Reversible inactivation of the tumor suppressor PTEN by H$_2$O$_2$

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SUMMARY

The tumor suppressor PTEN regulates cell migration, growth, and survival by removing the 3'-phosphate of phosphoinositides. Exposure of purified PTEN or of cells to H$_2$O$_2$ resulted in inactivation of PTEN in a time- and H$_2$O$_2$ concentration–dependent manner. Analysis of various cysteine mutants, including mass spectrometry of tryptic peptides, indicated that the essential Cys$^{124}$ residue in the active site of PTEN specifically forms a disulfide with Cys$^{71}$ during oxidation by H$_2$O$_2$. The reduction of H$_2$O$_2$-oxidized PTEN in cells appears to be mediated predominantly by thioredoxin. Thus, thioredoxin was more efficient than was glutaredoxin, glutathione, or a 14-kDa thioredoxin-like protein with regard to the reduction of oxidized PTEN in vitro; thioredoxin co-immunoprecipitated with PTEN from cell lysates; and incubation of cells with 2,4-dinitro-1-chlorobenzene (an inhibitor of thioredoxin reductase) delayed the reduction of oxidized PTEN, whereas incubation with buthioninesulfoximine (an inhibitor of glutathione biosynthesis) did not. These results suggest that the reversible inactivation of PTEN by H$_2$O$_2$ might be important for the accumulation of 3'-phosphorylated phosphoinositides, and that the uncontrolled generation of H$_2$O$_2$ associated with certain pathological conditions might contribute to cell proliferation by inhibiting PTEN function.
INTRODUCTION

Hydrogen peroxide (H$_2$O$_2$) is produced by all mammalian cells as a by-product of normal metabolism, including the oxidative phosphorylation of ADP and the conversion of arachidonic acid to leukotrienes, as well as by phagocytic cells in the host defense response to noxious stimuli. In addition, ultraviolet and gamma irradiation of cells results in the production of H$_2$O$_2$. A substantial increase in the intracellular concentration of H$_2$O$_2$ is generally associated with deleterious effects, including cell death by apoptosis or necrosis, in pathophysiological conditions such as inflammation and ischemia-reperfusion.

The generation of H$_2$O$_2$ also appears to be required, however, for many normal cellular functions, including propagation of receptor signaling (1,2). Growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF); cytokines such as transforming growth factor–β1 and tumor necrosis factor–α; and agonists of heterotrimeric GTP-binding protein (G protein)–coupled receptors such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) and angiotensin II (1,2). The essential role of H$_2$O$_2$ production in intracellular signaling triggered by PDGF (3,4), EGF (5), angiotensin II (6), and cell-cell contact (7) has been demonstrated by the observation that corresponding receptor-mediated events are abrogated by blocking the accumulation of H$_2$O$_2$ with enzymes such as catalase or small molecules such as N-acetylcysteine.

The addition of exogenous H$_2$O$_2$ or the intracellular production of this metabolite in response to receptor stimulation affects the function of a variety of proteins including transcription factors, protein kinases and phosphatases, phospholipases, ion channels, and G proteins (1,2). However, the mechanisms by which H$_2$O$_2$ achieves these effects remain unknown.
It is unlikely that $H_2O_2$ specifically binds proteins and thereby affects their functions. On the other hand, $H_2O_2$ is a mild oxidant that is able to oxidize cysteine residues in proteins to cysteine sulfenic acid or to disulfide, both of which are readily reduced back to cysteine by various cellular reductants. Because the $pK_a$ (where $K_a$ is the acid constant) of the sulphydryl group (Cys–SH) of most cysteine residues is ~8.5 (8), and because this group is less readily oxidized by $H_2O_2$ than is the cysteine thiolate anion (Cys–S$^-$), few proteins might be expected to possess a Cys–SH that is vulnerable to oxidation by $H_2O_2$ in cells. However, certain protein cysteine residues do exist as thiolate anions at neutral pH as a result of the lowering of their $pK_a$ values by charge interactions between the negatively charged thiolate and nearby positively charged amino acid residues (9).

