Protein Phosphatase 2A Subunit PR70 Interacts with pRb and Mediates Its Dephosphorylation[▽]

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The retinoblastoma tumor suppressor protein (pRb) regulates cell proliferation and differentiation via phosphorylation-sensitive interactions with specific targets. While the role of cyclin/cyclin-dependent kinase complexes in the modulation of pRb phosphorylation has been extensively studied, relatively little is known about the molecular mechanisms regulating phosphate removal by phosphatases. Protein phosphatase 2A (PP2A) is constituted by a core dimer bearing catalytic activity and one variable B regulatory subunit conferring target specificity and subcellular localization. We previously demonstrated that PP2A core dimer binds pRb and dephosphorylates pRb upon oxidative stress. In the present study, we identified a specific PP2A-B subunit, PR70, that was associated with pRb both in vitro and in vivo. PR70 overexpression caused pRb dephosphorylation; conversely, PR70 knockdown prevented both pRb dephosphorylation and DNA synthesis inhibition induced by oxidative stress. Moreover, we found that intracellular Ca²⁺ mobilization was necessary and sufficient to trigger pRb dephosphorylation and PP2A phosphatase activity of PR70 was Ca²⁺ induced. These data underline the importance of PR70-Ca²⁺ interaction in the signal transduction mechanisms triggered by redox imbalance and leading to pRb dephosphorylation.

Protein phosphatase 2A (PP2A) is a major cellular serine/threonine phosphatase that plays an important role in diverse cellular processes such as cell cycle regulation, DNA replication, transcription, and signal transduction (2, 20). Its complex composition and regulation are thought to provide the molecular basis for the appropriate regulation of these numerous cellular processes. The core structure comprises a 36-kDa catalytic subunit (PP2A-C) and a 65-kDa scaffolding subunit (PP2A-A). This core dimeric structure unit (PP2A_D) can exist independently or can be associated with a regulatory PP2A-B subunit to form a heterotrimeric holoenzyme. Various B subunits can be categorized into four different families on the basis of homology, namely B (B55 or PR55), B' (B56 or PR61), B'' (PR48/59/72/130), and B''' (PR93/110). B-type subunits are largely different, but they all share two motifs for PP2A-A subunit binding (26), the only exception is constituted by B''' subunits and their status as genuine PP2A-B subunits is currently under evaluation. It is believed that PP2A exercises regulatory flexibility and substrate specificity through the specific association of the core dimer with one of the regulatory B subunits. The diversity of possible combinations of PP2A subunits that exist as functional holoenzymes provides substrate specificity and intracellular localization.

The retinoblastoma family of growth-inhibitory proteins is an integral part of the mechanisms that control cell growth under normal conditions and after exposure to genotoxic stimuli. This family includes three members: the retinoblastoma protein (pRb)

and the related p107 and p130 (also known as p130^{Rb2}) (7, 30). Collectively, these proteins are called “pocket” proteins because they share a common domain, named the “pocket.” The structure of this region is characterized by two conserved functional domains identified as A and B pockets. Several cellular and viral proteins that possess the LXCXE peptide motif, such as oncoproteins from different tumor viruses and histone deacetylases, interact with the A and B pocket domains. However, members of the E2F transcription factors interact both with the A/B domains and with the C-terminal region of pRb (termed “C pocket”) (11, 16). Pocket proteins control the G₁ checkpoint of the cell cycle through their ability to bind and sequester members of the E2F family of transcription factors, which modulate the expression of genes involved in cell cycle progression (7, 30). The ability of pocket proteins to bind their interactors is abolished through cell cycle-regulated phosphorylation by cyclin-dependent kinases (CDKs). pRb is hypophosphorylated in early G₁ and becomes hyperphosphorylated in late G₁ prior to entry into S phase of the cell cycle. pRb phosphorylation increases even further as cells progress through S and G₂. p107 and p130 are regulated in a similar fashion (7, 30). Inhibitors of CDKs, Cip/Kip and INK4 families, provide another level of regulation (34). Indeed, increased levels of CDK inhibitors in response to stress or differentiative cues inhibit pRb phosphorylation and cause growth arrest (15).

The cyclin/CDK complexes and the inhibitors thereof that regulate pRb phosphorylation have been extensively studied. Conversely, little is known of the phosphatases that remove pRb serine/threonine phosphates. PP1 is involved in pRb dephosphorylation at mitotic exit (40), while PP2A_D regulates pRb phosphorylation in response to stress stimuli (3, 6, 14). However, the PP2A-B subunits responsible for these events and their regulation are still unknown.

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We previously demonstrated that PP2A_B interacts with pRb and is necessary for its rapid dephosphorylation in response to oxidative stress (6). In the present study, we identify a specific B regulatory subunit of PP2A, PR70, that is necessary and sufficient for pRb dephosphorylation. Moreover, we demonstrate a crucial role of intracellular Ca²⁺ in the signaling that activates pRb dephosphorylation by PR70 in response to oxidative stress.

MATERIALS AND METHODS

Cell cultures, transfections, and plasmids. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂–95% air. Human umbilical vein endothelial cells (HUVEC; Clonethics), U2OS osteosarcoma cells (American Type Culture Collection), and porcine aortic endothelial (PAE) cells were maintained in EGM-2 (Cambrex), Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum, and Ham's F-12 medium (Cambrex) containing 10% fetal bovine serum, respectively. Cells were transfected using Eugene 6 (Roche Applied Sciences) as previously reported (29). The following plasmids were used for transfection: pRc-CMV (cytomegalovirus), pCMV pRb (35), pRcCMV-HA (hemagglutinin) Rb (35), pCMVSPORT6 PR70 (IMAGE clone 5239974), and pCMVSPORT6 Bβ (IMAGE clone 4939981). pCDNA3-EE Aα and pCDNA3-HA Cα are kind gifts from Gernot Walter (University of California—San Diego) (45). pCMVSPORT6 PR70 EF1 mut, pCMVSPORT6 PR70 EF2 mut, and pCMVSPORT6 PR70 EF1/2 mut were generated by introducing, via PCR amplification, the relevant nucleotide substitution by standard techniques. The protein sequence of PR70 EF1, ³²⁷DTDHLLIDADDL³³⁹, was changed to ³²⁷ATDHLLIDADDL³³⁹. The PR70 EF2 sequence ⁴⁰¹DLGDGALSMFEL⁴¹³ was changed into ⁴⁰¹ALDGDGALSMFAL⁴¹³. (The appropriate residues before and after the change are highlighted in boldface.) The mutations were confirmed by DNA sequencing.

