Regulation of Peroxiredoxin I Activity by Cdc2-mediated Phosphorylation*

Received for publication, October 30, 2001, and in revised form, March 26, 2002
Published, JBC Papers in Press, May 1, 2002, DOI 10.1074/jbc.M110432200

Tong-Shin Chang‡§, Woorin Jeong‡§, Soon Young Choi‡¶, Shiqin Yu†, Sang Won Kang‡§, and Sue Goo Rhee‡**

From the ‡Laboratory of Cell Signaling, NHLBI, National Institutes of Health, Bethesda, Maryland 20892 and the §Center for Cell Signaling Research, Ewha Womans University, Seoul 120-750, Korea

Hydrogen peroxide is implicated as an intracellular messenger in various cellular responses such as proliferation and differentiation. Peroxiredoxin (Prx) I is a member of the peroxiredoxin family of peroxidases and contains a consensus site (Thr30-Pro-Lys-Lys) for phosphorylation by cyclin-dependent kinases (CDKs). This protein has now been shown to be phosphorylated specifically on Thr90 by several CDKs, including Cdc2, in vitro. Phosphorylation of Prx I on Thr90 reduced the peroxidase activity of this protein by 80%. The phosphorylation of Prx I in HeLa cells was monitored with the use of antibodies specific for Prx I phosphorylated on Thr90. Immunoblot analysis with these antibodies of HeLa cells arrested at various stages of the cell cycle revealed that Prx I phosphorylation occurs in parallel with the activation of Cdc2; Prx I phosphorylation was thus marked during mitosis but virtually undetectable during interphase. Furthermore, when Cdc2 expression was reduced by RNA interference with cognate small interfering RNAs, Prx I phosphorylation was not observed in the cells synchronized in mitotic phase. The cytosolic location of Prx I likely prevents its interaction with activated CDKs until after the breakdown of the nuclear envelope during mitosis, when Cdc2 is the CDK that is most active. Phosphorylation of Prx I on Thr90 both in vitro and in vivo was blocked by roscovitine, an inhibitor of CDKs. These results suggest that Cdc2-mediated phosphorylation and inactivation of Prx I and the resulting intracellular accumulation of H2O2 might be important for progression of the cell cycle.

Although H2O2 is generally considered a toxic by-product of respiration, recent evidence suggests that the transient generation of H2O2 is an important signaling event triggered by the activation of many cell surface receptors (1–4). H2O2 is an intracellular messenger that mediates various cellular functions, including the activation of transcription factors such as NF-κB (5) and AP-1 (6, 7) as well as the inactivation of protein-tyrosine phosphatases (8–12). The mechanism by which H2O2 propagates receptor-induced signaling is largely unknown. However, the inactivation of protein-tyrosine phosphatases by H2O2 produced in response to receptor activation is mediated by the specific oxidation of their essential cysteine residues. The oxidized phosphatases are re-activated by cellular reductants after the concentration of H2O2 returns to normal (9).

The timely elimination of second messengers, such as cyclic nucleotides and inositol 1,4,5-trisphosphate, after the completion of their functions is important for efficient signaling. Thus, in general, both the production and the elimination of second messengers are strictly controlled. Control of the elimination of H2O2 is likely especially important given that this molecule is readily converted to the destructive hydroxyl radical. Enzymes capable of eliminating H2O2 include catalase, glutathione peroxidase, and peroxiredoxin (Prx).1 There is no evidence to suggest that the activities of catalase and glutathione peroxidase are regulated.

Members of the Prx family of peroxidases are present in organisms from all kingdoms (13, 14). All Prx enzymes contain a conserved cysteine residue in the NH2-terminal region that is the primary site of oxidation by H2O2. At least six Prx enzymes are present in mammals, and these proteins can be divided into three subgroups referred to as 2-Cys, atypical 2-Cys, and 1-Cys (14–16). The 2-Cys isozymes (Prx I, II, III, and IV) contain an additional conserved cysteine in the COOH-terminal region, whereas the atypical 2-Cys (Prx V) and the 1-Cys (Prx VI) isozymes do not. The amino acid sequence homology among the three subgroups is low (<20% identity), whereas that among the members of the 2-Cys subgroup is relatively high (60–80% identity). Prx isoforms are distributed differentially within cells: Prx I and II are localized to the cytosol (17, 18); Prx III is synthesized with a mitochondrial-targeting sequence and is restricted to mitochondria; Prx IV is synthesized with an NH2-terminal signal sequence for secretion and is present in the endoplasmic reticulum as well as in the extracellular space; Prx V is expressed in long and short forms that are located in mitochondria and peroxisomes, respectively (16); and Prx VI is found in the cytosol (19, 20). When overexpressed in various cell types, Prx enzymes efficiently reduced the increase in the intracellular concentration of H2O2 induced by platelet-derived growth factor or tumor necrosis factor-α, inhibited the activation of NF-κB induced by tumor necrosis factor-α, and blocked apoptosis induced by ceramide (19, 21, 22), suggesting that they function in signaling cascades by removing H2O2.