Proteins with low-$pK_a$ cysteine residues include protein tyrosine phosphatases (PTPs) (10–12). All PTPs contain an essential cysteine residue ($pK_a$, 4.7 to 5.4) in the signature active site motif, HCXXGXXRS/T (where X is any amino acid residue), that exists as a thiolate anion at neutral pH (11). This thiolate anion contributes to formation of a thiol-phosphate intermediate in the catalytic mechanism of PTPs. The active site cysteine is the target of specific oxidation by various oxidants, including $H_2O_2$, and this modification is reversed by incubation with thiol compounds such as dithiothreitol (DTT) and reduced glutathione (GSH). The ability of intracellularly produced $H_2O_2$ to inhibit PTP activity was demonstrated by the observation that stimulation of A431 cells with EGF resulted in a selective reduction in the extent of subsequent labeling of the active site cysteine residue of PTP1B by $[^3H]$iodoacetic acid in cell lysates (12).

PTEN is a member of the PTP family and reverses the action of phosphoinositide (PI) 3-kinase by catalyzing the removal of the phosphate attached to the 3'-hydroxyl group of the PI inositol ring (13–15). By negatively modulating the PI 3-kinase–Akt signaling pathway, PTEN
functions as an important tumor suppressor (15–20).

We now show that $\text{H}_2\text{O}_2$ induces reversible inactivation of PTEN through oxidation of the essential Cys$^{124}$ and the formation by this residue of a disulfide with Cys$^{71}$, and that the PTEN disulfide is reduced by thioredoxin (Trx). Given that stimulation of various receptors induces $\text{H}_2\text{O}_2$ production, we propose that the receptor-mediated activation of PI 3-kinase may not be sufficient for the accumulation of 3'-phosphorylated PIs; the concomitant inactivation of PTEN by $\text{H}_2\text{O}_2$ produced in response to receptor stimulation might also be necessary for this effect.

**EXPERIMENTAL PROCEDURES**

*Materials*—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, penicillin, and streptomycin were from Life Technologies. Both PDGF-BB and rabbit polyclonal antibodies to PTEN were obtained from Upstate Biotechnology, and monoclonal antibodies to PTEN and to the hemagglutinin epitope (HA) tag were from Santa Cruz Biotechnology. Horseradish peroxidase–conjugated antibodies to mouse or rabbit immunoglobulin G were from Amersham Pharmacia Biotech. Recombinant Trx and glutaredoxin (Grx) were prepared as described (12). Thioredoxin reductase (TrxR) was prepared as described (21). Glucose oxidase, GSH, DTT, and $N$-ethylmaleimide (NEM) were from Sigma. Bovine xanthine oxidase and catalase were from Boehringer Mannheim, and xanthine was from Calbiochem. A 30% solution of $\text{H}_2\text{O}_2$ was from Fisher.

*Expression, purification, and assay of the activity of recombinant PTEN*—Human PTEN cDNA cloned in the pQE30 vector (Qiagen) for expression of the protein with a histidine tag at the NH$_2$-terminus was kindly provided by K. M. Yamada (National Institutes of Health).
The cDNAs encoding the PTEN mutants C71S, C83S, C105S, C124S, and C136S were generated by polymerase chain reaction–mediated site-directed mutagenesis and verified by DNA sequencing. The histidine-tagged wild-type and mutant proteins were expressed in *Escherichia coli* according to standard procedures and purified with the use of an immobilized nickel resin (Qiagen). The purified proteins were dialyzed against 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 10% glycerol, and 10 mM 2-mercaptoethanol, and were then stored at −80°C. The phosphatase activity of PTEN was assayed with 32P-labeled phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3] as described (13).

*Cell culture and transfection*—HeLa and NIH 3T3 cells were grown at 37°C under an atmosphere of 5% CO2 in DMEM supplemented with 10% fetal bovine serum.

The cDNAs encoding wild-type PTEN or the C124S mutant tagged at their NH2-termini with HA were subcloned into the pCGN plasmid (22), and the resulting vectors were introduced into NIH 3T3 cells by transfection with the use of Effectene (Qiagen).

*Identification of reduced and oxidized forms of PTEN by immunoblot analysis*—After stimulation, cells (1 × 10⁶ cells in 1 ml of DMEM) were scraped into 0.2 ml of ice-cold 50% trichloroacetic acid and transferred to microfuge tubes. The cell suspensions were briefly sonicated and then centrifuged at 2000 × g for 5 min. The supernatants were removed, and the pellets were washed with acetone and then solubilized in 0.2 ml of 100 mM Tris-HCl (pH 6.8) buffer containing 2% SDS and 40 mM NEM. Portions (5 μl) of the solubilized pellets were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) under nonreducing conditions, and the separated proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was then subjected to immunoblot analysis either with rabbit antibodies to PTEN or
with a monoclonal antibody to HA. Immune complexes were detected with horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech or Pierce). The intensity of PTEN bands was quantitated with an Image Station 440 instrument (Kodak Digital Science).