Drug treatments. H₂O₂ (30% [wt/wt] solution; Sigma) was administered to the cells as a 100 mM solution in phosphate-buffered saline (PBS). 1,2-Bis (2-aminophenoxy) ethane-*N,N,N',N'*-tetraacetic acid-acetoxymethyl ester (BAPTA-AM; Sigma) was resuspended in water. To load HUVEC with BAPTA-AM, cells were incubated in Ca²⁺-free PBS containing 10 mM HEPES (pH 7.4), 1 mM EGTA, 0.2 mg/ml bovine serum albumin (BSA), and the indicated concentrations of BAPTA-AM for 30 min. Once BAPTA-AM was loaded, the cells were then switched back to EGM medium to minimize toxicity and incubated with 400 μM H₂O₂ for 2 h. Thapsigargin (Sigma) was dissolved in dimethyl sulfoxide. Aphidicolin was resuspended in Me₂SO. For S-phase synchronization, cells were incubated with 2 μg/ml aphidicolin for 14 h, washed twice with PBS, and incubated for 1 h in prewarmed medium.

Western blotting. Cells were lysed in a buffer containing 100 mM Tris (pH 6.8), 20% glycerol, and 4% sodium dodecyl sulfate (SDS). Amounts of protein were determined by bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Then dithiothreitol (DTT; 200 mM) was added and lysates were boiled for 5 min. Proteins were separated in SDS-polyacrylamide gels and transferred to nitrocellulose by standard procedures. The following antibodies were used to detect the proteins of interest: anti-PR70 (C20; Santa Cruz), anti-pRb (G3-245; Pharmingen), anti-phospho-pRb-threonine 826 (44-576; Biosource International), anti-small t (PAb108; Pharmingen), and anti-α-tubulin (Ab-1; Oncogene Research Products).

Immunoprecipitation. Immunoprecipitations were performed as previously described (6). Cells were resuspended in lysis buffer containing 50 mM HEPES (pH 7.5), 250 mM NaCl, 1 mM DTT, 0.1% Tween 20, 10% glycerol, 5 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Na₃VO₄, 50 mM NaF, and protease inhibitor (complete EDTA-free protease inhibitor mixture tablets; Roche Applied Sciences). Immunoprecipitations were performed for 2 to 3 h at 4°C with protein A/G agarose and 1 μg of relevant antibodies. Immune complexes were resuspended in 2× Laemmli buffer, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and immunoblotted with relevant antibodies.

Immunoprecipitations/reimmunoprecipitations were performed as previously described (18). Briefly, cells were metabolically labeled for 3 h with 500 μCi of [³⁵S]methionine plus [³⁵S]cysteine (Redivue Promix; Amersham) per 10-cm dish. Then cells were harvested and immunoprecipitations were performed as described above, using 1 μg of control antibody, pRb antibody (C15; Santa Cruz), or PR70 antibody (C20; Santa Cruz). Immune complexes were then resuspended in a mixture of 1% SDS, 50 mM HEPES (pH 7.5), and 5 mM DTT and boiled for 5 min. Then samples were diluted with lysis buffer and reimmunoprecipitated overnight with 1 μg of either PR70 antibody or control antibody. Immune

complexes were resuspended in 2× Laemmli buffer, separated by SDS-PAGE, and detected by autoradiography.

Lentiviral infection. MISSION shRNA lentiviral constructs were purchased from Sigma. The mRNA regions targeted by shRNA sequences are as follows: nucleotides (nt) 238 to 258, nt 786 to 813, nt 1140 to 1162, and nt 1236 to 1256, starting from the ATG. Lentiviral supernatants were produced using standard procedures (13). HUVEC were infected for 2 h with lentiviral supernatants and then endothelial cells were allowed to recover in complete fresh medium for an additional 24 h. Afterwards, puromycin-containing medium (0.5 μg/ml; Sigma) was added to the cells.

Retroviral infection. Phoenix-ampho cells (American Type Culture Collection) were transfected with pBABE-puro vector control or the pBABE-SV40 (simian virus 40) small t wild type (wt). The medium containing the emerging retrovirus was harvested 48 h after transfection. HUVEC were then infected and selected with puromycin-containing medium (Sigma; 0.5 μg/ml).

GST pulldowns. Glutathione *S*-transferase (GST) and GST-Rb (amino acids [aa] 1 to 928, aa 379 to 928, aa 379 to 792, aa 792 to 928, and aa 792 to 882) proteins were produced in *Escherichia coli* BL21(pLys) cells following standard procedures and were purified on glutathione-Sepharose beads (Amersham-Pharmacia Biotech) according to the manufacturer's instructions. [³⁵S]methionine-labeled proteins were obtained using the TNT-coupled rabbit reticulocyte lysate system (Promega). Equal amounts of GST proteins were incubated with equal amounts of labeled proteins for 1 h at 4°C in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% Tween 20, and 1 mM PMSF. Beads were resuspended with 2× Laemmli buffer and boiled for 5 min. The eluted proteins were then fractionated by SDS-PAGE and detected by autoradiography.

Flow cytometry. HUVEC were incubated for 10 min with 20 μM bromodeoxyuridine (BrdU; Sigma) and then fixed with 70% ethanol. Cell cycle analysis was performed as previously described (6).

PP2A assay. U2OS cells were lysed in a buffer (pH 7.2) containing 50 mM HEPES; 150 mM NaCl; 10% glycerol; 1 mM PMSF; 1 mM DTT; 0.1% Tween 20; 5.55 mM EGTA; protease inhibitors (complete EDTA-free protease inhibitor mixture tablets; Roche Applied Sciences); and Ca²⁺ concentrations of 0, 1, 4, or 12 mM. Immunoprecipitation was performed as described above, using 0.5 μg of PR70 or control antibodies. Beads were resuspended in PP2A phosphatase buffer (50 mM imidazole [pH 7.2], 5.55 mM EGTA, 0.02% mercaptoethanol, 0.1 mg/ml BSA, and the Ca²⁺ concentrations mentioned above). Phosphatase activity was assayed by a serine/threonine phosphatase assay kit (Promega) according to the manufacturer's instructions, using a PP2A-specific substrate. PP2A activity was defined as the activity that was inhibited by 50 mM NaF. Furthermore, PP2A activity was normalized for the efficiency of PR70 immunoprecipitation, as assessed by loading 1/3 of each immunoprecipitate on SDS-PAGE, followed by Western blotting using an anti-PR70 antibody. The immunoprecipitates were also immunoblotted with antibody to PP2A-Aα (C20; Santa Cruz) and to PP2A-Cα (610555; BD Transduction Laboratories).