Although they are implicated in intracellular signaling, the

1 The abbreviations used are: Prx, peroxiredoxin; P-Prx I, Prx I phosphorylated on Thr90; CDK, cyclin-dependent kinase; siRNA, the small interfering RNA; Trx, thioredoxin.
activity of Prx enzymes has not previously been shown to be regulated in response to cell stimulation, as is the case with many other enzymes that catalyze the metabolism of second messengers. In the present study, we demonstrate that purified Prx I is phosphorylated on Thr90 by cyclin-dependent kinases (CDKs), including Cdc2 (also known as CDK1), resulting in a marked inhibition of the peroxidase activity of Prx I. Furthermore, Prx I phosphorylation in HeLa, HepG2, and NIH 3T3 cells was apparent during mitosis but not during interphase. These results suggest that Cdc2, the CDK that is activated during mitosis, is responsible for Prx I phosphorylation in intact cells and that the accumulation of H2O2 that results activated during mitosis, is responsible for Prx I phosphorylation in intact cells and that the accumulation of H2O2 that results in intact cells and that the accumulation of H2O2 that results

**EXPERIMENTAL PROCEDURES**

**Materials**—A monoclonal antibody to Cdc2 and rabbit antibodies to other CDKs (CDK2, CDK4, and CDK6) were obtained from Santa Cruz Biotechnology, and the purified recombinant Cdc2-cyclin B complex was from New England Biolabs. Roscovitine, okadaic acid, thymidine, and nocodazole were obtained from Calbiochem, propidium iodide was from Molecular Probes, histone H1 was from Invitrogen, DNase-free RNA-free water, 5′- and 3′-biotinylated primers, Q-Sepharose XL, and Sepharose 4B were from Amersham Biosciences. Rat thioridazine (Trx) (19), rat Trx reductase (23), Echerichia coli glutamine synthetase (24), recombinant 2-Cys Prx proteins (Prx I to IV) (19), and rabbit antisera to human Prx I (19) were prepared as described previously.

**Expression and Purification of Wild-type and Mutant Prx I**—The expression of a bacterial expression system for human Prx I (pET-Prx I) was described previously (19). Mutation of Thr90 to alanine (Prx I T90A) or to aspartate (Prx I T90D) was performed by polymerase chain reaction-mediated site-directed mutagenesis with pET-Prx I as the template. e coli BL21 (DE3) cells were transformed with the wild-type or mutant pET-Prx I plasmids, and the encoded proteins were purified from bacterial extracts as described (19).

**Preparation of Prx I Phosphorylated on Thr90 by Cdc2**—Recombinant Prx I (250 μg) was incubated for 6 h at 30°C with 100 ng of the purified Cdc2-cyclin B complex in a final volume of 50 μl containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 100 μM ATP, and 50 μCi of [γ-32P]ATP. Phosphorylated Prx I was separated from the unphosphorylated protein by diluting the reaction mixture with 50 μl of Tris-HCl (pH 8.5) and applying the resulting solution to a Q-Sepharose column that had been equilibrated with 50 mM Tris-HCl (pH 8.5) containing 0.5 M NaCl. After washing twice with 50 mM Tris-HCl (pH 8.5) containing 0.5 M NaCl, the column was washed with 50 mM Tris-HCl (pH 8.5) containing 0.5 M NaCl.

**Cell Culture and Synchronization**—HeLa cells were cultured in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 units/ml). The cells were arrested in the mitotic phase of the cell cycle by exposure to nocodazole as described (25). In brief, they were incubated with nocodazole (200 ng/ml) for 16 h, after which the culture dishes were shaken, and floating cells (~50% of total cells) were harvested. After washing twice with phosphate-buffered saline, the cells were cultured in nocodazole-free medium to induce release from mitotic arrest. HeLa cells were synchronized at the G1-S transition of the cell cycle by the thymidine double-block method (26) with some modifications. In brief, HeLa cells in the exponential growth phase were exposed to 2 mM thymidine for 16 h, released into thymidine-free medium for 8 h, and then released into thymidine-free medium. The block was released by transfer of cells to thymidine-free medium.