**HPLC and mass spectrometric analysis of PTEN peptides**—Oxidized PTEN (100 µg) was obtained by incubating the purified protein for 5 min at room temperature in a final volume of 20 µl containing 50 mM Hepes-NaOH (pH 7.0), 150 mM NaCl, and 1 mM H$_2$O$_2$; the reaction was stopped by the addition of 1 µg of catalase. The protein was then denatured with 6 M guanidine-HCl in 100 mM Tris-HCl (pH 9.0), and the free cysteine residues were alkylated by incubation for 2 h with 10 mM iodoacetamide in an anaerobic chamber. Alkylated PTEN was digested overnight at 37°C with Lys-C (0.5 µg/ml) in 100 mM Tris-HCl (pH 8.5) containing 10% acetonitrile. The digestion products were then fractionated by high-performance liquid chromatography (HPLC) on a C$_{18}$ column with a linear gradient (0 to 60%) of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. A peak that eluted at 30.5 min was exposed or not to 10 mM DTT for 10 min and then analyzed by MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) mass spectrometry.

**RESULTS**

In vitro inactivation of PTEN by H$_2$O$_2$ is accompanied by the formation of a disulfide between the active site Cys$^{124}$ and Cys$^{71}$—Purified human PTEN was inactivated by H$_2$O$_2$ in a concentration-dependent manner (Fig. 1A). PTEN was also inactivated on incubation with xanthine oxidase and xanthine, the combination of which results in the generation of superoxide
anions that are subsequently converted to H₂O₂ (Fig. 1B). The inactivation of PTEN by the
xanthine oxidase–xanthine system was inhibited by up to ~80% by catalase (Fig. 1B), suggesting
that PTEN oxidation was mediated predominantly by H₂O₂ rather than by superoxide anions.

PTEN that had been treated with H₂O₂ migrated faster on SDS-PAGE under nonreducing
conditions than did the untreated protein (Fig. 2A). This observation suggested that H₂O₂ induces
the oxidation of sulfhydryl groups of PTEN to a disulfide, a reaction that generally results in a
more compact protein structure. The PTEN protein comprises 403 amino acids, with the catalytic
domain being located in the NH₂-terminal region and a C2 domain present in the COOH-terminal
region (23). The catalytic domain of human PTEN contains cysteines at positions 71, 83, 105,
124, and 136 (15). To identify the cysteine residues that form a disulfide, we changed each of
these five cysteines in the catalytic domain individually to serine, thereby generating the mutants
C71S, C83S, C105S, C124S, and C136S. The mutant proteins were incubated with H₂O₂ and then
analyzed by gel electrophoresis. Like the wild-type protein, the C83S, C105S, and C136S
mutants migrated faster after exposure to H₂O₂, whereas the mobility of the C71S and C124S
mutants was not affected by H₂O₂ (Fig. 2A). These results thus suggested that, on oxidation with
H₂O₂, the Cys¹²⁴ residue in the active site of PTEN forms a disulfide bond with Cys⁷¹.

To demonstrate directly the formation of a disulfide bond between Cys⁷¹ and Cys¹²⁴ of
PTEN, we subjected the H₂O₂-treated protein first to alkylation under denaturing conditions with
iodoacetamide (in order to block free cysteines) and then to digestion with the endoproteinase
Lys-C. The resulting peptides were fractionated by reversed-phase HPLC. A peak that eluted at
30.5 min (data not shown) revealed several components on analysis by MALDI-TOF mass
spectrometry, including one of 4432.86 mass units (Fig. 2B); this size is highly similar to the
value of 4435.88 mass units calculated for a 37-residue fragment of PTEN in which a Cys\textsuperscript{71}-containing peptide and a Cys\textsuperscript{124}-containing peptide are bridged by a disulfide bond. When the HPLC peak fraction that eluted at 30.5 min was treated with DTT before analysis by mass spectrometry, no mass peak corresponding to the 37-residue fragment was detected; instead, fragments of 1696.37 and 2741.02 mass units, similar to the values of 1695.90 and 2741.98 mass units calculated for the 14-residue Cys\textsuperscript{71}-containing peptide and the 23-residue Cys\textsuperscript{124}-containing peptide, respectively, were apparent (Fig. 2B). These results thus demonstrate that Cys\textsuperscript{71} and Cys\textsuperscript{124} of PTEN form a disulfide on exposure to H\textsubscript{2}O\textsubscript{2}. This conclusion is consistent with the crystal structure of PTEN, which reveals that, among the four other cysteine residues in the catalytic domain, only Cys\textsuperscript{71} is sufficiently close (distance of 5.9 Å) to Cys\textsuperscript{124} to form a disulfide with this residue (23).