Calcium measurement. The variations in intracellular free Ca²⁺ concentration ([Ca²⁺]_i) were measured with a conventional fluorescence microscopy system and cell permeant Fura 2-acetoxymethyl ester (Fura 2-AM), as previously described (10).

RESULTS

PR70 and pRb interact in vitro. In order to determine which PP2A-B subunit mediated the interaction between PP2A_B and pRb, the ability of PP2A-B subunits, belonging to the B, B', and B'' classes, to bind pRb was assayed by an in vitro GST-pulldown assay. Luciferase protein and E1A, an adenoviral oncoprotein having a strong pRb binding affinity, were used as negative and positive controls, respectively. The most commonly used GST-Rb 379–928 protein, containing A, B, and C (A/B/C) pockets, was assayed for PP2A-B subunit interaction. Out of 13 PP2A-B isoforms tested, only PR48 bound specifically to GST-pRb 379–928 (Fig. 1A). PR48 has been demonstrated to be an N-terminal-truncation form of a protein named PR70 (PPP2R3B) (38), so we tested the full-length sequence for pRb binding. Figure 1B shows that PR70 bound to pRb as well, but with less efficiency than PR48. Interestingly, B56γ, a PP2A-B subunit involved in cell transformation (21), did not bind pRb specifically (Fig. 1A).

FIG. 1. PR70 and pRb interact in vitro. Bacterial recombinant GST or GST-Rb 379–792 was immobilized on glutathione-Sepharose beads and incubated with the indicated ^{35}S -labeled proteins produced by in vitro-coupled transcription-translation in reticulocyte lysates. After several washings, the resin-bound proteins were eluted with $2\times$ Laemmli buffer, separated by SDS-PAGE, and visualized by autoradiography. A fraction of labeled proteins used in this assay (INPUT) was loaded separately for SDS-PAGE. Luciferase (Luc) protein and E1A were used as negative and positive controls, respectively. (A) Among all PP2A-B subunits tested, only a B''/PR70 N-terminal-truncation form, PR48, was able to bind pRb. (B) Both PR48 and PR70 can bind pRb.

Successively, we tested GST-Rb full-length protein for PR70 binding. Figure 2A shows that PR70 binds to full-length GST-Rb and to GST-Rb 379–792 with the same efficiency. Finally, using GST-pRb proteins bearing different truncations, we assessed that both the pRb A/B pocket (aa 379 to 792) and pRb C pocket (aa 792 to 928) displayed the ability to bind PR70, although to different extents, and that the last 46 aa are necessary for PR70 binding by the pRb C pocket (Fig. 2B). PR72, another B'' PP2A-B isoform, served as a negative binding control.

PR70 and pRb interact in vivo. To assess whether pRb and PR70 interacted in vivo as well, plasmids encoding pRb and PR70 were transfected in U2OS cells. First, we analyzed the phosphorylation status of exogenous pRb under our experimental conditions. U2OS cells were transfected with pRb-HA either alone or together with the CDK inhibitor $p27^{\text{kip1}}$. In order to discriminate exogenous from endogenous pRb, an immunoprecipitation to HA tag was performed. Figure 3A shows that pRb-HA phosphorylation, as assessed by an antibody specific to pRb phosphorylated at threonine 826 (phospho-pRb antibody), was strongly diminished in the presence of $p27^{\text{kip1}}$. This result was confirmed by the accumulation of the fast-migrating hypophosphorylated form of pRb, which was detected using an antibody to HA tag. In agreement with previous reports (42), we concluded that exogenous pRb-HA transfected in U2OS cells was mostly in its hyperphosphorylated form.

Then we tested pRb-PR70 association. U2OS cells were transfected with plasmids encoding PR70 and an HA-tagged allele of pRb. Then cell extracts derived from these cells were immunoprecipitated with antibodies to HA or to an irrelevant target, followed by Western blotting to PR70. Figure 3B shows that PR70 coimmunoprecipitated with pRb-HA. Furthermore, the immunoblotting with phospho-pRb antibody revealed that the immunoprecipitated pRb-HA species was, at least in part, hyper-

FIG. 2. pRb binding site of PR70. (A) GST, GST-Rb, and GST-Rb 379–928 proteins were immobilized on glutathione-Sepharose beads and incubated with ^{35}S -labeled B''/PR70. Luciferase (Luc) protein and E1A were used as negative and positive controls, respectively. The resin-bound proteins were then separated by SDS-PAGE and visualized by autoradiography. A fraction of labeled proteins (INPUT) was loaded separately for SDS-PAGE. B''/PR70 binds equally to full-length GST-Rb and to GST-Rb containing only A, B, and C pockets. (B) GST and GST-Rb proteins containing the indicated amino acid regions were immobilized on glutathione-Sepharose beads and incubated with ^{35}S -labeled B''/PR70. ^{35}S -labeled B''/PR72 protein was used as a negative control. The resin-bound proteins were then separated by SDS-PAGE and visualized by autoradiography. A fraction of labeled proteins (INPUT) was loaded separately for SDS-PAGE. The pRb A/B pocket (aa 379 to 792) and pRb C pocket (aa 792 to 928) displayed the ability to bind PR70; the last 46 aa are necessary for PR70 binding by the pRb C pocket.