**Flow Cytometry**—Approximately 1 × 106 cells were washed twice with ice-cold phosphate-buffered saline and then fixed overnight at 4°C in 70% ethanol. Immediately before flow cytometry, the cells were resuspended in phosphate-buffered saline containing propidium iodide (50 μg/ml) and DNase-free RNase A (10 μg/ml). Flow cytometry was performed with a Coulter Epics XL instrument (Beckman Coulter).

**Preparation of HeLa Cell Lysates**—HeLa cells were washed twice with ice-cold phosphate-buffered saline and then lysed for 10 min at 4°C in a solution containing 25 mM Hepes-NaOH (pH 7.4), 2 mM EGTA, 25 mM β-glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM Na2VO3, 5 mM NaF, 1 mM 4-(2-aminoethyl)benzenesulfonil fluoride, aprotinin (10 μg/ml), leupeptin (10 μg/ml), and 25 mM microcinyst. The lysate was centrifuged at 15,000 × g for 10 min, and the protein concentration of the resulting supernatant was measured with a Bradford assay before experiments.

**In Vitro Assay of Kinase Activity**—Recombinant Prx I, II, III, or IV (2 μg each) was phosphorylated by the purified recombinant Cdc2-cyclin B complex (10 ng) in a final volume of 30 μl containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM EGTA, 1 mM dithiothreitol, 100 μM ATP, and 10 μCi of [γ-32P]ATP. The reaction mixture was incubated for the indicated times at 30°C, after which proteins were separated by SDS-PAGE on a 12% gel. The radioactivity associated with protein bands was quantified by PhosphorImager (Molecular Dynamics) analysis.

The phosphorylation of 1 μg of Prx I or histone H1 was also performed with immunoprecipitated CDKs under the same reaction conditions as those described above. HeLa cell lysates (0.1–1 mg of protein) were incubated for 1 h at 4°C with 15 μl of protein G-Sepharose beads and subsequent separation of the beads by centrifugation. The beads were washed twice with cell lysis buffer and twice with washing buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM EGTA) before use as the source of kinase.

**Preparation of Monospecific Antibodies to Prx I Phosphorylated on Thr90**—Antibodies were raised in rabbits with keyhole limpet hemocyanin conjugated to the phosphopeptide CHLAWVNTQPKKG, which corresponds to residues 83–95 of human Prx I. To remove antibodies that recognize the corresponding unphosphorylated peptide, we applied serum subsequently obtained from the rabbits to Sepharose 4B resin conjugated with the unphosphorylated peptide. The unbound fraction was further purified by collection of the antibodies that bound to Sepharose 4B conjugated with the phosphopeptide. The resulting monospecific antibodies were used for immunoblot analysis of phosphorylated Prx I.

**RNA Interference**—The small interfering RNAs (siRNAs) that target human Cdc2 were based on the coding regions 125–147 relative to the first nucleotide of the start codon as described previously (27), purchased from Dharmacon (Lafayette, CO) in purified and annealed form, and resuspended as described (28). Briefly, the day before transfection, HeLa cells were trypsinized, diluted with fresh medium without antibiotics, and transferred to 24-well plates (500 μl/well). Transient transfection of siRNAs was performed using OligofectAMINE (Invitrogen). 12 μl of OptiMEM medium/well (Invitrogen) and 3 μl of OligofectAMINE/well were preincubated for 10 min at room temperature. During the time for this incubation 50 μl of OptiMEM medium were mixed with 3 μl of 20 μM siRNA. The two mixtures were combined and incubated for 20 min at room temperature for complex formation. After the addition of 32 μl of OptiMEM medium to the mixture, the entire mixture was added to the cells in one well resulting in a final concentration of 100 nM for the siRNAs.

**Assay of Peroxidase Activity of Prx I**—The peroxidase activity of Prx I was measured as described (29). In brief, the initial rate of NADPH oxidation was monitored spectrophotometrically at 30°C in a reaction mixture (200 μl) containing 50 mM Hepes-NaOH (pH 7.0), 0.2 mM NADPH, 50 mM Trx reductase, 5 μM Trx, 0.1 mM H2O2, and 4 μg of Prx I.

