0.05$ was deemed statistically significant. Values are indicated ± S.E.

RESULTS

Oxidative Stress Induces Protein Hypophosphorylation—Oxidative stress has been shown to induce endothelial cell death and growth arrest. To investigate the possible role of pocket proteins in mediating the response to oxidative stress, pocket protein phosphorylation was analyzed in proliferating HUVEC treated with 800 μM H_2O_2 for increasing periods of time. Western blotting analysis of cell extracts derived from these cells showed that, upon 2 h of treatment with H_2O_2, the hyperphosphorylated, slower migrating forms of pRb disappeared with reciprocal increase of the hypophosphorylated, faster migrating forms. This pRb hypophosphorylation was already present after 30 min of treatment, was almost complete after 1 h of treatment (Fig. 1*A*), and was maintained for up to 24 h of incubation with H_2O_2 (not shown). Treatment of HUVEC with H_2O_2 concentrations ranging from 200 to 800 μM for 1 h demonstrated that the phenomenon was dose-dependent and as little as 200 μM H_2O_2 was sufficient to induce a partial, jet reproducible pRb hypophosphorylation (Fig. 1*B*).

The C-terminal 42-amino acid peptide of pRb has been shown to be specifically cleaved off during drug-induced or receptor-mediated apoptosis, generating an apoptosis-specific form of pRb that migrates in close proximity to the dephosphorylated form (29). This possibility was ruled out, using an antibody raised against the C-terminal 15-amino acid peptide of pRb, which recognizes the full-length but not the cleaved form of pRb. Moreover, pRb hypophosphorylation did not seem to be directly linked to apoptosis; Fig. 1*B* shows that following 1 h of incubation of HUVEC with 400 μM H_2O_2, pRb was largely hypophosphorylated. However, in keeping with previous results obtained in murine and human fibroblasts (30, 31), H_2O_2 concentrations up to 400 μM were sublethal and exposure to 800 μM H_2O_2 was necessary to induce cell death of most cells within 24 h (data not shown).

To analyze the phosphorylation of specific pRb sites upon H_2O_2 treatment, pRb phospho-specific antibodies were used. Two pRb phosphate acceptors, threonine 826 (Thr-826) and threonine 356 (Thr-356), are phosphorylated by CDK4-cyclin D1, whereas threonine 821 (Thr-821) is phosphorylated by CDK2-cyclin A or CDK2/cyclin E (32, 33). Immunoblot analysis of cell extracts derived from proliferating HUVEC treated with 200–800 μM H_2O_2 for 1 h showed that the signal detected using phospho-specific antibodies to Thr-356, Thr-821, and Thr-826 was greatly diminished in H_2O_2-treated cells (Fig 1*C*).

Then it was tested whether the phosphorylation of other members of the retinoblastoma gene family, namely p107 and p130, was regulated by H_2O_2. Following treatment of HUVEC with H_2O_2, a rapid, dose-dependent accumulation of the faster/hypophosphorylated forms of p107 and p130 was observed, with kinetics comparable with that of pRb (Fig. 1, *A* and *B*). Interestingly, pocket protein hypophosphorylation seems to be a specific event, because the phosphorylation patterns of E2F1 (Fig. 1*B*) and E2F4 (data not shown) were not altered by H_2O_2 treatment.

To assess whether pocket protein hypophosphorylation was caused by the H_2O_2-induced red/ox imbalance, the free radical scavenger *N*-acetyl-L-cysteine (NAC) was used. Indeed, preincubation of HUVEC with 10 mM NAC for 30 min totally prevented pRb hypophosphorylation induced by H_2O_2 treatment (not shown).

It was also tested whether pocket protein hypophosphorylation was caused by other interventions that cause intracellular red/ox imbalance. The alkylating agent 1,3-bis(2 chloroethyl)-1-nitrosourea (BCNU) is an inhibitor of glutathione reductase that blocks the conversion of oxidized to reduced glutathione (34, 35). Incubation of HUVEC with 0.5 mM BCNU for 2 h caused pocket hypophosphorylation; this phenomenon was inhibited by preincubation with 10 mM NAC. NAC treatment was shown to prevent the decrease in intracellular reduced glutathione induced by BCNU treatment (35), indicating a direct relationship between BCNU-induced red/ox imbalance and pocket protein hypophosphorylation (Fig. 1*D*). Taken together these experiments indicate that oxidative stress causes rapid pocket protein hypophosphorylation.

Pocket Protein Hypophosphorylation Is Not Associated with Cyclin/CDK Down-modulation and Is p53-independent—Next it was investigated whether pocket protein hypophosphorylation in response to H_2O_2 was mediated by a down-modulation of the cyclin/CDK targeting pocket proteins. When protein levels were measured by immunoblotting, it was found that one hour of H_2O_2 treatment (200–800 μM) induced a significant pRb hypophosphorylation in the absence of any modulation of the protein levels of CDK 1, 2, 4, and 6 and of cyclin E, A, D1,

[2] www.promega.com/tbs/tb218/tb218.pdf.