FIG. 3. PR70 and pRb interact in vivo. (A) U2OS cells were transfected with CMV vector or with plasmids encoding an HA-tagged allele of pRb either alone or in the presence of a plasmid encoding p27^{Kip1}, as indicated. Two days after transfection, cells were harvested and immunoprecipitated (Ip) with either an anti-HA antibody or an irrelevant isotypic antibody (negative control). Western blotting with a phospho-pRb antibody revealed that pRb-HA was mostly hyperphosphorylated and that p27^{Kip1} expression induced its hypophosphorylation, as expected. The asterisk indicates an aspecific band. (B) U2OS cells were transfected with plasmids encoding PR70 and an HA-tagged allele of pRb, as indicated. Two days after transfection, cells were harvested and immunoprecipitated with an anti-HA antibody or an irrelevant isotypic antibody as a negative control. Western blotting (WB) was performed with an antibody to PR70. The efficiency of immunoprecipitation was assessed with an anti-HA antibody. One-twentieth of the immunoprecipitated whole-cell extract (input) was loaded as a reference. PR70 was clearly detectable in immunoprecipitates to pRb-HA. Immunoblots with a phospho-pRb antibody revealed that the immunoprecipitated pRb-HA was, at least in part, in its hyperphosphorylated form. (C) U2OS cells were transfected with plasmids encoding PR70 and pRb or CMV vector alone, as indicated. Two days after transfection, cells were harvested and immunoprecipitated with anti-PR70 antibody or anti-goat IgG (negative isotypic control). Western blotting was performed with an antibody to pRb. The efficiency of immunoprecipitation was assessed with an anti-PR70 antibody. As a reference, 1/20 of each cell extract (input) was loaded; a shorter exposure is shown. pRb was readily detectable in PR70 but not in control immunoprecipitates. The immunoblots with a phospho-pRb antibody revealed that PR70 associated with the hyperphosphorylated form of pRb. The black arrowhead indicates the phospho-pRb-specific band; the asterisk indicates an aspecific signal. (D) HUVEC and SAOS2 cells were metabolically labeled with [³⁵S]methionine plus [³⁵S]cysteine. Afterwards, the cells were lysed and immunoprecipitated twice, sequentially (immunoprecipitation/reimmunoprecipitation) using control and pRb antibodies (shown as α IgG, α Rb, and α PR70) first (IP) and then control or PR70 antibodies (Re-IP), as indicated. As a positive control, one sample was immunoprecipitated twice using PR70 antibody both times and 1/4 of the immunoprecipitates were loaded. PR70 was clearly detectable in pRb immunoprecipitates that were reimmunoprecipitated with PR70 antibody but not with control antibody in HUVEC. The endogenous interaction between PR70 and pRb was not visible in pRb-null SAOS2 cells, which served as a further negative control.

phosphorylated. In order to test whether the reciprocal immunoprecipitation gave similar results, plasmids encoding pRb and PR70 were transfected in U2OS cells. Then cell extracts derived from these cells were immunoprecipitated with antibodies to PR70 or to an irrelevant target, followed by Western blotting to pRb. We found that pRb was clearly detectable in PR70 immunoprecipitates (Fig. 3C). Western blotting analysis with a phospho-specific pRb antibody revealed that hyperphosphorylated pRb associated with PR70.

We can conclude that PR70 was able to bind the hyperphosphorylated form of pRb.

Then we determined whether PR70-pRb complexes were detectable when these two proteins were expressed at the endogenous concentrations. Since the pRb pathway has been shown to be disrupted in most cancer cells (7), and we could not exclude that pRb-PR70 interaction was affected by the transformation process, primary cell cultures were used to detect endogenous pRb-PR70 complexes. Toward this end,

HUVEC were metabolically labeled with a mixture of [³⁵S]methionine and [³⁵S]cysteine and pRb was immunoprecipitated. After denaturation, the immunoprecipitated proteins were reimmunoprecipitated using an antibody to PR70. As shown in Fig. 3D, PR70 was clearly detectable in pRb immunoprecipitates, but not in control immunoprecipitates (immunoprecipitation to immunoglobulin G [IgG] and reimmunoprecipitation to PR70 and immunoprecipitation to pRb and reimmunoprecipitation to IgG). pRb was present in PR70 immunoprecipitates as well, although the band was difficult to visualize, possibly due to the lower efficiency of pRb labeling with ³⁵S-labeled amino acids (not shown). As a positive control, PR70 immunoprecipitates were reimmunoprecipitated with the same antibody (immunoprecipitation to PR70 and reimmunoprecipitation to PR70). As a further confirmation of the specificity of pRb-PR70 coimmunoprecipitation, we performed the same experiment in SAOS2 cells, which are pRb null (37). Figure 3D shows that PR70 was not present in pRb immunoprecipitates

FIG. 4. PR70 is necessary to induce pRb dephosphorylation. (A) Representative Western blot demonstrating a >70% knockdown of PR70 expression in HUVEC infected with a lentivirus encoding a PR70-specific shRNA sequence (PR70-a) compared to control virus-infected cells. Tub, α -tubulin. (B) HUVEC were infected with the lentiviral particles carrying the PR70-specific shRNA PR70-a or with the control (ctrl) virus. After 24 h, the cells were selected with puromycin and afterwards treated with 400 μ M H₂O₂ for 2 h. Then immunoblots were performed using a pan-pRb antibody or a phospho-pRb antibody. The knockdown of PR70 expression partially prevented pRb dephosphorylation upon H₂O₂ treatment. (C) HUVEC were infected with lentiviruses encoding two shRNA designed to target PR70 (PR70-b and -c) or a control. Western blotting analysis revealed that only shRNA PR70-c was effective at knocking down PR70 expression >70%. (D) HUVEC were infected with control virus or with shRNA PR70-b or -c. After 24 h, cells were selected with puromycin and afterwards treated with 600 μ M H₂O₂ for 1 h. Then immunoblots were performed using a pan-pRb antibody or a phospho-pRb antibody. The knockdown of PR70 expression by shRNA PR70-c prevented pRb dephosphorylation upon H₂O₂ treatment, whereas the ineffective shRNA sequence (PR70-b) failed to do so. Gray arrowheads indicate the hypophosphorylated form of pRb; black arrowheads indicate the hyperphosphorylated form.

(immunoprecipitation to pRb and reimmunoprecipitation to PR70) but was clearly detectable in the positive control (immunoprecipitation to PR70 and reimmunoprecipitation to PR70).