**RESULTS**

**Phosphorylation of Prx Enzymes by CDKs in Vitro**—We noticed that the amino acid sequence of the 2-Cys Prx enzymes from human, rat, and mouse all contain the consensus sequence, (Ser/Thr)-Pro-Xaa-(Lys/Arg), for phosphorylation by CDKs; these sequences are Thr-Pro-Lys-Lys for Prx I, Thr-Pro-Arg-Arg for Prx II and Prx III, and Thr-Pro-Arg-Arg for Prx IV. Neither Prx V nor Prx VI contains such a consensus sequence (30, 31). The putative phosphorylation site Thr, which in human Prx I is Thr90, is located 38 residues downstream of the NH2-terminal conserved cysteine in all four 2-Cys Prx enzymes. To determine whether CDKs are able to catalyze the phosphorylation of 2-Cys Prx enzymes, we phosphorylated purified recombinant Prx I, II, III, and IV (10 μg each) in a final volume of 25 μl with constant rotation. After removal of the beads by centrifugation, the resulting supernatants were incubated for 2 h at 4°C with 1 μg of a monoclonal antibody to Cdc2 or with 4 μg of rabbit antibodies to CDK2, CDK4, or CDK6. The immune complexes were recovered by further incubation for 1 h at 4°C with 15 μl of protein G-Sepharose beads and subsequent separation of the beads by centrifugation. The beads were washed twice with cell lysis buffer and twice with washing buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM EGTA) before use as the source of kinase.

**Preparation of Monospecific Antibodies to Prx I Phosphorylated on Thr90**—Antibodies were raised in rabbits with keyhole limpet hemocyanin conjugated to the phosphopeptide CHLAWVNTQPKKG, which corresponds to residues 83–95 of human Prx I. To remove antibodies that recognize the corresponding unphosphorylated peptide, we applied serum subsequently obtained from the rabbits to Sepharose 4B resin conjugated with the unphosphorylated peptide. The unbound fraction was further purified by collection of the antibodies that bound to Sepharose 4B conjugated with the phosphopeptide. The resulting monospecific antibodies were used for immunoblot analysis of phosphorylated Prx I.
Inhibitor of several CDKs including Cdc2 (33). To verify the site corresponding unphosphorylated peptide. The specificity of the bind to the phosphorylated 13-residue peptide but not to the C
Prx I T90A was undetectable (Fig. 1A). The similarity in phosphorylation of the wild-type protein, phosphorylation of purified Cdc2-cyclin B complex. In contrast to the marked phosphorylation of the wild-type protein, phosphorylation of the purified Cdc2-cyclin B complex (5 ng). All of the phosphorylation reactions were performed at 30°C in the presence of [γ-32P]ATP. The Prx proteins were separated by SDS-PAGE on a 12% gel and visualized by autoradiography (i[32P]Prx), staining with Coomassie Blue (Coomassie), or immunoblot analysis with antibodies to Prx I (αPrx I).

Phosphorylation of Prx I by immunoprecipitated Cdc2 was prevented by 2 μM roscovitine (Fig. 1B), a potent and selective inhibitor of several CDKs including Cdc2 (33). To verify the site of phosphorylation of Prx I by Cdc2, we subjected both wild-type Prx I and the mutant Prx I T90A, in which Thr90 is replaced by alanine, to the in vitro kinase assay with the purified Cdc2-cyclin B complex. In contrast to the marked phosphorylation of the wild-type protein, phosphorylation of Prx I T90A was undetectable (Fig. 1C), indicating that Thr90 is the only site of Prx I phosphorylated by Cdc2.

Phosphorylation of Prx I in HeLa Cells—To facilitate the monitoring of Prx I phosphorylation in intact cells, we prepared antibodies specific for Prx I phosphorylated on Thr90 by injecting rabbits with a 13-residue phosphopeptide corresponding to residues 83–95 of Thr90-phosphorylated human Prx I. The resulting antiserum was subjected to selection for antibodies that bind to the phosphorylated 13-residue peptide but not to the corresponding unphosphorylated peptide. The specificity of the purified antibodies was assessed by performing in vitro kinase reactions with purified Prx I and Cdc2-cyclin B in the absence or presence of [γ-32P]ATP. The resulting 32P-labeled Prx I and unphosphorylated Prx I were combined in various ratios, and then equal amounts of the Prx I protein combinations were subjected to autoradiography or to immunoblot analysis either with the monospecific antibodies to Thr90-phosphorylated Prx I (P-Prx I) or with antibodies to Prx I (αPrx I).

