Functional Interaction between the c-Abl and Arg Protein-tyrosine Kinases in the Oxidative Stress Response*

The Abl family of mammalian nonreceptor tyrosine kinases consists of c-Abl and Arg. Recent work has shown that c-Abl and Arg are activated in the cellular response to oxidative stress. The present studies demonstrate that reactive oxygen species (ROS) induce the formation of c-Abl and Arg heterodimers. The results show that the c-Abl SH3 domain binds directly to a proline-rich site (amino acids 567–576) in the Arg C-terminal region. Formation of c-Abl-Arg heterodimers also involves direct binding of the Arg Src homology 3 domain to the C-terminal region of c-Abl. The results further demonstrate that the interaction between c-Abl and Arg involves c-Abl-mediated phosphorylation of Arg. The functional significance of the c-Abl-Arg interaction is supported by the demonstration that both c-Abl and Arg are required for ROS-induced apoptosis. These findings indicate that ROS induce c-Abl-Arg heterodimers and that both c-Abl and Arg are necessary as effectors in the apoptotic response to oxidative stress.

The mammalian c-Abl and Arg nonreceptor tyrosine kinases are expressed widely in adult tissues (1–3). The N-terminal regions of c-Abl and Arg share ~90% identity and, as found in members of the Src family, contain tandem Src homology 3 (SH3),1 SH2, and tyrosine kinase (SH1) domains. Following the kinase domain, the next 135 amino acids of both proteins contain three conserved PXXP motifs that can serve as binding sites for SH3 domains (4, 5). The C-terminal regions of c-Abl and Arg share 29% identity and differ from other nonreceptor tyrosine kinases by the presence of globular and filamentous actin binding domains (6). In addition, the C-terminal region of c-Abl differs from that of Arg by the presence of a nuclear localization signal (7) and DNA binding sequences (8). In concert with these structural differences, c-Abl is expressed in both the nucleus and cytoplasm, whereas Arg has been found predominantly in the cytoplasm (5, 9).

The available evidence supports a role for c-Abl and Arg in regulating cytoskeletal dynamics. Mammalian c-abl and arg exhibit structural conservation with genes in the sea urchin (E-abl), fruit fly (D-abl), and nematode (N-abl) (10, 11). D-abl is expressed in neuronal axons (12) and functions in control of the axonal cytoskeleton (13). Other studies have demonstrated that D-abl interacts with the Notch transmembrane receptor to regulate axon extension (14). Mice with targeted disruption of the c-abl gene are born runted and exhibit head and eye abnormalities (14). Mice deficient in Arg develop normally and exhibit behavioral abnormalities (9). Moreover, embryos deficient in both c-Abl and Arg die before 11 days postcoitus with defects in neurulation (9). The finding that neuroepithelial cells from c-abl−/− arg−/− mice have an altered actin cytoskeleton has supported involvement of c-Abl and Arg in the regulation of actin microfilaments (9). Further support for interactions between c-Abl and the actin cytoskeleton has been obtained from the demonstration that clustering of integrins and thereby docking of actin stress fibers is associated with stimulation of c-Abl activity (15).

Other insights into a functional role for c-Abl have been derived from the findings that overexpression of c-Abl in fibroblasts induces cell cycle arrest (16, 17). Growth suppression is dependent on the nuclear localization sequences, an intact SH2 domain, and tyrosine kinase activity (17). Expression of C-Ab in Schizosaccharomyces pombe similarly induces growth arrest by a mechanism dependent on the c-abl kinase function (18). In mammalian cells, c-Abl-dependent growth arrest is mediated in part by interactions with p53 and the induction of p21 (19, 20). Nuclear c-Abl also associates with the DNA-dependent protein kinase complex (21, 22) and with the product of the gene mutated in ataxia telangiectasia (23, 24). Activation of these serinethreonine kinases in the response of cells to DNA damage is associated with induction of c-abl activity (21, 23–26). Activation of c-Abl contributes to DNA damage-induced apoptosis by mechanisms in part dependent on p53 and its homolog p73 (27–31). In contrast to the involvement of nuclear c-Abl in DNA damage-induced signaling, there is no known role for Arg in the cellular response to genotoxic stress.