PR70 is necessary and sufficient to induce pRb dephosphorylation. In order to assess whether PR70 was required for pRb dephosphorylation induced by H₂O₂, PR70 was knocked down by RNA interference (RNAi). HUVEC were infected with lentiviral particles carrying a PR70-specific short hairpin RNA (shRNA) sequence (PR70-a) or with a control virus. Figure 4A shows that PR70 expression was silenced efficiently, reducing the endogenous PR70 protein level more than 70%. The infected cells were then treated with 400 μ M H₂O₂ for 2 h. pRb phosphorylation was analyzed by Western blotting, both using a pRb phospho-specific antibody and measuring the abundance of the slower-migrating, hypophosphorylated form of pRb. Figure 4B shows that, while H₂O₂ induced a complete dephosphorylation of pRb in control cells, phosphorylated pRb was still detectable in cells with PR70 knocked down.

These results are unlikely due to off-target RNAi effects, since an independent shRNA (PR70-c) that effectively repressed PR70 expression efficiently prevented pRb dephosphorylation upon cell treatment with 600 μ M H₂O₂ for 1 h.

FIG. 5. PR70 is sufficient to induce pRb dephosphorylation. PAE cells were transfected with either 1.1 μ g of CMV vector or with 0.8 μ g of pRb-HA plasmid together with increasing amount of a plasmid encoding PR70, as indicated. Cells were also treated with 800 μ M H₂O₂ for 1 h as a positive control or cotransfected with 0.8 μ g of pRb-HA and 0.3 μ g of B55 β -encoding plasmids as a negative control. The immunoblots with a phospho-pRb antibody revealed that the overexpression of PR70 was sufficient to induce the dephosphorylation of exogenous pRb.

Conversely, an shRNA ineffective in PR70 knockdown (PR70-b) behaved like the control shRNA (Fig. 4C and D).

Taken together, these data indicate that PR70 is necessary for pRb dephosphorylation induced by oxidative stress.

Whether PR70 overexpression was able to induce pRb dephosphorylation was also tested.

Since the transfection procedure was highly toxic to HUVEC and induced pRb dephosphorylation per se, PAE cells were exploited. PAE cells overexpressing an HA-tagged allele of pRb showed a dose-dependent dephosphorylation of exogenous pRb in the presence of PR70 expression (Fig. 5). PAE cells treated with H₂O₂ were used as a positive control. To further confirm the specificity of PR70-induced pRb dephosphorylation, the overexpression of a PP2A-B subunit that was not able to bind pRb (B55 β) did not cause any change in pRb phosphorylation status.

We can conclude that PR70 is not only necessary, but also sufficient to induce pRb dephosphorylation.

Functional effects of PR70 knockdown during S phase. We and others previously demonstrated that pRb phosphorylation or loss and PP2A inhibition prevented DNA synthesis inhibition induced by H₂O₂ treatment and other stress stimuli (3, 6, 14, 25). Thus, DNA synthesis modulation was measured in cells in which oxidative stress-induced pRb dephosphorylation was prevented by PR70 knockdown. HUVEC were infected with lentiviruses encoding PR70-specific shRNA sequences or with control virus. Twenty-four hours later, cells were synchronized in early S phase and treated with H₂O₂ or solvent alone for 30 min. Cell cycle progression was monitored by a 10-min pulse of BrdU followed by bivariate fluorescence-activated cell sorter analysis of cells stained for BrdU incorporation and propidium iodide. As expected (3, 6, 14, 25), following H₂O₂ treatment, DNA synthesis rapidly decreased in control cells, reaching 30% of the control value. Conversely, in cells with PR70 knocked down, the inhibition of BrdU incorporation induced by H₂O₂ treatment was markedly attenuated; indeed, DNA synthesis was about 90% of the value for untreated control cells (Fig. 6). Taken together, these data indicate that PR70 depletion prevents DNA synthesis inhibition induced by oxidative stress.

FIG. 6. PR70 knockdown prevents DNA synthesis inhibition induced by H₂O₂. HUVEC were infected with a lentivirus encoding a PR70-specific shRNA (PR70-a) or with control virus. Twenty-four hours later, cells were treated with 2 μ g/ml aphidicolin for 14 h. Afterwards, cells were released for 1 h from aphidicolin arrest (0 min of treatment) and then treated with 400 μ M H₂O₂ or solvent alone (w/o H₂O₂) for 30 min. Cell cycle progression was monitored by a 10-min pulse of BrdU followed by bivariate fluorescence-activated cell sorter analysis of cells stained for BrdU incorporation and propidium iodide. The graph represents the percentage of S-phase cells exhibiting DNA synthesis (BrdU positive). Values are expressed as percentages of BrdU incorporation of each cell group at 0 min of treatment. PR70 knockdown markedly attenuated the inhibition of BrdU incorporation induced by H₂O₂ ($P < 0.02$).

In vivo association of PR70 EF-hand mutated proteins with pRb and PP2A-core dimer. H₂O₂ is known to induce intracellular Ca²⁺ mobilization mediating certain crucial aspects of cell response (10, 19). Moreover, PP2A-B' family members share the presence of two conserved EF-hand Ca²⁺-binding domains (termed EF1 and EF2) (22). In order to investigate the structural and functional role of PR70 EF-hand motifs, we constructed PR70 EF-hand singly mutated (EF1 mut and EF2 mut) and doubly mutated (EF1/2) proteins. First, we assayed these mutated proteins for pRb binding when expressed in vivo. Interestingly, we found that PR70 EF-hand mutated proteins were all able to bind pRb with the same efficiency as the wt protein (not shown). Then we assayed the ability of EF-hand mutated proteins to bind PP2A_D core dimer, consisting of PP2A-C and PP2A-A. U2OS cells were transfected with the expression constructs carrying either the PR70 wt or PR70 single or double mutant (EF1 mut, EF2 mut, and EF1/2 mut), together with plasmids encoding PP2A-C α and PP2A-A α . Then cells were lysed and immunoprecipitated with antibodies to PR70 or with control antibody. We found that EF2 mut and EF1/2 mut were not able to bind PP2A_D (Fig. 7A). In contrast, EF1 mut was able to bind to both PP2A-C and PP2A-A with a higher efficiency than the wt (Fig. 7B).

These data indicate that EF2-hand domain integrity is required for PR70 binding to the PP2A_D core dimer, the EF1 domain had a slight inhibitory effect on PP2A_D-binding ability, and EF-hand domain integrity was not necessary for pRb binding.