To examine whether Prx I is phosphorylated on Thr90 in intact cells, we took advantage of the fact that okadaic acid, an inhibitor of type 1 and type 2A serine-threonine protein phosphatases, activates Cdc2 by inducing phosphorylation of Cdc25 (34, 35). We also reasoned that okadaic acid might induce the accumulation of P-Prx I molecules by inhibiting their dephosphorylation. We therefore exposed HeLa cells to various concentrations (0–25 nM) of okadaic acid for 24 h, after which Cdc2 was immunoprecipitated from cell lysates and assayed for kinase activity with histone H1 as substrate. The presence of P-Prx I in the cell lysates was also evaluated by immunoblot analysis with the monospecific antibodies (Fig. 3A). Both the kinase activity of Cdc2 and the amount of P-Prx I immunoreactivity increased as the concentration of okadaic acid to which the cells were exposed increased.

Okadaic acid has previously been shown to block cell cycle progression during the G2-M phase (36, 37). We examined the effect of this agent on the cell cycle in HeLa cells by flow cytometric analysis of cellular DNA content. Control HeLa cells in the exponential phase of growth were distributed in the G1, S, and G2-M phases in the ratio 60:23:17. The cell cycle distribution remained virtually unchanged for cells exposed to 5 or 10 nM okadaic acid; however, the percentage of cells arrested in G2-M increased from 17% to 40% for those exposed to 0, 5, or 10 nM okadaic acid to 35, 58, and 57% for those treated with 15, 20, or 25 nM okadaic acid, respectively (Fig. 3B). The similarity in the dependence of Cdc2 activation, P-Prx I accumulation, and...
Cell Cycle-dependent Phosphorylation of Prx I on Thr\(^{90}\)—To investigate further the phosphorylation status of Prx I during cell cycle progression, we subjected proliferating HeLa cells to mitotic arrest with nocodazole. The cells were then released from mitotic arrest and, at various times thereafter, subjected to assay of the kinase activity of Cdc2, to immunoblot analysis of P-Prx I, and to flow cytometric analysis of DNA content.

The detached cells collected 16 h after the onset of nocodazole treatment appeared to be arrested in mitosis as evidenced by a high level of Cdc2 kinase activity and the observation that 93% of the cells had a fully replicated complement of DNA (Fig. 4A). Prx I in the mitotically arrested cells was highly phosphorylated on Thr\(^{90}\), whereas phosphorylation of this residue of Prx I was virtually undetectable in asynchronous cells (Fig. 4A). Release of the arrested cells by incubation in nocodazole-free medium resulted in cell cycle progression into G1 phase; the percentage of cells in G1 increased from 4% of the arrested cells to 6, 15, and 39% of cells 1, 2, and 4 h after release, respectively. As the cells progressed into G1, the kinase activity of Cdc2 as well as the amount of P-Prx I decreased, suggesting that Prx I phosphorylation occurs in the M phase but not in the G1 phase. Phosphorylation of Prx I on Thr\(^{90}\) was also observed in NIH 3T3 and HepG2 cells that had been subjected to mitotic arrest with nocodazole (data not shown).

We also subjected HeLa cells to synchronization at the G1-S transition by the thymidine double-block method and then released the synchronized cells from arrest by the addition of thymidine-free medium. Both the kinase activity of Cdc2 and the amount of P-Prx I were virtually undetectable in both asynchronous cells and cells arrested at G1-S. Furthermore, Cdc2 kinase activity and phosphorylation of Prx I remained undetectable in cells released from arrest, despite the fact that the percentage of cells in S phase increased from 31% of the arrested cells to 51, 54, and 69% of cells 1, 2, and 4 h after release, respectively. These results suggest that Prx I is not phosphorylated during S phase. The abundance of Prx I remained relatively constant during progression of cells through the various stages of the cell cycle (Fig. 4B).

The extent of Prx I phosphorylation was estimated in HeLa cells that had been synchronized in mitotic phase with nocodazole treatment. To prepare standard samples to which Prx I from the mitotic HeLa cells can be compared, purified recombinant Prx I was phosphorylated by Cdc2-cyclin B in vitro in the presence of [\(\gamma^{32}P\)]ATP. On the basis of the amount of [\(\gamma^{32}P\)]radioactivity associated with Prx I, the extent of phosphorylation was determined to be ~10% of the maximal level (data not shown). The HeLa cell lysates, along with in vitro phosphorylated Prx I, were subjected to immunoblot analyses with antibodies to P-Prx I and Prx I (Fig. 5). From comparison of their immunoblot intensities, the percentage of phosphorylated Prx I in the mitotic HeLa cells was estimated to be ~12%.