Recent work has shown that the cytoplasmic forms of c-Abl and Arg are activated in the response of cells to oxidative stress. Normal cellular metabolism is associated with the production of reactive oxygen species (ROS) and, as a consequence, damage to DNA and proteins (32, 33). Cytoplasmic c-Abl is activated in response to ROS production by a mechanism that depends on interactions with protein kinase C6 (34–36). Activation of cytoplasmic c-Abl by ROS is associated with targeting of c-Abl to mitochondria, release of cytochrome c, and induction of cell death (36, 37). Other studies have shown that Arg is activated by oxidative stress and that this response involves Arg-mediated phosphorylation of the pro-apoptotic Siva-1 protein (38). The finding that ROS-induced apoptosis is attenuated in arg−/− cells has supported a role for Arg in the cell death response to oxidative stress (38). Thus, the available evidence indicates that cytoplasmic c-Abl...
and Arg are both functional in the oxidative stress response. The present studies demonstrate that c-Abl forms heterodimers with Arg in response to oxidative stress. The functional significance of these findings is supported by the demonstration that both c-Abl and Arg are required for ROS-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293 cells, MCF-7, MCF-7/c-Abl(K-R) (27), and MCF-7/Arg(K-R) (38) cells, and mouse embryonic fibroblasts (MEFs; wild-type, c-abl<sup>−/−</sup>, and arg<sup>−/−</sup>) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Transient transfections were performed with LipofectAMINE (Invitrogen). Cells were treated with H<sub>2</sub>O<sub>2</sub> (Sigma), menadione (Sigma), exposure to 640 nM H<sub>2</sub>O<sub>2</sub> was associated with detectable increases in c-Abl<sup>−/−</sup> (Fig. 1B). The association between c-Abl and Arg was increased after exposure to 10, 40, and 160 µM H<sub>2</sub>O<sub>2</sub>, whereas treatment with 640 µM H<sub>2</sub>O<sub>2</sub> had little effect (Fig. 1B). To determine whether c-Abl and Arg interact in response to other inducers of oxidative stress, wild-type MEFs were treated with menadione, a redox-cycling agent that increases ROS generation (39). Like H<sub>2</sub>O<sub>2</sub>, treatment with 25 µM menadione increased the formation of c-Abl-Arg complexes, whereas exposure to higher concentrations had less of an effect (Fig. 1C, left). Similar results were obtained when cells were treated with 20 ng/ml TNF-α to induce an endogenous oxidative stress response (40, 41) (Fig. 1C, right). As a control, anti-c-Abl immunoprecipitates from c-abl<sup>−/−</sup> and arg<sup>−/−</sup> MEFs showed no detectable signals when probed with anti-Arg (Fig. 1D, left). As additional controls, anti-Arg reacted specifically with c-Abl, whereas anti-Arg reacted specifically with Arg in immunoblot analyses of arg<sup>−/−</sup> and c-abl<sup>−/−</sup> MEFs (Fig. 1D, right).

To extend these findings, 293 cells were transfected to express Myc-tagged c-Abl (Myc-c-Abl) and FLAG-tagged Arg (FLAG-Arg). Immunoblot analysis of anti-Myc immunoprecipitates with anti-FLAG demonstrated detection of complexes containing c-Abl and Arg (Fig. 2A). In the reciprocal experiment, immunoblot analysis of anti-FLAG immunoprecipitates with anti-Myc provided further support for the association of c-Abl and Arg in cells (Fig. 2B). To define the kinetics of the interaction between c-Abl and Arg, the parameters for binding of Arg were determined using GST-c-Abl immobilized to the sensor chip in a BIAcore. Arg bound to c-Abl with a dissociation constant (K<sub>d</sub>) of 0.05 µM (Fig. 2C). These findings demonstrate that c-Abl binds to Arg in the response to oxidative stress and that the interaction is direct.

