The c-Abl and Arg tyrosine kinases are activated in the cellular response to oxidative stress. The present studies demonstrate that c-Abl and Arg associate with glutathione peroxidase 1 (GPx1) and that this interaction is regulated by intracellular oxidant levels. The c-Abl and Arg SH3 domains bind directly to a proline-rich site in GPx1 at amino acids 132–145. GPx1 also functions as a substrate for c-Abl- and Arg-mediated phosphorylation on Tyr-96. The results further show that c-Abl and Arg stimulate GPx activity and that these kinases contribute to GPx-mediated protection of cells against oxidative stress. Our findings provide the first evidence that GPx1 is regulated by a signaling pathway that is activated in the oxidative stress response.

The mammalian c-Abl and Arg nonreceptor tyrosine kinases are widely expressed in adult tissues. The N-terminal regions of these kinases are ~90% identical and, as found in members of the Src family, contain tandem SH3, SH2, and tyrosine kinase domains (1, 2). Recent studies have shown that the cytoplasmic forms of c-Abl and Arg are activated in the cellular response to oxidative stress (3–5). Activation of c-Abl is associated with targeting of c-Abl to mitochondria, loss of mitochondrial transmembrane potential, and release of cytochrome c (4, 6). The finding that H2O2-induced apoptosis is attenuated in cells deficient in c-Abl or expressing a kinase-inactive c-Abl(K-R) mutant has supported a role for the c-Abl kinase in the apoptotic response to oxidative stress (4, 6). H2O2-induced apoptosis is also attenuated in cells deficient in Arg or expressing a kinase-inactive Arg(K-R) mutant (5). These findings and the demonstration that H2O2 induces c-Abl-Arg heterodimers indicate that both c-Abl and Arg are necessary in the apoptotic response to oxidative stress (7).

In mammalian cells, superoxide is metabolized to H2O2 by the 80-kDa tetrameric Mn-superoxide dismutase in mitochondria and the 32-kDa dimeric Cu/Zn-superoxide dismutase in the cytosol. Mice deficient in Mn-superoxide dismutase die as neonates and exhibit neurodegeneration (8). The finding that mice deficient in the cytosolic Cu/Zn-superoxide dismutase exhibit a normal phenotype has indicated that this isoform is less important in superoxide metabolism (9). Glutathione peroxidase (GPx) converts H2O2 to H2O in a reaction that oxidizes glutathione (GSH) to its disulfide form (GSSG). There are at least five glutathione peroxidases (GPx1–5) in mammalian cells: the cytosolic/mitochondrial selenoprotein GPx1, the gastrointestinal GPx2, the plasma GPx3, the phospholipid hydroperoxide GPx4, and the epidermal secretory GPx5 (10). The more abundant GPx1 is expressed in most cells (11). Mice deficient in GPx1 develop normally but exhibit sensitivity to H2O2 (11, 12). H2O2 is also converted to H2O in peroxisomes by the tetrameric catalase (13). Little is known about how superoxide dismutase, GPx, and catalase are regulated in the cellular response to oxidative stress. Recent studies, however, have demonstrated that catalase activity is stimulated by c-Abl- and Arg-mediated phosphorylation (14).

The present studies demonstrate that c-Abl and Arg associate with GPx1 and that GPx1 functions as a substrate for these kinases. The results also show that c-Abl and Arg regulate GPx1 activity and thereby the sensitivity of cells to oxidative stress.

MATERIALS AND METHODS

Cell Culture—293 cells, SH-SY5Y cells, and cell lines from mouse embryonic fibroblasts (wild-type and c-abl(−/−) c-abl(+/−)) (15) were grown in high glucose Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. selenium content under these growth conditions is ~20 μM (16). Cells were treated with H2O2 (Sigma), STI571 (Novartis, Basel, Switzerland), or 3-amino-1,2,4-triazole (3AT, Sigma). Transfections were performed in the presence of Lipofect-AMINE (Invitrogen).