The effect of roscovitine on Prx I phosphorylation was also examined in HeLa cells. HeLa cells that had been subjected to mitotic arrest with nocodazole were exposed for 1 h to various concentrations of roscovitine (Fig. 6). As expected, roscovitine inhibited the kinase activity of Cdc2 in a concentration-dependent manner. In parallel with its effect on Cdc2 activity, roscovitine also reduced the level of Prx I phosphorylation on Thr\(^{90}\). These results indicate that Prx I phosphorylation in mitotic cells is reversible and that once P-Prx I becomes dephosphorylated, its rephosphorylation is slower in roscovitine-treated cells than in untreated cells.

Effect of Cdc2 Knockdown on Prx I Phosphorylation—To provide more definite proof that Cdc2 phosphorylates Prx I during the M phase, we employed siRNA-mediated interference (28) to deplete endogenous Cdc2 protein in HeLa cells. Transfection with the siRNAs for Cdc2 reduced the level of Cdc2 protein to <10% of that of the mock transfected cells (Fig. 7) but did not affect the expression of Prx I or tubulin protein (data not shown). Importantly, unlike in the mock transfected cells, Prx I phosphorylation could not be observed when the siRNA-transfected cells were treated with nocodazole (Fig. 7). This result demonstrates that Cdc2 is involved in Prx I phosphorylation.

Effect of Thr\(^{90}\) Phosphorylation on Prx I Peroxidase Activity—To investigate the effect of phosphorylation of Prx I on its peroxidase activity, the in vitro phosphorylated Prx I, for which the extent of phosphorylation was shown to be ~10%, was subjected to chromatography on a Q-Sepharose column to separate phosphorylated and unphosphorylated enzymes (data not shown). Then peroxidase activities of phosphorylated and unphosphorylated Prx I enzymes were determined by monitoring the H\(_2\)O\(_2\)-dependent oxidation of NADPH in the presence of Trx and Txr reductase. Prx I catalyzes the reduction of H\(_2\)O\(_2\) to H\(_2\)O with the use of reducing equivalents provided by Trx, which in turn receives reducing equivalents from NADPH in the presence of Trx reductase (29). The peroxidase activity of P-Prx I was 20% of that of unphosphorylated Prx I (Fig. 8, left panel).

The effect of phosphorylation is often mimicked by replace-
ment of the target residue with an acidic amino acid such as aspartate or glutamate. The replacement of Thr90 of Prx I with aspartate (T90D) resulted in an 87% reduction in peroxidase activity, whereas replacement of the same residue by alanine resulted in only a 30% reduction in peroxidase activity (Fig. 8, right panel).

**DISCUSSION**

Cell cycle progression in mammalian cells depends on various CDKs, which form complexes with different cyclins and perform specific functions during each phase of the cycle. Cdc2 thus associates with cyclin B and regulates the transition from G2 to M; CDK2 interacts with cyclin A or cyclin E and regulates the transition from G1 to S; and CDK4 and CDK6 bind D-type cyclins and regulate progression through G1 (38–40). We have...
now shown that Prx I is phosphorylated on Thr\(^{90}\) by Cdc2 as well as by CDK2, CDK4, and CDK6 in vitro. Despite the broad susceptibility of Prx I to phosphorylation by different CDKs, the amount of P-Prx I in cells varied in parallel with the activity of Cdc2 during cell cycle progression, being high in cells in the G\(_2\) and M phases but not detectable in G\(_1\) or S phase cells. The cytosolic localization of Prx I likely prevents its interaction with activated CDKs during interphase; Prx I may thus come into contact with activated CDKs only after the breakdown of the nuclear envelope during mitosis (more specifically during prometaphase in the M phase). During mitosis, Cdc2 is fully active, whereas other CDKs are inactive. The RNA interference experiment also suggests that Cdc2 is involved in Prx I phosphorylation.

The peroxidase activity of P-Prx I was \(-20\%\) of that of the unphosphorylated enzyme. Because the phosphorylation reaction was incomplete, the phosphorylated and unphosphorylated enzymes were separated by ion exchange chromatography before measurement of peroxidase activity. However, the extent of phosphorylation estimated for the separated P-Prx I fraction on the basis of incorporated \(^{32}\)P radioactivity was substantially less than stoichiometric (data not shown). Given that Prx I exists as homodimers, the separated P-Prx I fraction might have contained Prx I dimers that comprise both phosphorylated and unphosphorylated subunits. It is therefore possible that the peroxidase activity observed with the P-Prx I fraction was attributable to the presence of unphosphorylated Prx I subunits associated with phosphorylated subunits.