Oxidation of PTEN in cells exposed to H\textsubscript{2}O\textsubscript{2}—We examined whether PTEN is oxidized in cells exposed to H\textsubscript{2}O\textsubscript{2}. NIH 3T3 cells were incubated for 5 min with various concentrations of H\textsubscript{2}O\textsubscript{2}, after which cell extracts were exposed to NEM to block free sulfhydryls and then subjected to immunoblot analysis with antibodies to PTEN. Exposure of cells to H\textsubscript{2}O\textsubscript{2} at concentrations as low as 50 µM resulted in the appearance of the higher-mobility (oxidized) form of PTEN, and the intensity of this band increased as the concentration of H\textsubscript{2}O\textsubscript{2} increased (Fig. 3A). The amount of the oxidized form of PTEN also increased with time of incubation of cells with 0.5 mM H\textsubscript{2}O\textsubscript{2}, reaching a maximum at 10 to 40 min and decreasing gradually thereafter (Fig. 3B). The amount of the reduced form of the protein decreased as the amount of the oxidized form increased and vice versa, suggesting that PTEN was inactivated by H\textsubscript{2}O\textsubscript{2} and then reactivated by cellular reductants as the concentration of H\textsubscript{2}O\textsubscript{2} declined. When extracts derived
from NIH 3T3 cells treated with H\textsubscript{2}O\textsubscript{2} were incubated with DTT before gel electrophoresis, only the lower-mobility PTEN band was detected (Fig. 3A, B), consistent with the notion that the higher-mobility form of the protein contains a disulfide. To demonstrate that Cys\textsuperscript{124} contributes to the disulfide responsible for the increase in PTEN mobility, we transfected NIH 3T3 cells separately with vectors encoding either the C124S mutant of human PTEN tagged with HA or the HA-tagged wild-type protein. Exposure of the transfected cells to H\textsubscript{2}O\textsubscript{2} revealed that the HA-tagged wild-type protein, but not the HA-tagged C124S mutant, underwent reversible oxidation (Fig. 3C), confirming that Cys\textsuperscript{124} forms the disulfide bond responsible for the mobility shift of PTEN.

The reversible inactivation of PTEN was also apparent in HeLa cells exposed to 1.5 mM H\textsubscript{2}O\textsubscript{2} (Fig. 4A). When HeLa cells were incubated in a medium in which H\textsubscript{2}O\textsubscript{2} was produced continuously as a result of the presence of glucose oxidase (which converts molecular oxygen to H\textsubscript{2}O\textsubscript{2} with electrons derived from glucose also present in the medium), the oxidized form of PTEN persisted (Fig. 4B).

*Reduction of oxidized PTEN*—Two prominent physiological electron donors for protein reduction are GSH and Trx (24). GSH reduces proteins either directly or indirectly through the regeneration of Grx after its reaction with oxidized proteins. Oxidized Trx is reduced by TrxR with the use of electrons supplied by NADPH. Purified PTEN that had been inactivated by exposure to glucose and glucose oxidase was incubated with electron donors and the extent of its reduction was monitored on the basis of the mobility shift in nonreducing gels (Fig. 5A). The most rapid reduction was achieved with the nonphysiological electron donor DTT (5 mM). The Trx system, comprising 5 µM Trx and saturating concentrations of TrxR and NADPH, was
almost as efficient as DTT; the Trx system devoid of Trx was completely inactive (data not shown). GSH (5 mM) was markedly less effective than was the Trx system, and the presence of 5 µM Grx in addition to 5 mM GSH (the Grx system) did not substantially increase the efficiency of the latter (Fig. 5A). Similar results were obtained when PTEN reduction was monitored on the basis of enzyme activity (data not shown).