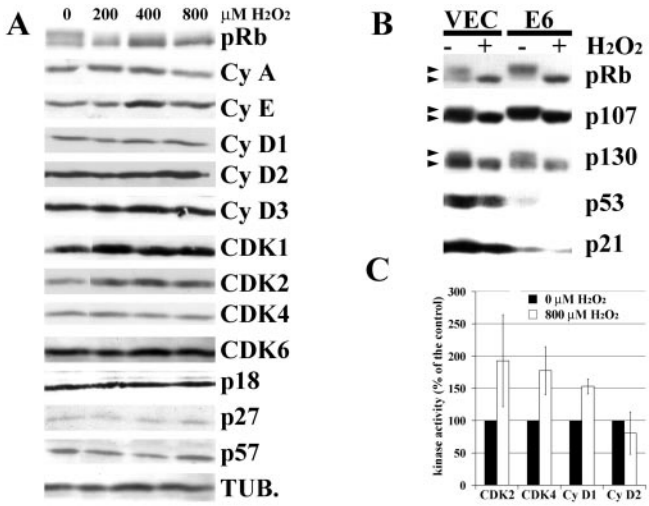

FIG. 2. **Pocket protein hypophosphorylation occurs in the absence of cyclin/CDK down-modulation and is p53-independent.** *A*, HUVEC were incubated with 200, 400, or 800 μM H_2O_2 for 1 h. Protein detection was performed by immunoblotting using specific antibodies to the indicated proteins (see "Materials and Methods"). *B*, pRb hypophosphorylation precedes p21 and p53 induction and is p53-independent. HUVEC infected with HPV16-E6-encoding retrovirus (*E6*) or backbone vector alone (*VEC*) were exposed to 800 μM H_2O_2 for 1 h. Next, immunoblots were performed using specific antibodies raised against pRb, p107, p130, p53, and p21. *Arrowheads* indicate different phosphorylation forms of each protein. *C*, kinase activity of CDK2, CDK4, and cyclin D1 and D2 is not down-modulated by H_2O_2 treatment. Cell lysates derived from HUVEC incubated with 800 μM H_2O_2 for 1 h were immunoprecipitated using antibodies to CDK2, CDK4, cyclin D1, D2, or total mouse IgGs (negative control). Next, kinase assays using opportune substrates were performed, and data were plotted as percentage of ^{32}P labeling of the substrates, indicating the value of the untreated cells as 100%. *Error bars* represent S.D. Differences in CDK2, CDK4, and cyclin D1 kinase activity observed between H_2O_2-treated and untreated cells were statistically significant ($p < 0.04$).

-2, and -3 (Fig. 2*A*). In agreement with previous observations (30), cyclin D1 and D2 accumulation decreased at later time points, but this event clearly followed pocket protein hypophosphorylation (not shown).

CDKs are negatively regulated by two classes of CDK inhibitors, the CIP/KIP and the INK4 families of proteins (10). It was determined whether the protein levels of CDK inhibitors were modulated by oxidative stress. Expression of $p16^{INK4a}$, the first isolated member of the INK4 family of inhibitors, was barely detectable in HUVEC (not shown), in keeping with other low passage primary cells (10). $p18^{INK4c}$, another INK4 inhibitor, was readily detectable by Western blotting, but its expression was not modulated in response to H_2O_2 treatment (Fig. 2*A*). $p27^{kip1}$ and $p57^{kip2}$, cyclin/CDK inhibitors of the CIP/KIP family, were not modulated as well. It has been shown that oxidating agents can induce p21 up-regulation, via both p53-dependent and -independent mechanisms (10–12, 30). However, under our experimental conditions, no p53 or p21 up-regulation was observed after 1 h of H_2O_2 treatment (Fig. 2*B*). Positive regulation of p53 or p21 was indeed observed following longer H_2O_2 treatment, but it clearly followed pocket protein hypophosphorylation (not shown). To further investigate the role of p53 and p21, pocket protein phosphorylation was examined in cells that do or do not express the viral oncoprotein E6 of human papilloma virus 16 (HPV16-E6), which can bind p53 and induce its degradation (36). Therefore, HUVEC were infected with a retroviral vector encoding HPV16-E6 or the backbone vector alone; whereas p53 protein was readily detectable in cells infected with backbone virus, it was undetectable in HPV16-E6 expressing cells. In these cells, as reported in other systems (37, 38), p21 steady-state protein levels were strongly down-modulated as a consequence of p53 low levels of expression. Following incubation with 800 μM H_2O_2 for 1 h, pocket proteins accumulated in their hypophosphorylated form with similar efficiencies both in HPV16-E6 and in backbone virus-infected cells (Fig. 2*B*). Similar results were obtained in SV40 T antigen expressing HUVEC and in HEK-293 cells, where p53 is inactivated by SV40 large T (39) and adenoviral E1B (40), respectively, and in HL-60 cells, which are p53 null (41) (not shown).