PR70/PP2A phosphatase activity increases with Ca²⁺ concentration. In the following experiments, whether PR70-associated phosphatase activity was modulated by Ca²⁺ concentration was examined. Toward this end, U2OS cells were cotransfected with plasmids encoding PR70, PP2A-A α , and PP2A-C α subunits. Then cell extracts derived from these cells were immunoprecipitated with antibodies to PR70 or to control antibody and the phosphatase activity associated with

FIG. 7. In vivo association of PR70 EF-hand mutated proteins with PP2A core dimer. (A) U2OS cells were transfected (Transf.) with expression constructs carrying either PR70 wt (wt) or a PR70 EF2 singly mutated (EF2 mut) or doubly mutated (EF1/2 mut) protein together with plasmids encoding PP2A-A α and PP2A-C α . Two days after transfection, cells were lysed and immunoprecipitated (Ip) with antibodies to PR70 or to IgG (negative isotypic control). Both PR70 mutated proteins did not bind to PP2A core dimer, whereas the wild type coimmunoprecipitates with both A α and C α subunits. WB, Western blot. (B) U2OS cells were transfected with either the expression constructs for PR70 wt (wt) or a PR70 EF1-hand singly mutated protein (EF1 mut) together with plasmids encoding PP2A-A α and PP2A-C α . After 36 h, cells were lysed and immunoprecipitated with antibodies to PR70 or to IgG (negative isotypic control). EF1 mut was able to bind to the core dimer with a higher efficiency than the wt.

these immunoprecipitates was assayed in the presence of different Ca²⁺ concentrations. Interestingly, the phosphatase activity associated with PR70 increased with Ca²⁺ concentration, in a dose-dependent manner (Fig. 8A).

Afterwards, we functionally investigated the effect of EF-hand domain mutations on phosphatase activity associated with PR70. Toward this end, we performed a PP2A phosphatase assay using PR70 wt and EF-hand singly and doubly mutated proteins in the absence or presence of Ca²⁺. In keeping with the lack of PP2A_D binding, we found that the EF2 and EF1/2 mutations abolished the phosphatase activity; on the contrary, in agreement with the increased PP2A_D binding efficiency, EF1 mutated protein showed a twofold-higher phosphatase activity than the wt, which further increased in the presence of Ca²⁺ (Fig. 8B).

These data indicate that the Ca²⁺ increase, at least in the range tested, positively modulated PR70-associated phosphatase activity and that EF2-hand domain integrity was necessary for both basal and Ca²⁺-induced activity. Conversely, the EF1-hand domain seems to have a slight inhibitory action.

H₂O₂-induced pRb dephosphorylation is Ca²⁺ dependent. We demonstrated the importance of Ca²⁺ on PR70-associated phosphatase activity and the requirement of this subunit for

FIG. 8. PP2A phosphatase activity associated with PR70 increases with Ca^{2+} concentration. (A) U2OS cells were transiently cotransfected with plasmids encoding PR70, PP2A-A α , and PP2A-C α . After 36 h, cells were lysed and immunoprecipitated with antibodies to PR70 or to IgG (negative isotypic control). PP2A phosphatase activity associated with these immunoprecipitates was assayed in the presence of the indicated Ca^{2+} and EGTA concentrations, corresponding to 0 nM, 30 nM, 360 nM, and 6.4 mM free Ca^{2+} concentrations, respectively. The activity associated with PR70 increased in a dose-dependent manner with Ca^{2+} concentration ($P < 0.02$). (B) U2OS cells were transfected with expression constructs carrying either PR70 wt (wt) or PR70 EF-hand singly mutated (EF1 mut and EF2 mut) or doubly mutated (EF 1/2 mut) protein, together with plasmids encoding PP2A-A α and PP2A-C α . After 36 h, cells were lysed and immunoprecipitated with antibodies to PR70 or to IgG (negative isotypic control). The phosphatase activity associated with each EF mutated protein was assayed in the presence or absence of 12 mM Ca^{2+} and 5.55 mM EGTA. EF2 and EF1/2 mutations abolished the phosphatase activity, whereas EF1 mutated protein showed a twofold-higher phosphatase activity than the wt, which further increased in the presence of Ca^{2+} ($P < 0.03$).

pRb dephosphorylation upon H_2O_2 treatment. Since H_2O_2 treatment is known to induce an increase in $[\text{Ca}^{2+}]_i$ (10, 19), we consequently wondered whether H_2O_2 -induced pRb dephosphorylation was Ca^{2+} dependent.

First, we assessed whether H_2O_2 treatment induced an increase of ($[\text{Ca}^{2+}]_i$) under the adopted experimental conditions. HUVEC were loaded with Fura 2-AM and then assayed by time-resolved fluorescent microscopy. In keeping with previous results (10, 19), HUVEC incubation with both 400 and 800 μM H_2O_2 induced a slowly developing rise in $[\text{Ca}^{2+}]_i$ that started about 10 min after H_2O_2 addition and lasted for more than 30 min (Fig. 9A).

Then we tested whether cell treatment with BAPTA-AM, an intracellular Ca^{2+} chelator, prevented pRb dephosphorylation upon H_2O_2 exposure. Figure 9B shows that BAPTA-AM significantly prevented pRb dephosphorylation induced by H_2O_2 , as assessed with both a pan-pRb antibody and phospho-pRb antibody. Conversely, extracellular Ca^{2+} chelation by EGTA was not sufficient to prevent pRb dephosphorylation induced

by H_2O_2 , at least in the short time frame of our experimental conditions (Fig. 9C).

To determine whether the increase in $[\text{Ca}^{2+}]_i$ was sufficient to induce pRb dephosphorylation in the absence of oxidative stress, HUVEC were treated with thapsigargin, a SERCA inhibitor that elicits $[\text{Ca}^{2+}]_i$ increase. Indeed, HUVEC treatment with thapsigargin induced an increase in $[\text{Ca}^{2+}]_i$ that reached its maximum between 5 and 10 min and declined afterwards (Fig. 10A). Figure 10B shows that thapsigargin rapidly induced pRb dephosphorylation that was already detectable at a 5 nM concentration.

In order to understand if the Ca^{2+} -mediated pRb dephosphorylation was indeed PP2A mediated, we took advantage of SV40 small t. In fact, this viral oncoprotein has the ability to replace B subunits in the PP2A holoenzyme and to inhibit PP2A phosphatase activity toward most substrates (32, 36). Figure 10C shows that SV40 small t completely prevented pRb dephosphorylation induced by Ca^{2+} . In conclusion, although it is possible that other pathways contribute to pRb dephosphorylation, the present results demonstrated that H_2O_2 -induced $[\text{Ca}^{2+}]_i$ increase plays a pivotal role in PP2A-mediated dephosphorylation of pRb.