Vectors—c-Abl, c-Abl(K-R), Arg, and Arg(K-R) were expressed in pSRa vectors. To achieve expression of the human GPx1 selenoprotein in bacteria, the selenocysteine residue (Sec-47) was mutated to cysteine (Sec-47 → Cys) (17). The GPx1(Sec-47 → Cys) mutant was also used for overexpression of GPx1 in mammalian cells. FLAG-tagged c-Abl, Arg, and GPx1(Sec-47 → Cys) mutants were expressed by cloning into the pcDNA3.1-based FLAG vector. His/Exp-tagged constructs were prepared by cloning into pcdNA4HisMAX, which contains N-terminal His and Express tags (Invitrogen). Myc-tagged c-Abl was prepared by cloning into the pCMV-Myc vector (Clontech). Glutathione S-transferase (GST) fusion proteins were generated by expression of pGEX4T2-based vectors in Escherichia coli BL21(DE3).

Immunoprecipitation and Immunoblot Analysis—Cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 10 mM sodium fluoride, and 10 μM aprotinin, leupeptin, and pepstatin A) containing 1% Nonidet P-40. Soluble protein was subjected to immunoprecipitation with anti-Myc (Santa Cruz Biochemicals, Santa Cruz, CA), anti-FLAG M2, Sigma), anti-GPx (MBL Ltd., Japan), or anti-c-Abl (K-12, Santa Cruz Biochemicals). Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-FLAG (Sigma), anti-Myc (Santa Cruz Biochemicals), horseradish peroxidase-conjugated anti-Exp (Invitrogen), anti-c-Abl (2411, Santa Cruz Biochemicals), sheep anti-GPx (Novus Biologicals), or horse-radish peroxidase-conjugated anti-TyrP (4G10, Upstate Biotechnology). The antigen-antibody complexes were visualized by chemiluminescence (ECL, Amersham Biosciences).

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Binding Assays—Cell lysates were incubated with 2 μg of GST or GST fusion proteins bound to glutathione beads. After treatment for 2 h at 4 °C, the adsorbates were washed with lysis buffer and then subjected to immunoblot analysis. Purified GST or GST fusion proteins were also incubated with in vitro translated 35S-labeled FLAG-c-Abl or GPx1. The adsorbates were analyzed by SDS-PAGE and autoradiography.

Kinase Assays—Purified GPx1(Sec-47 → Cys) or GPx1(Sec-47 → Cys/Tyr-96 → Phe) expressed in E. coli (2 μg) was incubated with immunoprecipitated FLAG-c-Abl or FLAG-Arg in kinase buffer (20 mM HEPES, pH 7.5, 75 mM KCl, 10 mM MgCl2, 10 mM MnCl2) containing 2.5 μCi of [γ-32P]ATP for 30 min at 30 °C. The reaction products were analyzed by SDS-PAGE and autoradiography.

Measurement of GPx Activity—Cell lysates were assayed with the GPx cellular assay kit (Sigma) at 25 °C and pH 8.0 according to the manufacturer’s instructions.

Apoptosis Assays—DNA content was assessed by staining ethanol-fixed and citrate buffer-permeabilized cells with propidium iodide and monitoring by FACScan (BD Biosciences). The numbers of cells with sub-G1 DNA content were determined with a MODFIT LT program (5).