Despite the fact that pocket protein hypophosphorylation in response to H_2O_2 clearly preceded any protein level modulation of the cyclins and their inhibitors, it was determined whether the kinase activity associated with CDK4, CDK2, and cyclin D1 and D2 was down-modulated after 1 h of H_2O_2 treatment. Surprisingly, although the kinase activity associated to cyclin D2 was not modulated, the phosphorylation levels of specific substrates of CDK2, CDK4, and cyclin D1 immunocomplexes exhibited a slight increase upon H_2O_2 treatment (Fig. 2*C*). Although the functional relevance of this increase is not clear to date, the lack of a measurable inhibition of cyclin/CDK protein levels and kinase activities and the lack of any significant modulation of the CDK inhibitors suggested that pocket protein hypophosphorylation was because of the activity of a phosphatase.

Phosphatase Inhibitors Prevent Pocket Protein Hypophosphorylation Induced by H_2O_2—To test whether pRb, p107, and p130 hypophosphorylation after H_2O_2 treatment was because of the induction of a red/ox-sensitive phosphatase, it was determined whether serine/threonine-specific phosphatase inhibitors would block the effect of H_2O_2 on pocket proteins. HUVEC were pretreated with the cell-permeable phosphatase inhibitors OA, CL-A (PP1 and PP2A inhibitors), or cyclosporin A (PP2B inhibitor) (15) for 30 min and then incubated either with H_2O_2 or solvent alone. Cell extracts collected after 1 h were then Western-blotted with antibodies specific for pRb, p107, and p130. It was found that 1 μM OA (Fig. 3*A*) and 45 nM CL-A (Fig. 3*B*) prevented pocket protein accumulation in their hypophosphorylated forms induced by H_2O_2. Conversely, up to 1 μM cyclosporin A (42) had no effect on H_2O_2-induced pocket protein hypophosphorylation (data not shown). These observations strongly suggest that the rapid hypophosphorylation of pocket proteins is the result of a dephosphorylation event mediated by a specific phosphatase responsive to H_2O_2. It has been reported that incubation of Balb/c3T3 fibroblasts with OA and CL-A induced pRb hypophosphorylation (43). However, incubation of HUVEC for 90 min with OA and CL-A in the absence of H_2O_2 had only minor effects on the phosphorylation status of the pocket proteins (Fig. 3, *A* and *B*).

To extend the previous observations and provide further evidence that pocket protein hypophosphorylation in response to H_2O_2 was mediated by a phosphatase, this phenomenon was studied in cell-free conditions. Specifically, conditions to study pRb dephosphorylation were set up. Whole cell extracts prepared from HUVEC after a short exposure to H_2O_2 were incubated for 4 h at 30 °C in the absence or presence of sodium fluoride, a general serine/threonine phosphatase inhibitor. Fig. 3*C* shows that in cell extracts of H_2O_2-treated cells incubated at 30 °C, endogenous pRb was dephosphorylated and this phenomenon was prevented by the presence of sodium fluoride. This event was clearly H_2O_2-dependent, because no pRb dephosphorylation was observed in cell extracts derived from cells that were not incubated with H_2O_2. Thus, treatment of HUVEC with H_2O_2 induces a phosphatase activity targeting pRb.

Pocket Dephosphorylation after H_2O_2 Treatment Is PP2A-dependent—The previously shown experiments (Fig. 3, *A* and *B*)

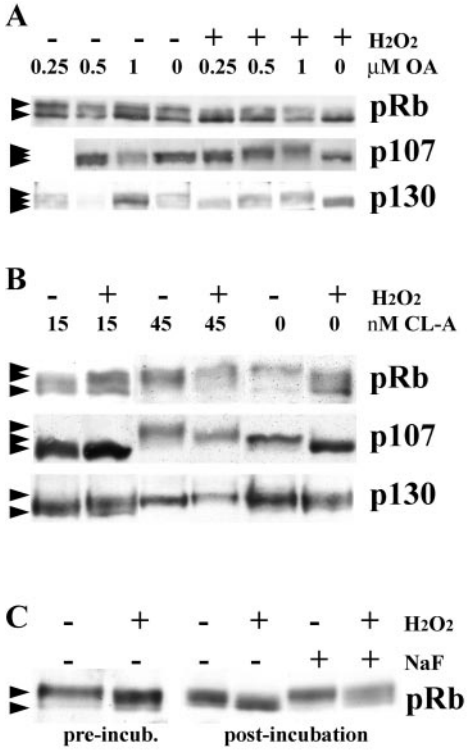

FIG. 3. **Hypophosphorylation of pocket proteins is the result of a dephosphorylation event.** PP2A and PP1 inhibitors OA and CL-A prevent pocket protein hypophosphorylation following H_2O_2 treatment. HUVEC were pretreated with the indicated concentrations of either (*A*) okadaic acid (*OA*) or (*B*) calyculin A (*CL-A*) for 30 min before adding 400 μM H_2O_2. After one more hour of incubation, cell lysates were prepared and used for Western blotting with antibodies to pRb, p107, and p130. It is worth noting that in cells treated with 45 nM CL-A, p107 migrates slower than in control cells, suggesting that p107 is partially dephosphorylated in steady-state conditions. *C*, whole cell extracts were prepared from HUVEC cells immediately after treatment with 800 μM H_2O_2 for 15 min. The cell extracts were incubated for 4 h at 30 °C in the presence or absence of phosphatase inhibitor (50 mM NaF), followed by Western blotting using an antibody raised against pRb. *Arrowheads* indicate different phosphorylation forms.