DISCUSSION

The aim of this study was to identify the regulatory B subunit of PP2A that mediates pRb dephosphorylation upon cell exposure to oxidative stress. We assayed several PP2A-B subunits, and among these only B"/PR48 was able to bind pRb in vitro, although we cannot exclude that other B subunits are able to interact with pRb. It has been demonstrated that PR48 is a partial clone representing an N-terminal truncation of the full-length protein PR70 (38). When we tested this full-length protein for pRb binding, we found that PR70 was able to bind pRb, albeit with less efficiency than PR48. This could be due to the presence of negative binding domains in the N-terminal site of PR70 protein or to conformational changes following protein truncation. The PR70-pRb association was also confirmed in vivo, both in overexpression and at the endogenous level. This interaction occurred with hyperphosphorylated pRb, as demonstrated by the in vivo experiments. However, given that bacterially produced GST-pRb is not phosphorylated, GST pulldowns demonstrated that PR70 interacted also with the nonphosphorylated form of pRb.

Furthermore, it was demonstrated that PR70 was necessary and sufficient for pRb dephosphorylation. In fact, the overexpression of PR70 was able to induce pRb dephosphorylation and PR70 knockdown by RNAi prevented pRb dephosphorylation induced by oxidative stress. Although the PR70 depletion via RNAi we achieved was relatively modest, there was a significant impact on pRb phosphorylation. However, it is not completely unusual that a 50% decrease in the expression of specific genes is insufficient to bring about a wt condition, leading to an abnormal or diseased state (haploinsufficiency). The relatively low levels of PR70 knockdown may also explain the reason why pRb dephosphorylation, induced by oxidative stress, was prevented only in part. Nevertheless, we cannot rule out that other B subunits may intervene in pRb dephosphorylation upon exposure to oxidative stress.

It has been shown that PP2A is involved in the regulation of

FIG. 9. Intracellular but not extracellular Ca^{2+} chelation prevents pRb dephosphorylation induced by H_2O_2 . (A) HUVEC were loaded with Fura 2-AM, and $[\text{Ca}^{2+}]_i$ variations were measured. $[\text{Ca}^{2+}]_i$ was expressed as the time-resolved ratio between fluorescence images obtained at 340 and 380 nm excitation wavelengths. H_2O_2 treatment induces a slowly developing increase in $[\text{Ca}^{2+}]_i$. A representative trace for both H_2O_2 concentrations is shown. (B) HUVEC were incubated for 30 min with Ca^{2+} -free medium containing the indicated concentrations of intracellular Ca^{2+} chelator BAPTA-AM. Once BAPTA-AM is loaded into the cells, the ester bond is cleaved by cytosolic esterase, trapping the active chelator BAPTA intracellularly. Then cells were refed with Ca^{2+} -containing medium to minimize toxicity and incubated with 400 μM H_2O_2 for 2 h. Afterwards, cells were harvested and protein immunoblots were performed using a pan-pRb antibody and a phospho-pRb antibody. w/o H_2O_2 , without H_2O_2 . (C) HUVEC were incubated in Ca^{2+} -free PBS containing 10 mM HEPES (pH 7.4), 1 mM EGTA, and 0.2 mg/ml BSA for 25 min. Then cells were treated with 400 μM H_2O_2 or solvent alone for 1 h. Western blotting analysis of protein extracts derived from these cells showed that pRb was dephosphorylated upon H_2O_2 treatment. Gray arrowheads indicate the hypophosphorylated form of pRb; black arrowheads indicate the hyperphosphorylated form.

p107 and p130 phosphorylation (6, 27, 42, 43). Whether PR70 affects their phosphorylation as much as pRb phosphorylation remains to be elucidated. Voorhoeve et al. showed that another B' subunit identified in the mouse, named PR59, binds and dephosphorylates p107 but not pRb (42), suggesting that different pocket proteins are targeted by distinct PP2A-B subunits. In keeping with this interpretation, PR59 and PR70 diverge significantly both at their N terminus and at the C terminus, a region where most differences between the different B' subunits are located (38). However, PR59 does not have a clear human homologue, indicating that further studies are needed to identify functional homologues between mice and humans. Moreover, whether pocket dephosphorylation is regulated in the same way in these two species needs to be clarified.

The simplest scenario emerging from our study is that PR70 recruits the PP2A catalytic subunit to pRb and PP2A directly catalyzes pRb phosphate removal. In keeping with this model, biochemical studies with purified PP2A showed that in vitro phosphorylated pRb served as a substrate for PP2A (1). However, the present data are also compatible with PP2A activating a second phosphatase that in turn targets pRb phosphorylated residues.

Moreover, while pRb dephosphorylation by PR70-PP2A seems to be a fast-acting mechanism to induce pRb hypophosphorylation upon stress exposure, other mechanisms such as D-cyclin degradation and p21^{Waf1/Cip1/Sdi1} induction may represent reinforcement mechanisms that help to keep pRb dephosphorylated over long periods of time (10).

PP2A-B' family members share the presence of two well-

conserved EF-hand Ca^{2+} -binding domains (termed EF1 and EF2) (22). These motifs are very often present in tandem in many Ca^{2+} -binding proteins like calmodulin (17). Ca^{2+} binding to these domains results in conformational changes within EF-hand proteins that often modulate their biological activities. To assess whether Ca^{2+} was important for PR70 conformation and function, we generated PR70 EF-hand mutated proteins. In these proteins, the glutamate and aspartate residues at positions 1 and 12 of the EF-hand domains were changed into alanines, since these sites are both necessary for Ca^{2+} coordination in many different EF-hand proteins (17). Indeed, the same mutations in B'/PR72 EF-hand domains abolished Ca^{2+} binding completely (22).