We also measured the rate of PTEN reactivation in the presence of various concentrations of Trx (Fig. 5B). Half-maximal reactivation was apparent at 1 µM Trx. Furthermore, immunoblot analysis revealed that Trx was co-immunoprecipitated with PTEN from NIH 3T3 cell extracts (Fig. 6). Estimation of the relative amounts of PTEN and Trx in the immunoprecipitates by comparison of the band intensities with those of protein standards yielded a molar ratio of PTEN to Trx of 1: 0.07.

Mammalian cells contain a 14 kDa protein, which is 20% identical to Trx in amino acid sequence. This Trx-related protein, named TRP14, contains a WCXXC motif, which is conserved among the members of the thiol-disulfide oxidoreductase superfamily that includes Trx, Grx, and protein disulfide isomerases. The reduction potentials of TRP14 (-0.257 V) and Trx (-0.274 V) are similar, and oxidized TRP14 and Trx were reduced with equal efficiency by TrxR. As such, we tested whether TRP14 can reactivate oxidized PTEN in the presence of TrxR and NADPH. TRP14 was nearly as ineffective as GSH, further suggesting the specificity of electron donor molecules (Fig. 7).

To evaluate the relative importance of GSH and Trx with regard to reactivation of PTEN in cells, we incubated HeLa cells either with 2,4-dinitro-1-chlorobenzene (DNCB), which prevents recycling of Trx by inhibiting TrxR activity (25), or with buthioninesulfoximine (BSO),
which inhibits GSH biosynthesis (26), prior to exposure to H$_2$O$_2$. Treatment of cells with 100 µM DNCB for 15 min resulted in the accumulation of >80% of Trx in its oxidized form, and treatment with 300 µM BSO for 24 h resulted in the depletion of GSH by >90% (data not shown). Whereas BSO did not substantially affect the reduction of H$_2$O$_2$-oxidized PTEN, DNCB induced a marked delay in PTEN reduction (Fig. 8), suggesting that the reduction of PTEN in cells is mediated predominantly by Trx. DNCB is known to be toxic and induce cell death upon incubation for several hours (27). Incubation of HeLa cells with 100 µM DNCB for 15 min, however, did not cause any detectable cell death measured by the Trypan blue exclusion assay, and detectable levels of cell death were apparent after incubation for 90 min (not shown).

**DISCUSSION**

A variety of cellular stimuli induce the activation of PI 3-kinase, resulting in the conversion of PI(4,5)P$_2$ to PI(3,4,5)P$_3$ (28,29). The latter lipid is dephosphorylated back to PI(4,5)P$_2$ by PTEN (13) or to PI(3,4)P$_2$ by 5-phosphatases such as SHIP (30). There are at least nine distinct mammalian 5-phosphatases (31), the catalytic cores of all of which contain two signature active site motifs, (F/I)WXGDXN(F/Y)R and (R/N)XP(S/A)(W/Y)(C/T)DR; these motifs, unlike the active site motif of the 3-phosphatase PTEN, do not possess a conserved cysteine residue, however. The 3'-phosphorylated PIs bind and modulate numerous proteins that regulate a variety of cellular functions (15,29). One of the best characterized targets of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ is the protein kinase Akt. Both PI(3,4,5)P$_3$ and PI(3,4)P$_2$ activate Akt (20), although their relative contribution to this process *in vivo* remains unclear (32). Activated Akt promotes cell survival by phosphorylating the pro-apoptotic factors BAD and caspase-9 and thereby inhibiting their
functions (33,34). Unchecked activation of Akt may thus lead to tumor formation (17,35). PTEN opposes this proliferation signaling pathway by reducing the cellular abundance of PI(3,4,5)P$_3$ and PI(3,4)P$_2$ and blocking Akt activation (17,35).

Exogenous H$_2$O$_2$ induces the activation of Akt (6,36,37). However, the 3'-phosphorylated PI that accumulates in response to H$_2$O$_2$ is almost exclusively PI(3,4)P$_2$ rather than PI(3,4,5)P$_3$ (24,38). Given that H$_2$O$_2$ activates receptor-type protein tyrosine kinases such as the PDGF receptor and the EGF receptor (37,39,40), it also likely activates PI 3-kinase, resulting in the production of PI(3,4,5)P$_3$. In support of this notion, H$_2$O$_2$-dependent activation of Akt is blocked by the PI 3-kinase inhibitor wortmannin (6,37,41). We have now shown that PTEN is inactivated by H$_2$O$_2$. This observation, together with the fact that PTEN, but not 5-phosphatases, contains an oxidizable cysteine residue at its active site, suggests that, in cells exposed to H$_2$O$_2$, PI(3,4,5)P$_3$ synthesized by PI 3-kinase activity is rapidly dephosphorylated by 5-phosphatases that are insensitive to H$_2$O$_2$, whereas dephosphorylation at the 3' position is markedly inhibited as a result of inactivation of PTEN by H$_2$O$_2$.