**c-Abl SH3 Interacts with the Arg C Terminus**—To define the interaction between c-Abl and Arg, lysates from cells expressing FLAG-Arg were incubated with GST or GST fusion proteins containing the c-Abl SH3 or SH2 domains. The results show that FLAG-Arg associates with GST-c-Abl SH3 and not GST-c-Abl SH2 (Fig. 3A). To assess whether the interaction between c-Abl SH3 and Arg is direct, the GST fusion proteins were incubated with in vitro translated<sup>35</sup>S-FLAG-Arg. The finding that GST-c-Abl SH3 associates with<sup>35</sup>S-FLAG-Arg supported a direct interaction (Fig. 3B). Moreover, the finding that there is no detectable binding of<sup>35</sup>S-FLAG-Arg with the c-Abl SH2 domain supported specificity of the c-Abl SH3-Arg interaction (Fig. 3B). Other studies were performed with<sup>35</sup>S-labeled Arg(1–501) or Arg(532–1182) to define regions of Arg responsible for binding to c-Abl SH3. The results demonstrate that GST-c-Abl SH3 binds to Arg(532–1182) and not Arg(1–501) (Fig. 3C). Arg(532–1182) contains six proline-rich sequences that could function as binding sites for the c-Abl SH3 domain (Fig. 3D). To define the Arg site(s) responsible for the c-Abl SH3 interaction, FLAG-Arg proteins mutated at each of the PXXP sequences were incubated with GST-c-Abl SH3. Analysis of the adsorbates with anti-FLAG demonstrated that binding of c-Abl SH3 is decreased, but not completely abrogated, with the Arg(570A/573A) mutant (Fig. 3D). By contrast, the other Arg mutants had little if any effect on c-Abl SH3 binding (Fig. 3D).
These findings demonstrate that the c-Abl SH3 domain interacts, at least in part, with the Arg proline-rich site at amino acids 567–576.

To assess binding in vivo, lysates from 293 cells expressing FLAG-Arg(1–501) or FLAG-Arg(532–1182) were subjected to immunoprecipitation with anti-c-Abl. Immunoblot analysis of the precipitates with anti-c-FLAG demonstrated that c-Abl associates with Arg(1–501) (Fig. 4A, left) and Arg(532–1182) (Fig. 4A, right). In the reciprocal analysis, coexpression of Arg and c-Abl(1–486) or c-Abl(487–1130) demonstrated that Arg associates with the c-Abl N- and C-terminal regions (Fig. 4B). In studies performed with 293 cells expressing Exp-c-Abl(1–486) and Arg(532–1182), immunoblot analysis of anti-Exp immunoprecipitates with anti-Arg also demonstrated association of the c-Abl N-terminal and the Arg C-terminal regions (Fig. 4C). By contrast, when anti-FLAG immunoprecipitates from 293 cells expressing FLAG-Arg(1–501) and Exp-c-Abl(1–486) were subjected to immunoprecipitation with anti-Exp, there was no detectable interaction between these N-terminal regions of c-Abl and Arg (data not shown). Moreover, in similar experiments performed on 293 cells expressing FLAG-Arg(532–1182) and Exp-c-Abl(487–1130), there was no apparent association of these c-Abl and Arg C-terminal regions (data not shown).

Taken together with the in vitro binding data, the results demonstrate that the c-Abl SH3 domain interacts with the Arg C-terminal region and that the c-Abl C-terminal region interacts with the Arg N-terminal region.

**The Arg SH3 Domain Binds Directly to the c-Abl C Terminus**—To determine whether the Arg SH3 domain binds to c-Abl, GST-Arg SH3 was incubated with in vitro translated 35S-labeled FLAG-c-Abl. The results demonstrate that Arg SH3 binds directly to c-Abl (Fig. 5A). To extend this finding, we generated a c-Abl C-terminal (amino acids 487–1130) fragment. The results demonstrate that GST-Arg SH3 binds to 35S-labeled c-Abl(487–1130) (Fig. 5B). These findings indicate that, in addition to binding of c-Abl SH3 to the Arg C terminus, a direct interaction between Arg SH3 and the c-Abl C-terminal region contributes to the formation of c-Abl-Arg complexes (Fig. 5C).