indicated that OA and CL-A prevented pRb, p130, and p107 dephosphorylation at concentrations that have been reported to specifically inhibit PP2A, but not PP1, PP2B, PP2C, and PP7 (43–46). These results suggest that the pocket protein phosphatase is PP2A or a PP2A-related enzyme. To confirm this hypothesis, the ability of SV40 small t to replace B subunits in the PP2A heterotrimer and to inhibit PP2A phosphatase activity toward most substrates was exploited (47, 48). The ability of SV40 small t to inhibit PP2A activity is markedly diminished following substitution with serine of the cysteine at position 103 (small t C103S) (22). Therefore, it was investigated whether SV40 small t wt expression could prevent pocket protein dephosphorylation in response to H_2O_2 and whether small t C103S allele failed to do so.

HUVEC were infected with defective adenoviruses that expressed LacZ, small t wt, or small-t C103S under the control of the cytomegalovirus promoter. Eighteen hours after infection, cells were treated either with H_2O_2 or solvent alone and incubated for an additional 2 h. Western blot analysis of the extracts showed that, upon H_2O_2 treatment, pocket protein dephosphorylation occurred in cells infected with LacZ- and small t C103S-encoding adenoviruses. In contrast, small t wt completely prevented pRb, p107, and p130 dephosphorylation (Fig. 4).

It has been shown in different cell systems that SV40 small t overexpression induces cyclin D1, raising the possibility that

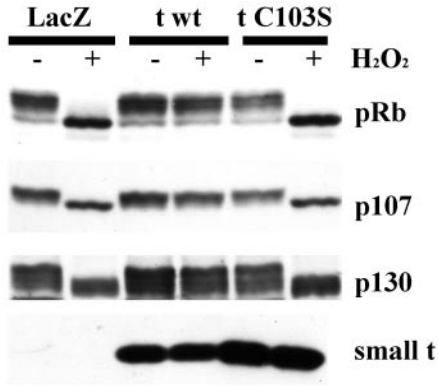

FIG. 4. **SV40 small t expression prevents pocket protein dephosphorylation**. HUVEC were infected with defective adenoviruses that expressed LacZ, small t wt (*t wt*), or small t C103S mutant (*t C103S*) under the control of the cytomegalovirus promoter. Eighteen hours after infection, cells were treated with either 800 μM H_2O_2 (+) or solvent alone (−) and incubated for one additional hour. Next, cells were harvested, and cell lysates were used for Western blotting with antibodies raised against pRb, p107 and p130 and SV40 small t. Pocket protein dephosphorylation is prevented in cells expressing small t wt.

prevention of pocket protein hypophosphorylation was because of increased cyclinD1/CDK kinase activity (49). However, in the experimental conditions used in the present study, no induction of cyclin D1 protein was observed upon infection with SV40 small t-encoding virus, possibly because of the short period of small t overexpression (not shown).

These results indicate that inhibition of PP2A with specific drugs and alteration of the PP2A oloenzyme composition abolish the effect of H_2O_2 and implicate PP2A in mediating the dephosphorylation of pocket proteins following H_2O_2 treatment.

Association of Pocket Proteins with PP2A—Given the short period of time needed for pocket protein dephosphorylation after H_2O_2 treatment, the possibility that pocket proteins were a direct target of PP2A was investigated. To this aim, it was assessed whether pocket proteins physically interacted with PP2A-C and PP2A-A, components of the PP2A core enzyme. Both PP2A-C (Fig. 5*A*) and PP2A-A (Fig. 5*B*) were readily detectable in pRb immunoprecipitates but not in control immunoprecipitates derived from HUVEC treated with 0, 400, or 800 μM H_2O_2 for 1 h. Similar results were obtained with an antibody to p107 (Fig. 5, *A* and *B*). PP2A-C and PP2A-A were detectable in immunoprecipitates of p130 as well; however, because the antibody that was used cross-reacted with p107 in immunoprecipitation experiments, an univocal interpretation was not possible (data not shown).

PP2A Phosphatase Activity Associated with Endogenous pRb Is Positively Modulated by H_2O_2—Although pocket protein association with PP2A core enzyme was readily detectable, PP2A-A and PP2A-C levels, both pocket-associated (Fig. 5, *A* and *B*) and total (not shown), were not regulated by H_2O_2. Thus, it was tested whether pRb-associated PP2A was catalitically active and whether PP2A activity was modulated by oxidative stress. PP2A activity was assayed using a buffer with no Ca^{2+} and Mg^{2+} and a synthetic phosphorylated peptide as substrate, RRApTVA (26, 27), that is a very poor substrate for PP1.[2] These conditions strongly favor PP2A over PP1, PP2B, and PP2C phosphatase activities (14, 15). It has been shown that OA doses necessary to inhibit PP2A in cell-free systems are significantly lower than those necessary *in vivo* and that 5 nM OA is sufficient to completely inhibit PP2A phosphatase activity when assayed *in vitro* (14, 15, 43, 44). Henceforth, PP2A activity was defined as the phosphopeptide phosphatase activity inhibited by 5 nM OA.