The H$_2$O$_2$-dependent inhibition of the lipid phosphatase activity of the tumor suppressor PTEN suggests that H$_2$O$_2$ produced under pathological conditions, such as during chronic inflammation, might contribute to inhibition of apoptosis, to hyperplasia, and to tumor formation. Indeed, many normal cells exposed to H$_2$O$_2$ exhibit increased proliferation and express growth-related genes (42–44). Furthermore, given that tumor cells produce large amounts of H$_2$O$_2$ (45,46), this metabolite might also promote tumor cell proliferation. Previously, the tumor-promoting activity of reactive oxygen species has been explained mainly in terms of the mutagenic activity of hydroxyl radicals derived from them.
A variety of cell surface receptor ligands induce the production of H$_2$O$_2$, which is necessary for the receptor-mediated cellular events such as chemotaxis, DNA synthesis, and cell cycle progress (3, 47). Like exogenous H$_2$O$_2$, the receptor-produced H$_2$O$_2$ also likely causes PTEN inactivation and contributes to the accumulation of 3'-phosphorylated PIs. Thus, the receptor-mediated activation of PI 3-kinase may not be sufficient for the accumulation of 3'-phosphorylated PIs and the concomitant inactivation of PTEN by receptor-produced H$_2$O$_2$ might also be necessary for this effect.

Despite numerous studies demonstrating the importance of PTEN in cell growth, survival, and migration, the mechanisms by which the activity of this protein is regulated have remained unclear (15). PTEN contains tandem phosphatase and C2 domains, which share an extensive interface (23). The phosphatase domain exhibits substantial structural homology to protein tyrosine phosphatases, but it contains an enlarged active site with several basic residues that are important for the accommodation of the bulky inositol trisphosphate moiety. PTEN also contains a putative PDZ domain–binding motif at its COOH-terminus. Although this notion remains controversial (48-50), the C2 domain and the PDZ domain–binding motif have been proposed to mediate the translocation of PTEN from the cytosol to sites near to PI(3,4,5)P$_3$ (23,51-53).

Phosphorylation of PTEN by casein kinase II has also been shown to increase the susceptibility of the protein to proteasome-mediated degradation (54). How these modes of regulation might be affected by the activation of cell surface receptors, however, is not known. Although in vitro measurements of its lipid phosphatase activity suggest that PTEN might be constitutively active, no mechanism for its negative regulation has previously been revealed (15). Constitutive activity of PTEN is likely required to prevent accumulation of PI(3,4,5)P$_3$ and unwanted triggering of
proliferation signals in quiescent cells. The oxidative inactivation of PTEN in response to H$_2$O$_2$ generation might therefore be an important determinant of the timing and localization of the production of this important lipid messenger elicited by receptor stimulation.

Oxidized PTEN is gradually converted back to the reduced form by intracellular reducing agents, indicating that the reversible oxidation and reduction of the active site cysteine residue is an important mechanism for the regulation of PTEN. The best candidate for the intracellular reducing agent is Trx, given that oxidized PTEN was reduced much more effectively by Trx than by Grx or GSH in vitro. Furthermore, depletion of reduced Trx, but not of GSH, markedly reduced the rate of reactivation of oxidized PTEN in cells. The association of Trx with PTEN revealed by the co-immunoprecipitation of these proteins suggests that the reduction reaction might be governed by a specific interaction between oxidized PTEN and reduced thioredoxin. Such a specific interaction is also supported by the observation that a 14-kDa Trx-related protein (TRP14) was not able to reduce oxidized PTEN despite the fact that it contains a WCXXC motif, which is conserved among members of the thiol-disulfide oxidoreductase superfamily, and that TRP14 and Trx exhibit similar redox potentials and reactivity toward TrxR.

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REFERENCES


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FOOTNOTES

1 Abbreviations used are: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; fMLP, N-formyl-methionyl-leucyl-phenylalanine; PTP, protein tyrosine phosphatase; DTT, dithiothreitol; PI, phosphoinositide; Trx, thioredoxin; DMEM, Dulbecco’s modified Eagle’s medium; HA, hemagglutinin epitope; Grx, glutaredoxin; TrxR, thioredoxin reductase; NEM, N-ethylmaleimide; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; DNBC, 2,4-dinitro-1-chlorobenzene; BSO, buthioninesulfoximine.