**c-Abl-mediated Phosphorylation of Arg**—To determine whether Arg is a substrate for c-Abl, in vitro translated/heat-inactivated Arg was incubated with a 45-kDa kinase-active Abl and γ-32P-ATP. Analysis of the reaction products demonstrated that Abl phosphorylates the 145-kDa Arg protein (Fig. 6A). To assess tyrosine phosphorylation of Arg in vivo, a kinase-inactive FLAG-Arg(K-R) mutant was expressed in 293...
cells. Immunoblot analysis of anti-FLAG immunoprecipitates with anti-Tyr(P) demonstrated that ectopically expressed Arg is constitutively phosphorylated on tyrosine (Fig. 6B). To confirm that Arg is phosphorylated by c-Abl in cells, c- abl/H11002 cells and c- abl/H11001 cells were infected with a retrovirus expressing FLAG-Arg(K-R). Immunoblot analysis of anti-FLAG immunoprecipitates with anti-Tyr(P) showed that tyrosine phosphorylation of FLAG-Arg(K-R) is substantially higher in c- abl/H11001 compared with c- abl/H11002 cells (Fig. 6C). These findings indicate that Arg is phosphorylated, at least in large part, by a c-Abl-dependent mechanism.

c-Abl and Arg Are Required for ROS-induced Apoptosis—To assess involvement of c-Abl and Arg in the response of cells to ROS, cells were studied for H2O2-induced apoptosis. Compared with MCF-7 cells expressing the empty vector, treatment of MCF-7/c-Abl(K-R) cells with 250 μM H2O2 resulted in an attenuated apoptotic response (Fig. 7A). Similar findings were obtained in MCF-7 cells stably expressing Arg(K-R) (Fig. 7A). In studies of MEFs, c- abl−/− cells exhibited little if any apoptosis in response to treatment with 40 or 250 μM H2O2 (Fig. 7B). The finding that stable expression of c-Abl in the c- abl−/− cells reconstitutes the apoptotic response to ROS demonstrates dependence on c-Abl (Fig. 7B). The arg−/− cells were also less sensitive to ROS-induced apoptosis compared with arg+ cells (Fig. 7B). The results further demonstrate that compared with MCF-7 cells expressing the empty vector, menadione-induced apoptosis is attenuated in MCF-7/c-Abl(K-R) and MCF/

Fig. 2. A and B, 293 cells were transfected to express Myc-c-Abl and FLAG-Arg. Lysates were subjected to immunoprecipitation (IP) with anti-Myc (A) or anti-FLAG (B). The immunoprecipitates were analyzed by immunoblotting (IB) with anti-FLAG or anti-Myc. C, a sensor chip was conjugated with GST-c-Abl. Arg was injected over the chip at concentrations ranging from 20 to 80 nM. Raw binding data were analyzed by BIAevaluation software 3.0 and fit to a 1.1 Langmuir binding model.

Fig. 3. Direct interaction between the c-Abl SH3 domain and Arg in vitro. A, lysates from 293 cells expressing FLAG-Arg were incubated with GST or the indicated GST fusion proteins. The adsorbates were analyzed by immunoblotting (IB) with anti-FLAG. Lysate was included as a control. B, GST or the indicated GST fusion proteins were incubated with in vitro translated 35S-labeled FLAG-Arg. The adsorbates were analyzed by SDS-PAGE and autoradiography. C, GST or GST-c-Abl SH3 were incubated with 35S-labeled FLAG-Arg(1–501) (left panel) or 35S-labeled Arg(532–1182) (right panel). The adsorbates were analyzed by SDS-PAGE and autoradiography. D, proline-rich sequences in Arg(532–1182) are listed with their respective mutants. Lysates from 293 cells expressing wild-type FLAG-Arg or the indicated mutants were incubated with GST-c-Abl SH3. The adsorbates were analyzed by immunoblotting with anti-FLAG (left panel). Lysates not incubated with GST-c-Abl SH3 were analyzed with anti-FLAG as controls for expression of the Arg proteins (right panel).