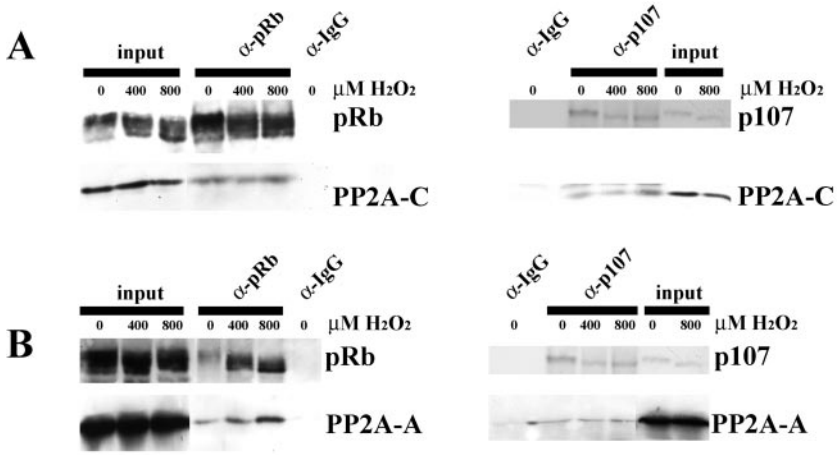

FIG. 5. **PP2A physically interacts with pRb and p107.** HUVEC were incubated for 1 h with 0, 400, and 800 μM H_2O_2, followed by immunoprecipitation with an antibody to pRb (*α-pRb*), an antibody to p107 (*α-p107*), or an antibody to mouse IgG (*α-IgG*, negative control). Western blotting was performed with antibodies to pRb, p107, PP2A-C (*A*), or antibodies to pRb, p107, and PP2A-A (*B*). As a reference, 1/20 of the immunoprecipitated whole cell extract (*input*) was loaded. Both in pRb and in p107, but not in control immunoprecipitates, PP2A-C and -A were readily detectable. Similar results were obtained by immunoprecipitating extracts with a different antibody to p107.

Cell extracts were derived from HUVEC followed by immunoprecipitation with antibody to pRb, PP2A-C, or to an irrelevant control protein. When PP2A activity associated with these immunoprecipitates was assayed, the activity associated with both pRb and PP2A immunoprecipitates significantly exceeded the activity associated with the control immunoprecipitate (Fig. 6*A*). Incubation of pRb immunoprecipitate with 5 nM OA almost completely inhibited phosphatase activity associated with pRb, indicating that this activity was largely because of PP2A or a PP2A-like phosphatase.

Then it was assessed whether the PP2A activity associated with pRb was modulated by cell treatment with H_2O_2 for 30 min. Fig. 6*B* shows that the pRb-associated phosphatase activity increased in a dose-dependent fashion following H_2O_2 treatment and pRb-associated PP2A activity was almost 2-fold higher in cells treated with 800 mM H_2O_2 for 30 min than in untreated cells. Interestingly, whole cell PP2A activity was not modulated by H_2O_2 treatment (not shown); the phosphatase activity associated with the PP2A-C immunoprecipitate was not modulated by 30 min of incubation of HUVEC with H_2O_2, indicating that the sub-pool of PP2A associated with pRb is specifically activated by oxidative stress.

Functional Effects of pRb Dephosphorylation during S-phase—DNA damage induces checkpoints to prevent damaged cells from progressing deleteriously through the cell cycle (11). Rb is a critical component of these checkpoints; pRb has been shown to be a necessary component of the G_1/S checkpoint (32, 50), and more recently its involvement in a S-phase checkpoint has been demonstrated (24). Indeed, when primary fibroblasts are exposed to cisplatin or H_2O_2 during S-phase, pRb is hypophosphorylated/activated. Although Rb is dispensable for the G_2/M block induced either by cisplatin or H_2O_2, it is strictly required for the cessation of S-phase progression in primary fibroblasts[3] (24). To evaluate the functional significance of rapid pRb dephosphorylation induced by oxidative stress, the modulation of DNA synthesis was measured in cells where pRb dephosphorylation was prevented by the overexpression of SV40 small t.