RESULTS

c-Abl and Arg Associate with Glutathione Peroxidase—Studies using the yeast two-hybrid system demonstrated that c-Abl associates with a 3’-terminal fragment of GPx1 containing amino acids 70–201 (data not shown). In this Matchmaker Gal4 cDNA library (Clontech), the full-length cDNA or a 3’-terminal fragment is fused to the activation domain of the Gal4 gene (Gal4AD). To determine whether c-Abl interacts with GPx1 in cells, lysates from 293 cells expressing Myc-c-Abl and FLAG-GPx1(Sec-47 → Cys) were subjected to immunoprecipitation with anti-Myc. Immunoblot analysis of the precipitates with anti-FLAG demonstrated that c-Abl associates with GPx1 (Fig. 1A, lane 1). By contrast, GPx1 was undetectable in immunoprecipitates prepared with control IgG (Fig. 1A, lane 2). In the reciprocal experiment, anti-FLAG immunoprecipitates from cells expressing Exp-c-Abl and FLAG-GPx1(Sec-47 → Cys) were analyzed by immunoblotting with anti-Exp. The results confirmed an association between c-Abl and GPx1 (Fig. 1B, lane 1). Whereas c-Abl and GPx1 are effectors in the response to oxidative stress, similar experiments were performed after treatment with increasing concentrations of H2O2. Exposure to 0.16 mM H2O2 was associated with a decrease in c-Abl-Arg complexes (Fig. 1B, lanes 3 and 4). As a control, there was no detectable c-Abl in the IgG immunoprecipitates (Fig. 1B, lanes 5–7). To determine whether Arg, like c-Abl, associates with GPx1, anti-FLAG immunoprecipitates from cells expressing Exp-Arg and FLAG-GPx1(Sec-47 → Cys) were analyzed by immunoblotting with anti-Exp. As found for c-Abl, Arg associates with GPx1 (Fig. 1C, lane 1). Treatment with increasing concentrations of H2O2 also resulted in decreases in Arg-GPx1 complexes (Fig. 1C, lanes 2–4). In addition, there was no detectable Arg in the control IgG immunoprecipitates (Fig. 1C, lanes 5–7). These findings demonstrate that c-Abl and Arg associate with GPx1 in cells.

c-Abl and Arg SH3 Domains Confer Binding to GPx1—To further define the interaction between c-Abl and GPx1, lysates from 293 cells were incubated with GST or a GST-GPx1(Sec-47 → Cys) fusion protein. Immunoblot analysis of the adsorbates with anti-c-Abl demonstrated binding of c-Abl to GST-GPx1(Sec-47 → Cys) and not GST (Fig. 2A). To assess whether the interaction is direct, GST and GST-GPx1(Sec-47 → Cys) were incubated with 35S-labeled FLAG-c-Abl. Analysis of the adsorbates by SDS-PAGE and autoradiography showed binding of c-Abl to GST-GPx1(Sec-47 → Cys) and not to GST (Fig. 2B). c-Abl and Arg bind to other proteins through their SH3 and SH2 domains. To determine whether these domains are involved in the interaction between c-Abl/Arg and GPx1, 35S-labeled GPx1(Sec-47 → Cys) was incubated with GST fusion proteins containing the c-Abl/Arg SH3 or SH2 domains. Analysis of the adsorbates demonstrated that GPx1 binds to the c-Abl SH3 but not to the c-Abl SH2 domain (Fig. 2C). Binding of GPx1 was also detectable with the Arg SH3 but not the Arg SH2 domain (Fig. 2C). SH3 domains bind to proline-rich sites with a consensus PXXPF motif. Whereas several prolines but not a consensus motif are present in GPx1 at amino acids 132–145 (PAPSDDATALMTDP), we generated GPx1(Sec-47 → Cys/Pro-132 → Ala) and GPx1(Sec-47 → Cys/Pro-145 → Ala) mutants. Another Pro → Ala mutation at position 99 was prepared as a control. Immunoblot analysis of lysates from cells expressing the GPx1 mutants demonstrated equal levels of protein (Fig. 2D, lanes 6–9). Incubation of the lysates with GST-c-Abl SH3 demonstrated binding of GPx1(Sec-47 → Cys) and the
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GPx1 Is Phosphorylated on Tyr-96 by c-Abl and Arg—To determine if GPx1 is a potential substrate for c-Abl in vivo, anti-GPx1 immunoprecipitates from SH-SY5Y cells were first subjected to immunoblotting with anti-c-Abl and anti-Tyr(P). As found previously (16, 18, 19), GPx1 was expressed at detectable levels in the absence of selenium supplementation (Fig. 3A). The results also demonstrate that endogenous c-Abl binds to GPx1 and that GPx1 is phosphorylated on tyrosine (Fig. 3A). To further assess tyrosine phosphorylation of GPx1, FLAG-GPx1(Sec-47 → Cys) was expressed with kinase-active c-Abl or the kinase-inactive c-Abl(K-R) mutant (Fig. 3B, left). Immunoblot analysis of anti-FLAG immunoprecipitates with anti-Tyr(P) demonstrated tyrosine phosphorylation of GPx1 by c-Abl and not c-Abl(K-R) (Fig. 3B, left). Tyrosine phosphorylation of GPx1 was also mediated by Arg, but not Arg(K-R) (Fig. 3B, left). In concert with these results, treatment with STI571 inhibited c-Abl- and Arg-mediated phosphorylation of GPx1 (Fig. 3B, right and data not shown). GPx1 contains a potential c-Abl/Arg phosphorylation site at amino acids 96–99 (YVRP). To determine whether that site is involved in c-Abl phosphor-ylation, GPx1(Sec-47 → Cys) and a GPx1(Sec-47 → Cys/ Tyr-96 → Phe) mutant were incubated with FLAG-c-Abl in the presence of [γ-32P]ATP. Analysis of the reaction products demonstrated that c-Abl phosphorylation of GPx1 is abrogated by the Tyr-96 → Phe mutation (Fig. 3C). To determine whether the GPx1 Tyr-96 site is phosphorylated by c-Abl in vivo, anti-FLAG immunoprecipitates from cells expressing FLAG-GPx1(Sec-47 → Cys) or GPx1(Sec-47 → Cys/Tyr-96 → Phe) were analyzed by immunoblotting with anti-Tyr(P) (Fig. 3D). The results demonstrate that c-Abl-mediated phosphorylation of GPx1 in cells is abrogated by the Tyr-96 → Phe mutation (Fig. 3D). Arg-mediated phosphorylation of GPx1 was also undetectable with the GPx1(Sec-47 → Cys/Tyr-96 → Phe) mutant (Fig. 3D). These findings demonstrate that c-Abl and Arg phosphorylate GPx1 on Tyr-96 in vitro and in vivo.