The sulphydryl group of the conserved NH\(_2\)-terminal Cys\(^{52}\) residue (Cys\(^{52}\)-SH) of Prx I is the primary site of oxidation by \(\mathrm{H}_2\mathrm{O}_2\), and the resulting Cys\(^{52}\)-SOH group forms an intermolecular disulfide linkage with the conserved COOH-terminal Cys\(^{173}\)-SH of the paired subunit in the homodimer. The crystal structure of Prx I indicates that the reactivity of Cys\(^{52}\)-SH is attributable to stabilization of its ionized state by the positively charged environment provided by Arg\(^{128}\), Arg\(^{151}\), and the di- and tri-oligomers of helices \(\alpha_2\) and \(\alpha_5\) (41). The thiolate anion of cysteine is markedly more susceptible to oxidation than is protonated cysteine. The introduction of a negative charge by phosphorylation of Thr\(^{90}\), which is also located close to Cys\(^{52}\), might therefore disturb the charge-charge or charge-dipole interactions, rendering Cys\(^{52}\)-SH insensitive to \(\mathrm{H}_2\mathrm{O}_2\). This notion is consistent with the observation that replacement of Thr\(^{90}\) with aspartate reduced the peroxidase activity of Prx I by 87%.

P-Prx II was also phosphorylated, albeit to a lesser extent than was P-Prx I, by Cdc2 in vitro. P-Prx II is also a cytosolic protein, and Cdc2-mediated phosphorylation and inactivation of P-Prx II are therefore also likely to occur during mitosis. The percentage of phosphorylated Prx I in the mitotic HeLa cells was estimated to be 12%. Because the intracellular concentration of \(\mathrm{H}_2\mathrm{O}_2\) is the net result of production and destruction, inhibition of Prx I activity by 12% might lead to a significant increase in \(\mathrm{H}_2\mathrm{O}_2\) concentration. It is also possible that the phosphorylation of P-Prx I is a local phenomenon restricted to where activated Cdc2 encounters P-Prx I. In this case, the percentage of phosphorylated P-Prx I in certain areas could be much higher. The significance of a transient increase in \(\mathrm{H}_2\mathrm{O}_2\) concentration during mitosis is not clear. One potential target of \(\mathrm{H}_2\mathrm{O}_2\) signaling is Cdc25C, a dual specificity phosphatase that is an important regulator of Cdc2 (42–46). Cyclin B, although necessary, is not sufficient to activate Cdc2. Once bound to cyclin B during the G\(_2\) phase, Cdc2 is phosphorylated initially on Thr\(^{14}\) and Tyr\(^{15}\) residues by an inhibitory kinase and then on Thr\(^{161}\) by a stimulatory kinase. This triply phosphorylated form of Cdc2 is still inactive. At the onset of mitosis, Cdc25C activates Cdc2 by catalyzing the dephosphorylation of Thr\(^{14}\) and Tyr\(^{15}\) (40, 47, 48). Cdc25C is weakly active during interphase and fully activated during mitosis. The activity of Cdc25C is also highly regulated by phosphorylation and dephosphorylation; Cdc25C is phosphorylated and activated by several kinases including Cdc2 and is inactivated by okadaic acid-sensitive phosphatases (49). A low level of Cdc2 activity generated as a result of the activation of Cdc25C is therefore expected to increase further the activities of both Cdc25C and Cdc2 in a positive feedback loop.

Like other protein-tyrosine phosphatases, Cdc25 isoforms contain an essential cysteine residue in the His-Cys-Xaa-Xaa-Xaa-Xaa-Xaa-Arg motif (50, 51). The active site cysteines of protein-tyrosine phosphatases are sensitive to oxidation by \(\mathrm{H}_2\mathrm{O}_2\) because their ionization is promoted by the conserved histidine and arginine residues (9, 52, 53). Protein-tyrosine phosphatase 1B is reversibly inactivated by \(\mathrm{H}_2\mathrm{O}_2\) produced in response to epidermal growth factor in A431 cells (9). Cdc25C is also sensitive to oxidation and requires the presence of reducing agents for its activity (42, 54). Furthermore, the crystal structures of Cdc25A and Cdc25B have revealed that the catalytic cysteine residue of these proteins forms an intramolecular disulfide bond with another conserved cysteine (50, 51), suggesting that the active site cysteine is sensitive to oxidation.