HUVEC were infected with adenoviruses encoding LacZ, small t wt, or small t C103S. Four hours later, cells were synchronized in early S-phase incubating cells with the DNA polymerase inhibitor aphidicolin for an additional 14 h. Cells were then released from aphidicolin arrest and, 60 min after release, treated with either H_2O_2 or solvent alone for an additional 30, 60, or 120 min. Cell cycle progression was monitored by a 10-min pulse of BrdUrd followed by bivariate fluorescence-activated cell sorter analysis of cells stained for BrdUrd incorporation and propidium iodide. Both LacZ- and SV40 small t-expressing cells incorporated BrdUrd with similar efficiency upon release from aphidicolin block (not shown). BrdUrd incorporation was steady for the next 60 min and slightly declined after an additional 60 min (3 h from aphidicolin-block release), when some cells exited from S-phase (Fig. 7). Following H_2O_2 treatment, DNA synthesis rapidly decreased in LacZ- and small t C103S-expressing cells and was almost undetectable after 60 min of H_2O_2 treatment (Fig. 7). Conversely, in small t wt-expressing cells, inhibition of BrdUrd incorporation induced by H_2O_2 treatment was markedly attenuated. Cells treated with H_2O_2 for 30 min incorporated almost as much BrdUrd as untreated control cells, and after 120 min of H_2O_2 treatment, the rate of DNA synthesis was almost 40% of the control. These data indicate that pRb dephosphorylation may trigger an intra-S-phase response leading to a reduction of the rate of DNA synthesis.

DISCUSSION

In this study the role of retinoblastoma family proteins in mediating the cellular response to oxidative stress was examined at the molecular level. Treatment with H_2O_2 of endothelial cells induced a rapid dephosphorylation of pRb, p107, and p130. This phenomenon was not limited to H_2O_2 because a red/ox imbalance obtained by glutathione reductase inhibition or, to a lower extent by cisplatin treatment (not shown), achieved the same effect.

H_2O_2-induced pocket protein dephosphorylation required neither p53 nor p21 because their up-regulation followed pRb, p107, and p130 dephosphorylation. Moreover, pocket proteins were readily dephosphorylated upon H_2O_2 treatment in cells where p53 was disabled in a variety of manners and where greatly diminished levels of p21 were expressed. Indeed, the relatively short time (30–60 min) required to achieve pocket hypophosphorylation implies that this effect is not likely mediated by increased rates of p21 transcription and translation.

When the kinase activity associated with CDK2, CDK4, and cyclin D1 was measured, a paradoxical increase was found within the first hour of treatment with H_2O_2. The physiological significance of this increase is not clear; however, these data

[3] F. Martelli and M. C.Capogrossi, unpublished results.

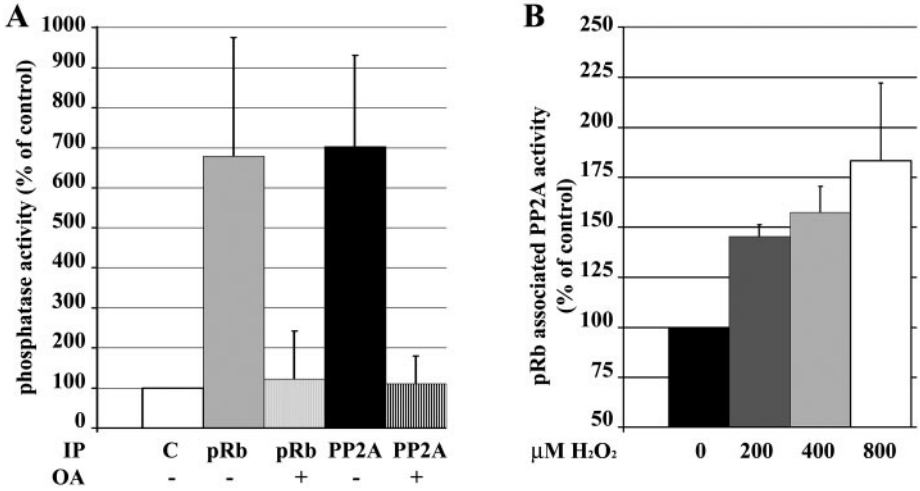

FIG. 6. **A PP2A-like phosphatase activity associated with pRb is positively modulated by H_2O_2.** *A*, pRb co-immunoprecipitates with a PP2A-like phosphatase activity. Cell extracts of HUVEC were immunoprecipitated (*IP*) with antibodies to pRb, PP2A-C, or to IgG (*C*, negative control). The phosphatase activity associated with these immunoprecipitates was then assayed in the absence (−) or presence (+) of 5 nM okadaic acid (*OA*). Values are represented as % of the phosphatase activity associated with the negative control. *Error bars* are S.E. The activity associated with both pRb and PP2A immunoprecipitates was about 7-fold higher than the activity associated with the control immunoprecipitate ($p < 0.04$). Low efficiency in immunoprecipitating PP2A by the antibody used may explain the relatively low phosphatase activity detected in PP2A-C immunoprecipitates. *B*, PP2A activity associated with pRb is up-regulated by H_2O_2. HUVEC were treated with 200–800 μM H_2O_2 for 30 min followed by immunoprecipitation with an antibody to pRb. The phosphatase activity associated with these immunoprecipitates was then assayed in the absence (−) or presence (+) of 5 nM okadaic acid (*OA*). The fraction of phosphatase activity that could be inhibited by 5 nM OA (PP2A activity) was then calculated; values are represented as % of the pRb-associated PP2A activity present in untreated cells. pRb-associated PP2A activity increased significantly ($p < 0.03$) in cells treated with H_2O_2 in a dose-dependent manner.