GPx Activity Is Regulated by c-Abl and Arg—To assess the effects of c-Abl on GPx activity, lysates from control and STI571-treated SH-SY5Y cells were assayed for the oxidation of GSH to GSGS. The finding that STI571 partially inhibits GPx activity indicated that c-Abl and Arg contribute to GPx activation (Fig. 4A). In concert with this observation, lysates from cells expressing c-Abl exhibited a substantial increase in GPx activity compared with control cells expressing the empty vector (Fig. 4B). By contrast, expression of c-Abl(K-R) was associated with a decrease in GPx activity (Fig. 4D). A decrease in GPx activity was also observed in cells deficient in c-Abl and Arg compared with that in wild-type cells (Fig. 4C). Catalase and GPx are major effectors in the cellular response to H2O2. To assess in part the contribution of GPx activity, cells were treated with 3AT, an irreversible inhibitor of catalase, and then exposed to H2O2. 3AT treatment alone had no apparent effect on apoptosis of wild-type or c-abl−/− arg−/− cells (Fig. 4D). Treatment with 3AT and H2O2, however, was associated with a low level of apoptosis in wild-type cells and a pronounced apoptotic response in the c-abl−/− arg−/− cells (Fig. 4D). These findings demonstrate that c-Abl and Arg stimulate GPx activity and that c-Abl/Arg contribute to GPx-mediated protection of cells against oxidative stress.

DISCUSSION

Dual Roles for c-Abl and Arg in the Response to Oxidative Stress—The available evidence indicates that c-Abl and Arg are functionally important in the cellular response to oxidative stress. c-Abl and Arg are activated in cells treated with H2O2, and both are required for apoptosis in response to increased oxidant levels (4, 5). More recent work has demonstrated that treatment of cells with non-lethal concentrations of H2O2 induces the formation of c-Abl-Arg heterodimers (7). Notably, higher concentrations of H2O2 that induce apoptosis are not effective in inducing the heterodimerization of c-Abl and Arg (7). These findings have suggested that c-Abl and Arg may confer different functions that are dependent on the level of...
oxidative stress. Specifically, activation of c-Abl and Arg at non-lethal concentrations of H₂O₂ appears to play an anti-apoptotic function in protecting cells against increased oxidant levels. Conversely, at lethal concentrations of H₂O₂, c-Abl and Arg function as pro-apoptotic effectors based on the demonstration that cells deficient in either kinase exhibit an attenuated apoptotic response (4, 5). In concert with c-Abl and Arg playing dual roles in the oxidative stress response is the observation that cells deficient in both c-Abl and Arg exhibit substantial increases in intracellular oxidant levels compared with wild-type cells or those lacking only one of these kinases (20).