Arg(K-R) cells (Fig. 7C). Similar results were obtained when these cells were treated with TNF-α (Fig. 7C). By contrast, apoptosis induced by stabilization of microtubules with pacli-
taxel was unaffected by a expression of c-Abl(K-R) or Arg(K-R) (Fig. 7C). These findings collectively support a model in which both c-Abl and Arg are required for the apoptotic response to oxidative stress.

DISCUSSION

c-Abl Interacts with Arg—Recent findings that c-Abl and Arg are both activated in the cellular response to oxidative stress suggested that these related proteins may share similar functions (36, 38). The present studies were thus performed to determine whether c-Abl and Arg interact in ROS-induced signaling. The results of coimmunoprecipitation studies demonstrate that endogenous c-Abl and Arg associate in the response to oxidative stress. These findings were confirmed by showing the association of ectopically expressed c-Abl and Arg in 293 cells. The results further demonstrate that c-Abl and Arg interact directly. The in vitro findings support direct binding of the c-Abl SH3 domain to a proline-rich site (amino acids 567–576) in the Arg C-terminal region. By contrast, there was no detectable binding of the c-Abl SH3 domain to the Arg N-terminal region. In concert with these results, expression of c-Abl(1–486) and Arg(572–1182) confirmed that the c-Abl N-terminal region associates with the Arg C-terminal region in cells. In addition, binding of c-Abl to the Arg(P570A/P573A) mutant was decreased compared with that found for wild-type Arg. The results also demonstrate that the Arg SH3 domain associates with the c-Abl C-terminal region. In concert with an interaction between c-Abl and Arg, we demonstrate that Arg functions as an in vitro substrate for c-Abl phosphorylation. Moreover, the results show that tyrosine phosphorylation of Arg in cells expressing c-Abl is substantially higher than that found in c-abl/H11002/H11002 cells. These findings demonstrate that c-Abl forms heterodimers with Arg in vivo by mechanisms involving intermolecular binding of the respective SH3 domains and C-terminal regions (Fig. 5C) and that Arg is phosphorylated by a c-Abl-dependent mechanism.

Induction of c-Abl/Arg Heterodimers in the Oxidative Stress Response—Certain insights into the involvement of c-Abl in the response of cells to oxidative stress came from the finding that ROS induce tyrosine phosphorylation and activation of protein kinase C (34, 35). In an apparent feedback mechanism, protein kinase C activates c-Abl, and, in turn, c-Abl phosphorylates protein kinase C on Tyr-512 (35, 36). Activation of c-Abl is associated with targeting to mitochondria, release of mitochondrial cytochrome c, and induction of apoptosis (36, 37). Arg is also activated in the oxidative stress response and contributes to the induction of apoptosis by interacting with the proapoptotic Siva-1 protein (38). The present results demonstrate that oxidative stress induces the formation of c-Abl(Arg) heterodimers. Moreover, the findings show that binding of c-Abl...
and Arg is dependent on the concentration of H$_2$O$_2$. Thus, treatment with 10 to greater than 160 $\mu$M H$_2$O$_2$ was associated with increases in c-Abl/Arg heterodimers, whereas treatment with 640 $\mu$M H$_2$O$_2$ resulted in binding of c-Abl and Arg at a level found in control cells. The finding that menadione and TNF-$\alpha$ also induce the formation of c-Abl/Arg heterodimers is in concert with the effects of these agents on redox cycling and ROS generation (39, 41, 42). Thus, c-Abl and Arg form heterodimers in response to diverse agents that induce oxidative stress.