It has been demonstrated that the PR72 EF2-hand motif is required for PP2A-A subunit interaction, whereas EF1 is dispensable (22). We obtained similar results for PR70. In fact, PR70 EF2-hand domain integrity was required for the binding to PP2A_D core dimer, whereas the EF1 domain displayed a slight inhibitory effect on PP2A_D-binding ability. Another remarkable result was the dose-dependent increase of PR70/PP2A phosphatase activity with Ca^{2+} concentration. As expected, EF2-hand domain integrity was necessary for both the basal and Ca^{2+} -stimulated activity; conversely, the EF1 domain slightly inhibits the basal phosphatase activity but does not seem to be required for Ca^{2+} responsiveness. Thus, EF-hand domains are both involved, although to different extents, in PR70 structural and functional regulation, underlying the importance of $[\text{Ca}^{2+}]$ on PR70/PP2A phosphatase activity.

Moreover, while the aim of the presented experiments was simply to assess whether in vitro PP2A activity of PR70 was

FIG. 10. Intracellular Ca^{2+} increase is sufficient to induce pRb dephosphorylation. (A) HUVEC were loaded with Fura 2-AM, and $[\text{Ca}^{2+}]_i$ variations were measured by time-resolved fluorescent microscopy. HUVEC treatment with thapsigargin (Tg) induced an increase of $[\text{Ca}^{2+}]_i$, that reached its maximum between 5 and 10 min and declined afterwards. A representative trace for thapsigargin is shown. (B) HUVEC were treated for 2 h with increasing concentrations of thapsigargin, as indicated. Western blotting analysis was performed with antibody against pRb and phospho-pRb. (C) HUVEC infected with retroviruses encoding SV40 small t wt (wt) or backbone vector alone (vec) were exposed to 1 μM thapsigargin or solvent alone for 2 h. Afterwards, cells were lysed and proteins were immunoblotted with antibody raised against pRb and small t. Thapsigargin-induced pRb dephosphorylation was markedly inhibited in cells expressing small t wt. Gray arrowheads indicate the hypophosphorylated form of pRb; black arrowheads indicate the hyperphosphorylated form.

influenced by Ca^{2+} , it is worth noting that most Ca^{2+} concentrations used in this study are within—or very close to—the physiological range (10, 19).

Calcium is a ubiquitous second messenger controlling a broad range of cellular functions, including growth and proliferation (4). The duration, amplitude, and frequency of Ca^{2+} rises and the spatiotemporal compartmentalization are the distinguishing parameters that enable a cell to use Ca^{2+} signaling for a wide variety of physiological responses. Different types of calcium signals are able to induce either proliferation or cell cycle arrest maintenance in quiescent cells, activating two alternative pathways (28). Reactive oxygen species (ROS), which include H_2O_2 , superoxide anion and hydroxyl radicals, are produced in response to many different stresses, such as hypoxia and hyperglycemia. Both stresses are known to evoke a cell cycle arrest and an intracellular Ca^{2+} increase (33, 39).

Thus, a Ca^{2+} -induced PR70 activation leading to pRb dephosphorylation, in both hypoxia and hyperglycemia, could be proposed. Moreover, it is tempting to speculate that PR70 activation could be part of a more general response elicited by different stimuli involving an H_2O_2 -like Ca^{2+} signal, characterized by a slowly developing and sustained Ca^{2+} mobilization (10, 19).

ROS are also known to play a major role in cellular senescence, a permanent withdrawal from cell cycle in response to stress (12). Signaling that induces senescence has been extensively studied, and two major tumor suppressor cascades have been unraveled—one involving the pRb pathway and another p53—and both orchestrate the exit from cell cycle (5). One might speculate that PR70 may be involved in the onset of senescence as well, given a PR70 requirement for pRb dephosphorylation and DNA synthesis arrest in response to H_2O_2 -induced oxidative stress.

Another potential implication comes from the fact that PR48, the truncated form of PR70, was first identified as a Cdc6-interacting protein (44). Cdc6 is an ATPase required for the initiation of DNA replication that acts by recruiting the prereplicative complexes to the origins of replication (9). Thus, PR48 directs PP2A activity to the DNA replication machinery through an interaction with Cdc6 (44). Notably, it has been demonstrated that, in primary fibroblasts, DNA synthesis appears in specific nuclear foci, which contain replication proteins and members of the retinoblastoma protein family (24). Furthermore, it has been shown that during S phase, the pRb pool associated with chromatin is underphosphorylated and that PP2A activity licenses the recruitment of pRb to chromatin sites. After DNA damage, an intra-S-phase arrest is evoked and pRb relocates to selected replication control sites in order to suppress abnormal, postdamage rereplicative activity (3, 25). One might speculate that PR70/PP2A recruits pRb to the replication origins and together with the replication proteins is part of the prereplicative complex that controls DNA replication following DNA damage. In agreement with this hypothesis, a pRb function in DNA synthesis inhibition was demonstrated during S phase, in response to DNA damage induced by chemotherapeutics, such as cisplatin (25) and following H_2O_2 treatment (6). As a further confirmation, we demonstrated that the inhibition of DNA synthesis induced by H_2O_2 was markedly attenuated in cells with PR70 knocked down compared to control cells. These data underlie the importance of PR70 for the regulation of pRb phosphorylation and consequently for the control of DNA synthesis upon oxidative stress exposure.

PP2A has been demonstrated to be involved in the development of cancer (21). Since okadaic acid, a potent inhibitor of PP2A, is a tumor promoter (8), PP2A has been proposed as a negative regulator of cellular growth. The tumor suppressor function of PP2A has been attributed to specific subunits of this complex enzyme, PP2A-A being one of these, since several studies reported cancer-associated mutations in this subunit (21). The most characterized subunit involved in PP2A tumor suppressor function is a member of the B' family of subunits, B56 γ . An N-terminal truncation of this subunit is highly expressed in metastatic melanoma cells, and its down-modulation mimics virus-induced transformation (2, 21, 41). PP2A inhibitors seem to play an important role as well: PP2A activity

is inhibited by the BCR/ABL-induced expression of the PP2A inhibitor SET (31), and CIP2A is involved in the inhibition of PP2A activity toward *c-myc* (23). One might speculate that PP2A tumor suppressor function could be mediated also by the PR70 B' subunit. We can hypothesize that cells lacking PR70 function could possibly give rise to tumor formation, owing to a lack of pRb dephosphorylation. This idea is also supported by the fact that the Rb gene itself is mutated at a relatively low frequency in tumors, but it is inactivated in the majority of them by alterations of its phosphorylation (7, 30). Thus, PP2A inactivation or loss could provide a further mechanism for pRb functional impairment.

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