On the basis of these various observations, we propose that, as mitosis progresses to prometaphase and the nuclear envelope breaks down, Prx I and likely P-Prx II are phosphorylated by Cdc2 that was activated at the G\(_2\)-M transition. The phosphorylation of these two cytosolic Prx enzymes results in their inactivation and the consequent intracellular accumulation of \(\mathrm{H}_2\mathrm{O}_2\). The resulting \(\mathrm{H}_2\mathrm{O}_2\) induces inactivation of Cdc25C, which in turn halts the positive feedback loop formed by Cdc25C and Cdc2. Thus, \(\mathrm{H}_2\mathrm{O}_2\) may function as an inhibitor of Cdc25C activity throughout the stages of mitosis and may affect cell cycle progression in the M phase. It is important to note that a recent report indicates that sublethal doses of \(\mathrm{H}_2\mathrm{O}_2\) induces a transient multi-phase cell cycle arrest through up-regulation of p21\(^{wp1}\) and down-regulation of cyclin D expression (55). Regardless of whether or not Cdc25C is a target of \(\mathrm{H}_2\mathrm{O}_2\) during the cell cycle, our results demonstrate that the peroxidase activity of Prx I is regulated by phosphorylation. Our data thus provide the first example of posttranslational modification of an \(\mathrm{H}_2\mathrm{O}_2\)-eliminating enzyme (such modification has not been demonstrated for catalase, glutathione peroxidase, or other Prx isozymes). The phosphorylation of Prx I is consistent with the notion that the intracellular concentration of \(\mathrm{H}_2\mathrm{O}_2\) is strictly regulated through control of the production and elimination of this agent.

REFERENCES
1. Rhee, S. G. (1999) Exp. Mol. Med. 31, 53–59
2. Rhee, S. G., Bae, Y. S., Lee, S.-R., and Kwon, J. (2000) Science’s STKE, www.stke.org/cgi/content/full/OC_sigtrans/2000/53/pe1
3. Finkel, T. (1999) Curr. Opin. Cell Biol. 10, 248–253
4. Thannickal, V. J., and Fanburg, B. L. (2000) Am. J. Physiol. 279, L1005–L1028
5. Schreck, R., Rieber, P., and Baeuerle, P. A. (1991) EMBO J. 10, 2247–2258
6. Pahl, H. L., and Baeuerle, P. A. (1994) Biosci. Rep. 14, 497–502
7. La, Y. Y., and Cruz, T. F. (1993) J. Biol. Chem. 270, 11727–11730
8. Barrett, W. C., DeGnore, J. P., Keng, Y. F., Zhang, Z. Y., Yin, M. B., and Chock, P. B. (1999) J. Biol. Chem. 274, 34543–34546
9. Lee, S. R., Kwon, R. S., Kim, S. R., and Rhee, S. G. (1998) J. Biol. Chem. 273, 15966–15972
10. Mahadev, K., Zilberberg, A., Zhu, L., and Goldstein, B. J. (2001) J. Biol. Chem. 276, 21938–21942
11. Meng, T. C., Fukada, T., and Tenks, N. K. (2002) Mol. Cell 9, 387–399
12. Blanchetot, C., Tertoolen, L. G., and den Hertog, J. (2002) EMBO J. 21, 493–503
13. Chen, H. Z., Chung, S. J., and Rhee, S. G. (1994) J. Biol. Chem. 269, 27670–27678
14. Rhee, S. G., Kang, S. W., Chang, T.-S., Jeong, W., and Kim, K. (2001) J. Exp. Biol. 204, 7–17
15. Kang, S. W., Baines, I. C., and Rhee, S. G. (1998) J. Biol. Chem. 273, 6303–6311
16. Seo, M. S., Kang, S. W., Kim, K. Baines, I. C., Lee, T. H., and Rhee, S. G. (2000) J. Biol. Chem. 275, 20346–20354
Regulation of Peroxiredoxin I Activity by Cdc2-mediated Phosphorylation
Tong-Shin Chang, Woojin Jeong, Soon Young Choi, Shiqin Yu, Sang Won Kang and Sue Goo Rhee

J. Biol. Chem. 2002, 277:25370-25376.
doi: 10.1074/jbc.M110432200 originally published online May 1, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M110432200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 54 references, 16 of which can be accessed free at
http://www.jbc.org/content/277/28/25370.full.html#ref-list-1