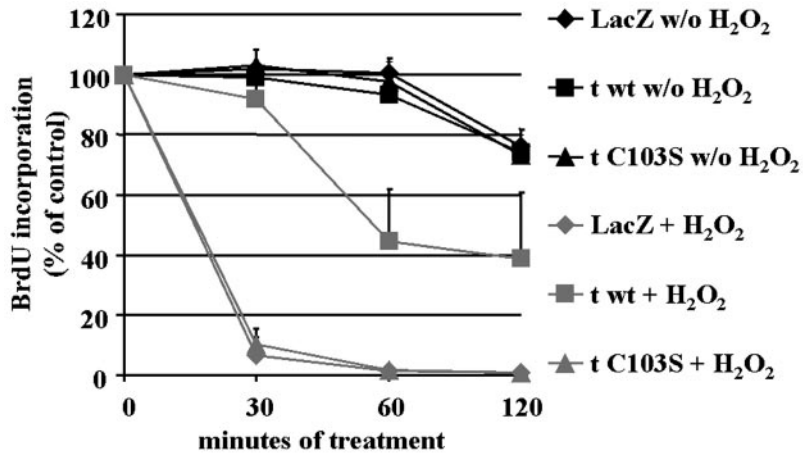

FIG. 7. **SV40 small t expression prevents DNA synthesis inhibition induced by H_2O_2.** HUVEC were infected with defective adenoviruses that expressed LacZ, small-t wt (*t wt*), or small-t C103S mutant (*t C103S*) under the control of the cytomegalovirus promoter. Four hours later, cells of the three groups were incubated with 2 μg/ml aphidicolin for a further 14 h. Following treatment, cells were released from aphidicolin arrest and 60 min later (0 min of treatment) incubated with 800 μM H_2O_2 or solvent alone for a further 30, 60, or 120 min. Cell cycle progression was monitored by a 10-min pulse of BrdUrd followed by bivariate fluorescence-activated cell sorter analysis of cells stained for BrdUrd incorporation and propidium iodide. Graph represents the percentage of S-phase cells exhibiting DNA synthesis (BrdUrd-positive). Values are expressed as % of the BrdUrd incorporation of each cell group at 0 min of treatment. *Error bars* indicate S. E. Differences between small t wt and both LacZ- and small t C103S mutant-expressing cells are statistically significant at all time points of H_2O_2 treatment ($p < 0.05$). In small t wt-expressing cells, inhibition of BrdUrd incorporation induced by H_2O_2 treatment was markedly attenuated.

further confirm that pocket protein hypophosphorylation cannot be attributed to a decreased rate of pocket protein phosphorylation. At later time points, cyclin D1 and D2 protein levels were down-modulated upon H_2O_2 treatment; however, these modulations clearly followed pocket protein hypophosphorylation and might represent reinforcement mechanisms that help to keep pocket proteins dephosphorylated over long periods of time (30, 31, 51).

Although the rate of pocket protein phosphorylation was not changed, two lines of evidence indicate that pocket proteins are actively dephosphorylated upon cell exposure to oxidative stress. 1) Both OA and CL-A, two cell-permeant inhibitors of PP1 and PP2A phosphatases, prevented pocket protein hypophosphorylation induced by H_2O_2. 2) The dephosphorylation detected in H_2O_2-treated cells could be reproduced in cell-free conditions. These observations are in agreement with previous studies showing the activation of pocket protein phosphatases by different stress stimuli. For example, the dephosphorylation of pRb following treatment with anticancer drugs or diethylmaleate is mediated by the activation of a phosphatase (52, 53), and exposure to UV has been shown to evoke a phosphatase activity, and this correlated with the dephosphorylation of p107 (16, 17).

Further, it was demonstrated that pocket protein dephosphorylation was dependent on the activity of PP2A or a closely related phosphatase. In fact, whereas the PP2B inhibitor cyclosporin A was ineffective, OA and CL-A inhibited pocket protein dephosphorylation at concentrations that specifically

inhibit PP2A but not most of the other cellular serine/threonine phosphatases, namely PP1, PP2B or PP2C, PP7 (14, 15, 43–46). It is worth noting that when living cells are treated with OA and CL-A, significantly higher concentrations of these phosphatase inhibitors are necessary to inhibit PP2A compared with *in vitro* conditions, possibly because of their relatively inefficient accumulation in the intracellular compartment (44).

Moreover, overexpression of SV40 small t prevented pocket protein dephosphorylation. SV40 small t specifically targets PP2A, replacing the B subunits in the PP2A heterotrimer and inhibiting its phosphatase activity toward most substrates (47, 48). Thus, a specific alteration of the PP2A subunit composition inhibited pocket protein dephosphorylation. The recently identified PP5 and the PP2A-like PP4/PPX phosphatases are as sensitive to OA as PP2A (54, 55). However, to the best of our knowledge, their binding to SV40 small t has never been described.