Regulation of GPx1 by c-Abl and Arg—The results of the present work demonstrate that c-Abl and Arg interact with GPx1, an important effector in regulating intracellular H₂O₂ levels. The findings show that c-Abl and Arg associate constitutively with GPx1 and that exposure of cells to lethal concentrations (≥0.5 mM) of H₂O₂ disrupts these complexes. GPx1 also functions as a substrate for c-Abl and Arg. Other studies have demonstrated that bovine erythrocyte GPx is activated by incubation with the catalytic subunit of c-AMP-dependent protein kinase (21). Moreover, epidermal growth factor receptor signaling is associated with activation of both c-Abl (22) and GPx (23). Thus, c-Abl may function in transducing signals from activated growth factor receptors to GPx1. The functional significance of the interaction with c-Abl and Arg is supported by the demonstration that inhibition of these kinases with STI571 decreases GPx activity. Expression of kinase-active c-Abl increases GPx activity, whereas the dominant-negative

![Figure 3](image-url)

**Fig. 3.** c-Abl and Arg phosphorylate GPx1 on Tyr96 in vitro and in vivo. A, lysates from SH-SY5Y cells were immunoprecipitated with anti-GPx1 or IgG. The precipitates were analyzed by immunoblotting with anti-c-Abl, anti-Tyr(P), and anti-GPx1. B, anti-FLAG immunoprecipitates (IP) from 293 cells expressing FLAG-GPx1(Sec-47 → Cys) and the indicated c-Abl or Arg vectors were analyzed by immunoblotting with anti-Tyr(P) and anti-FLAG. 293 cells expressing c-Abl and FLAG-GPx1(Sec-47 → Cys) or FLAG were incubated in the absence or presence of 10 μM STI571 for 12 h. Anti-FLAG immunoprecipitates were analyzed by immunoblotting with anti-Tyr(P) and anti-FLAG. C, GST-GPx1(Sec-47 → Cys) and GST-GPx1(Sec-47 → Cys/Tyr-96 → Phe) were incubated with FLAG-c-Abl and [γ-³²P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography (upper panel). The GST fusion proteins were stained with Coomassie Blue (lower panel). D, anti-FLAG immunoprecipitates from cells expressing the indicated vectors were analyzed by immunoblotting with anti-GPx1 and anti-FLAG. IB, immunoblot.
c-Abl(K-R) has an inhibitory effect. Moreover, GPx activity is decreased in c-abl−/−arg−/− cells. Taken together, these findings indicate that c-Abl- or Arg-mediated phosphorylation of GPx1 increases its activity. The demonstration that complexes of GPx1 with c-Abl or Arg decrease upon exposure to lethal concentrations of H2O2 further indicates that regulation of GPx1 activity may be down-regulated once the cells have committed to undergo apoptosis.

GPx1 Functions in the Apoptotic Response to Oxidative Stress—A role for GPx1 in protecting cells against oxidant-induced apoptosis is supported by the increased sensitivity of GPx1-deficient mice to H2O2 exposure (11, 12). The present results also support the regulation of GPx1 by c-Abl and Arg as being of potential importance to the regulation of oxidation-induced apoptosis. In cells treated with 3AT to inhibit catalase, exposure to H2O2 was associated with the induction of a low level of apoptosis. By contrast, treatment of c-abl−/−arg−/− cells with 3AT and H2O2 was associated with a substantial increase in the percentage of apoptotic cells. These findings indicate that, in the absence of catalase, loss of GPx1 regulation by a deficiency of both c-Abl and Arg results in increased oxidant-induced apoptosis. High intracellular oxidant levels have been shown to directly disrupt mitochondrial function and thereby contribute to further increases in reactive oxygen species. Under these circumstances, the pro-apoptotic functions of c-Abl and Arg in response to oxidative stress would be dispensable as high oxidant levels can activate release of pro-apoptotic factors from mitochondria or induce necrosis.

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