2 Jeong, W, and Rhee, S. G., unpublished data.
FIGURE LEGENDS

Fig. 1. Oxidative inactivation of PTEN by H$_2$O$_2$. Purified recombinant human PTEN (200 µg/ml) was incubated either for 10 min at 25°C in the presence of 20 mM Bis-Tris–HCl (pH 6.7) and the indicated concentrations of H$_2$O$_2$ (A) or for the indicated times at 25°C with 2 mM xanthine (X) and 150 µM xanthine oxidase (XO) in 50 mM Hepes-NaOH (pH 7.2) either in the absence or presence of catalase (CAT) (B). The PI(3,4,5)P$_3$-hydrolyzing activity of PTEN was then determined and is expressed as a percentage of that apparent for the untreated protein. The data represent the average of two separate experiments.

Fig. 2. Formation of a disulfide bond between Cys$^{71}$ and Cys$^{124}$ in oxidized PTEN. (A) Effect of mutation of various cysteine residues on the H$_2$O$_2$-induced mobility shift of PTEN. Wild-type (WT) PTEN and the indicated mutants were incubated with 1 mM H$_2$O$_2$ as in Fig. 1A, after which the proteins were subjected to nonreducing SDS-PAGE on a 10% gel followed by immunoblot analysis with antibodies to PTEN. The positions of the reduced and oxidized proteins are indicated. Data are representatives of three independent experiments. (B) Mass spectrometric detection of a fragment of oxidized PTEN containing a disulfide bond between Cys$^{71}$ and Cys$^{124}$. Purified PTEN was oxidized with H$_2$O$_2$, denatured, alkylated with iodoacetamide, digested with Lys-C, and subjected to HPLC. Components of a peak that eluted at 30.5 min were exposed (bottom panel) or not (upper panel) to DTT and were then analyzed by mass spectrometry (m/z, mass/charge ratio). Peaks corresponding either to peptides containing Cys$^{71}$ or Cys$^{124}$ or to both peptides linked by a disulfide bond are indicated.

Fig. 3. Oxidation of PTEN in NIH 3T3 cells exposed to H$_2$O$_2$. (A and B) Oxidation of
endogenous PTEN in NIH 3T3 cells exposed to H$_2$O$_2$. Cells were incubated either for 5 min with the indicated concentrations of H$_2$O$_2$ (A) or for the indicated times with 0.5 mM H$_2$O$_2$ (B). Cellular protein extracts were then alkylated with NEM and subjected to nonreducing SDS-PAGE followed by immunoblot analysis with antibodies to PTEN. One protein sample (rightmost lane) from each experiment was treated with 100 mM DTT for 5 min before electrophoresis. (C) Oxidation of wild-type PTEN, but not of the C124S mutant, in NIH 3T3 cells exposed to H$_2$O$_2$. Transfected cells expressing either HA-tagged wild-type PTEN or the HA-tagged C124S mutant were incubated with 0.5 mM H$_2$O$_2$ for the indicated times, after which cellular protein extracts were exposed to NEM and subjected to nonreducing SDS-PAGE and immunoblot analysis with a monoclonal antibody to HA. All blot data are representatives of at least three separate experiments.

**Fig. 4.** Oxidation of PTEN in HeLa cells exposed to H$_2$O$_2$. HeLa cells were incubated for the indicated times either with 1.5 mM H$_2$O$_2$ (A) or with an H$_2$O$_2$-generating system comprising glucose (4.5 mg/ml) and glucose oxidase (1 U/ml) (B). Cellular protein extracts were then analyzed as in Fig. 3A. All blot data are representatives of at least three separate experiments.

**Fig. 5.** Reduction of oxidized PTEN. (A) PTEN (250 µg/ml) that had been oxidized by exposure to the glucose–glucose oxidase system was incubated for the indicated times at 25°C in 50 mM Hepes-NaOH (pH 7.2) buffer in the absence (None) or presence of either the Trx system (5 µM Trx, 0.1 µM TrxR, 200 µM NADPH), the Grx system (5 µM Grx, 5 mM GSH), 5 mM GSH, or 5
mM DTT. The reaction mixture was then subjected to immunoblot analysis with antibodies to PTEN, and the intensity of the band corresponding to reduced PTEN was determined as a percentage of the sum of the intensities of the bands corresponding to the oxidized and reduced proteins. (B) Oxidized PTEN was incubated for 5 min as in (A) either with 5 mM DTT or with the Trx system containing 0.1 µM TrxR, 200 µM NADPH, and the indicated concentrations of Trx. The reaction mixture was then assayed for PI(3,4,5)P₃ phosphatase activity, and data are expressed as a percentage of the activity apparent with the DTT-treated protein. Results in (A) and (B) are means of triplicates from representative experiments.