Taken together, these observations indicate that pocket protein dephosphorylation is PP2A-dependent, suggesting that PP2A directly dephosphorylates pocket proteins. In agreement with this hypothesis, PP2A core enzyme interacted with pRb and p107 and a PP2A phosphatase activity co-immunoprecipitated with pRb. Moreover, biochemical studies with purified PP2A showed that *in vitro*-phosphorylated pRb served as a substrate for PP2A (56). Voorhoeve *et al.* (16) identified a PP2A regulatory B subunit that interacts with p107, showing that p107 also is a direct substrate for PP2A. Following H_2O_2 treatment, the activity of pRb-associated PP2A increased, inducing the accumulation of pRb in its hypophosphorylated form. In keeping with our results, Barnouin *et al.* (30) recently demonstrated that H_2O_2-induced cell cycle arrest in mouse fibroblasts can be effectively prevented by expression of Herpes virus cyclin K. This viral D-like cyclin is capable of phosphorylating pRb (57), and its overexpression may possibly increase the kinase activity toward pocket proteins to such a level that it is sufficient to override PP2A-dependent dephosphorylation.

In addition, PP2A may regulate pocket proteins in unchallenged cells. Indeed, it has been shown that pRb, p107, and p130 are associated specifically with early S-phase chromatin in primary cells (58) and that SV40 small t inhibits the stable association of hypophosphorylated pRb with chromatin.[4]

The best characterized phosphatase targeting pRb is PP1, which dephosphorylates specific pRb sites at mitotic exit (13). In particular, *in vitro* assays show that Thr-821 is not targeted efficiently by PP1 and pRb phosphorylation at Thr-821 does not disappear at M/G_1 transition (59). Although PP2A efficiency in dephosphorylating pRb at Thr-821 was not tested, pRb was rapidly dephosphorylated at Thr-821 following oxidative stress, further strengthening the model proposing that pRb is directly dephosphorylated by PP2A. However, PP2A and PP1 may, at least in part, cooperate in pRb dephosphorylation. It has been shown that PP1α complexed to pRb requires inhibitory phosphorylation at Thr-320 by CDK2 in order to prevent untimely dephosphorylation of pRb (60). PP1 may not be directly activated by oxidative stress, but it may be dephosphorylated at Thr-320 by pRb-associated PP2A and then PP1 and PP2A may cooperatively contribute to pRb dephosphorylation.

DNA damage activates regulatory mechanisms that stop a proliferating cell in the G_1, S, or G_2 phase of the cell cycle, contributing to the maintenance of genome integrity (11). In particular, cisplatin and H_2O_2 damage trigger an intra-S-phase response leading to a reduction of the rate of DNA synthesis (24, 30). This S-phase response associates with pRb hypophosphorylation, and although an intact Rb is required (24), p107 and p130 may cooperate in the DNA synthesis inhibition. Indeed, a pocket protein activation mechanism that leads to pRb dephosphorylation in 1 h or less might play a crucial role in this S-phase checkpoint, given the relatively short period of time that the cells spend in S-phase.

It has been shown that pRb interacts both physically and functionally with replication factor C (61) and that pocket proteins are associated with early S-phase chromatin (58). Further, the E2F-Rb complex interacts with the *Drosophila* chorion origin of replication, and *Drosophila* mutants of Rb fail to limit DNA replication (62, 63). We speculate that, upon cell treatment with H_2O_2, pRb family members might bind in their dephosphorylated form directly to the DNA replication origin, inhibiting their firing. Indeed, upon gamma irradiation, pRb binds to select DNA replication origins, and SV40 small t inhibits this interaction.[4]

The ability of PP2A to regulate S-phase progression via pRb suggests that cells that are deficient in PP2A-dependent pRb dephosphorylation may become genetically unstable following oxidative injury. Indeed, OA can promote tumor formation (64), and SV40 small t has oncoprotein activity (48). Moreover, loss-of-function mutations in the *PPP2R1A* and *PPP2R1B* genes, encoding two isoforms of the A subunit of PP2A, have been found in multiple human tumors (65–69), and potentially oncogenic human proteins that inhibit PP2A function have been reported, including the Hox11 (70) and the SET/PHAP-II/TAF1β oncogenes (28). Taken together, these data strongly suggest that PP2A has tumor suppressor activity. Given the fact that Rb is a frank tumor suppressor, one might wonder whether at least part of the putative tumor suppressor activity of PP2A is mediated by modulation of pRb phosphorylation status following oxidative damage.

In conclusion, this report shows that hypophosphorylation of the retinoblastoma family proteins induced by H_2O_2 was because of the activity of PP2A and that pRb dephosphorylation may induce an intra-S-phase response that leads to a reduction of the rate of DNA synthesis.

These findings may contribute to the understanding of the molecular events underlying numerous pathologies, including tissue ischemia and reperfusion, cancer, diabetic vasculopathy, atherosclerosis, Alzheimer's disease, and pulmonary fibrosis.

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