Regulation of the Apoptotic Response to Oxidative Stress by c-Abl and Arg—ROS have been implicated in the regulation of both mitogenic and apoptotic signaling pathways. Mitogenic signals induced by growth factors or activated Ras are mediated through ROS production (42, 43). Other work has indicated that ROS induce topoisomerase II-mediated cleavage of chromosomal DNA and thereby apoptosis (44). The p66$^{shc}$ adaptor protein (45) and the p85 subunit of phosphatidylinositol 3-kinase (46) have also been implicated in the apoptotic response to oxidative stress. Moreover, p53-induced apoptosis is mediated by ROS-dependent mechanisms (44, 47, 48). The present results provide support for involvement of both c-Abl and Arg in the apoptotic response to oxidative stress. Stable expression of either kinase-inactive c-Abl(K-R) or Arg(K-R) blocked H$_2$O$_2$-induced apoptosis of MCF-7 cells. In concert with these findings, the apoptotic response of MEFs to H$_2$O$_2$-induced oxidative stress was attenuated by targeted disruption of either c-abl or arg. Menadione- and TNF-$\alpha$-induced apoptosis was also attenuated by expression of c-Abl(K-R) or Arg(K-R). The present results also demonstrate that, in contrast to oxidative stress, both c-Abl and Arg are dispensable for paclitaxel-induced apoptosis. These findings thus provide the first evidence that c-Abl and Arg form heterodimers and that both c-Abl and Arg are required for the apoptotic response to oxidative stress.

Acknowledgments—We thank Dr. Tony Koleske for helpful discussions, for the c-abl$^{--}$/arg$^{--}$ MEFs, and for the recombinant Arg protein. We also thank Kamal Chauhan for excellent technical support.

---

**Fig. 6.** c-Abl phosphorylates Arg. A, in vitro translated FLAG-Arg was immunoprecipitated (IP) with anti-FLAG and heat inactivated (HI) at 80 °C for 20 min. The immunoprecipitates were incubated with Abl and [$\gamma$-32P]ATP. The reaction products were analyzed by SDS-PAGE and autoradiography (upper panel). The immunoprecipitates were also subjected to immunoblotting (IB) with anti-FLAG (lower panel). B, anti-FLAG immunoprecipitates from 293 cells expressing FLAG-Arg(K-R) were analyzed by immunoblotting with anti-Tyr(P) or anti-FLAG. C, Anti-FLAG immunoprecipitates from c-abl$^{--}$/arg$^{--}$ and c-abl$^{++}$/arg$^{--}$ MEFs infected with a retrovirus expressing FLAG-Arg(K-R) were analyzed by immunoblotting with anti-Tyr(P) or anti-FLAG.

**Fig. 7.** c-Abl and Arg regulate the apoptotic response to oxidative stress. A, MCF-7 cells stably expressing empty vector, c-Abl(K-R), or Arg(K-R) were left untreated (solid bars) or treated with 250 $\mu$M H$_2$O$_2$ (open bars) for 24 h. B, c-abl$^{--}$/arg$^{--}$, c-abl$^{++}$/arg$^{--}$, and arg$^{++}$/arg$^{--}$ MEFs were left untreated (solid bars) or treated with 40 $\mu$M (hatched bars) or 250 $\mu$M (open bars) H$_2$O$_2$ for 24 h. The percentage of cells with sub-G$_1$ DNA was determined by flow cytometry. The results are expressed as the mean ± S. D. of three experiments. C, MCF-7 cells stably expressing the empty vector (open bars), c-Abl(K-R) (hatched bars), and Arg(K-R) (solid bars) were treated with 25 $\mu$M menadione for 24 h, 20 ng/ml TNF-$\alpha$ + 10 $\mu$g/ml cycloheximide for 36 h, or 0.1 $\mu$M paclitaxel (Taxol) for 36 h. Control cells (C) were left untreated. The percentage of cells with sub-G$_1$ DNA is expressed as the mean ± S. D. of three experiments.
Functional Interaction between the c-Abl and Arg Protein-tyrosine Kinases in the Oxidative Stress Response
Cheng Cao, Yumei Leng, Chufang Li and Donald Kufe

J. Biol. Chem. 2003, 278:12961-12967.
doi: 10.1074/jbc.M300058200 originally published online February 4, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M300058200

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 48 references, 22 of which can be accessed free at http://www.jbc.org/content/278/15/12961.full.html#ref-list-1