Fig. 6. Co-immunoprecipitation of Trx with PTEN. Immunoprecipitates (IP) prepared from NIH 3T3 cell lysates with a monoclonal antibody to PTEN (αPTEN) were subjected to immunoblot analysis (IB) with rabbit antibodies either to Trx or to PTEN. The indicated amounts of purified Trx and PTEN were also analyzed for the purpose of quantitation. The data are representatives of three separate experiments.

Fig. 7. Reduction of oxidized PTEN by Trx, GSH, and TRP14. PTEN that had been oxidized as described in Fig. 5 was incubated for 10 min at 25°C in 50 mM Hepes-NaOH (pH 7.2) buffer in the absence (None) or presence of either 5mM DTT, the Trx system (5 µM Trx, 0.1 µM TrxR, 200 µM NADPH), 5 mM GSH, or the TRP14 system (5 µM TRP14, 0.1 µM TrxR, 200 µM NADPH). The reaction mixture was then assayed for PI(3,4,5)P₃ phosphatase activity, and data are expressed as a percentage of the activity apparent with the DTT-treated protein. Results are means ± S.E. of three independent experiments.
**Fig. 8.** Effects of DNCB and BSO on the reduction of oxidized PTEN.  (A) HeLa cells were incubated first for 15 min with 100 μM DNBC (right lanes) or for 24 h with 300 μM BSO (middle lanes) and then for the indicated times with 1.5 mM H₂O₂. As a control, cells were incubated with H₂O₂ without pretreatment (left lanes). Cell extracts were then subjected to nonreducing SDS-PAGE followed by immunoblot analysis with antibodies to PTEN (upper panels) and to reducing SDS-PAGE followed by immunoblot analysis with antibodies to PTEN (middle panels) and with antibodies to GAPDH (bottom panels). (B) The intensity of the bands corresponding to reduced PTEN in the upper panels of (A) was determined and presented as a percentage of the sum of the intensities of the bands corresponding to the oxidized and reduced proteins. Data are the average of two separate experiments.
A  

\[ \text{H}_2\text{O}_2 \text{ (mM)}: \quad 0 \quad 0.05 \quad 0.1 \quad 0.5 \quad 1 \quad 2 \quad 1 \]

\[ \text{DTT :} \quad - \quad - \quad + \quad + \quad + \]

B  

\[ \text{Time (min)}: \quad 0 \quad 2 \quad 5 \quad 10 \quad 20 \quad 40 \quad 60 \quad 80 \quad 10 \]

\[ \text{DTT :} \quad - \quad - \quad + \quad + \quad + \]

C  

\[ \text{Time (min)}: \quad 0 \quad 5 \quad 10 \quad 40 \quad 80 \]

\[ \text{WT} \quad \text{C124S} \]

\[ \text{Reduced} \quad \text{Oxidized} \]

Lee et al. Fig. 3
A  Time (min) :  0  5  10  30  60

[Image of gel with labeled bands]

→ reduced
→ oxidized

B  Time (min) :  0  2  5  10  20  40

[Image of gel with labeled bands]

→ reduced
→ oxidized

Lee et al. Fig. 4
Lee et al. Fig. 5
Lee et al. Fig. 6

IB: αPTEN

IP: αPTEN

PTEN (ng)
5 10 20

IB: αTrx

IP: αPTEN

Trx (ng)
7 14 28
PTEN activity (% of control)

Control  GSH  Trx  Trp14

Lee et al. Fig. 7
A

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αPTEN

αPTEN

αGAPDH

B

Reduced PTEN (%)

Time (min)

H₂O₂

BSO, H₂O₂

DNFB, H₂O₂

Lee et al. Fig. 8
Reversible inactivation of the tumor suppressor PTEN by HO
Seung-Rock Lee, Kap-Seok Yang, Jayul Kwon, Chunghee Lee, Woojin Jeong and Sue Goo Rhee

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