Activation of the Cytoplasmic c-Abl Tyrosine Kinase by Reactive Oxygen Species*

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The ubiquitously expressed c-Abl protein tyrosine kinase localizes to both the nucleus and cytoplasm. The nuclear form of c-Abl is activated in the cellular response to genotoxic stress. Here we show that cytoplasmic c-Abl is activated by oxidative stress. The results also demonstrate that mitochondrial cytochrome c is released in the cellular response to H2O2 and that this effect is mediated by a c-Abl-dependent mechanism. In concert with these results, we show that H2O2-induced apoptosis is attenuated in c-Abl-deficient cells. These findings demonstrate that cytoplasmic c-Abl is involved in the apoptotic response of cells to oxidative stress.

Normal cellular metabolism is associated with the production of reactive oxygen species (ROS)1 and, as a consequence, damage to DNA and proteins (1, 2). The generation of ROS is also known to induce apoptosis; however, the molecular mechanisms responsible for ROS-induced apoptosis are unclear. Studies have indicated that ROS induce activation of topoisomerase II-mediated cleavage of chromosomal DNA and thereby apoptosis (3). Other work has suggested that ROS-induced apoptosis is p53-dependent (4, 5) and that p53-induced apoptosis (3). Other work has suggested that ROS-induced apoptosis is p53-dependent (4, 5) and that p53-induced apoptosis is mediated by ROS (6–8). In addition, the p66hsc adaptor protein (5) and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (4) have been implicated in the apoptotic response to oxidative stress.

The nuclear form of the c-Abl tyrosine kinase is activated in the cellular response to genotoxic stress (9). Nuclear c-Abl has been implicated in the apoptotic response to DNA damage by mechanisms in part dependent on p53 and its homolog, p73 (10–14). c-Abl also functions as an upstream effector of the proapoptotic SAPK/JNK and p38 mitogen activated protein kinase (MAPK) pathways in the genotoxic stress response (9, 15, 16). Other studies have demonstrated that c-Abl phosphorylates p85 and thereby inhibits PI3K activity in the apoptotic response to DNA damage (17). Additional evidence supporting a role for c-Abl in apoptosis has been provided by the findings that cells deficient in c-Abl or expressing a dominant-negative c-Abl mutant exhibit an attenuated apoptotic response to genotoxic agents (18, 19).

Recent work has shown that c-Abl phosphorylates protein kinase C (PKC) δ in cells treated with H2O2 (20). The present results demonstrate that the cytoplasmic, and not the nuclear, form of c-Abl is activated in the cellular response to H2O2. We also show that H2O2 induces mitochondrial cytochrome c release and apoptosis by a c-Abl-dependent mechanism.

MATERIALS AND METHODS

Cell Culture—COS7 cells and MEFs derived from wild-type and c-Abl−/− mice (21) were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. DLD1 cells were grown as described (7). Cells were treated with H2O2 (Sigma), 30 μM N-acetyl-t-cysteine (NAC; Sigma), or 10 μM cis-platinum (Sigma).

Analysis of Kinase Activity—Cell lysates were prepared in lysis buffer (10 μM Tris-HCl, pH 7.5, 10 μM NaCl, 1 mM dithiothreitol, 0.1 μM EDTA, 3 mM MgCl2, 0.5 mM phenylmethysulfonyl fluoride, 5 μg/ml leupeptin) containing 0.5% Nonidet P-40 and subjected to immunoprecipitation as described (22) with anti-c-Abl (sc-23; Santa Cruz Biotechnology) or mouse IgG (Santa Cruz). The immunoprecipitates were released in kinase buffer (20 μM HEPES, pH 7.4, 10 mM MgCl2, 10 mM MnCl2) containing 2.5 μCi of [γ-32P]ATP and GST-Crk (120–212) for 20 min at 30 °C. The reaction products were analyzed by SDS-PAGE and autoradiography.

Isolation of Cytoplasts and Nuclear Fractions—Cells were disrupted in lysis buffer containing 0.05% Nonidet P-40. The cytoplasmic and nuclear fractions were prepared as described (24).

Preparation of Cytoplasts—Enucleated cells were prepared by density centrifugation as described (25). Cells were incubated in 21 mM cytochalasin B for 1 h at 37 °C, layered over a discontinuous Ficoll gradient, and centrifuged at 80,000 × g for 1 h. Cytoplasts were collected at the 12.5–15% Ficoll interface. Cytoplast purity was assessed by staining with 0.5 μg/ml 4′,6-diamino-2-phenylindole (DAPI) and greater than 95% free of whole cells.

Immunoblot Analysis—Proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with anti-c-Abl, anti-IC80 (sc-847; Santa Cruz) or anti-cytochrome c (26). The antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech).

Apoptosis Assays—DNA content was assessed by staining ethanol-fixed cells with propidium iodide and monitoring by FACScan (Becton-Dickinson).

RESULTS AND DISCUSSION

To determine whether c-Abl is activated by ROS, lysates from COS7 cells exposed to H2O2 were subjected to immunoprecipitation with mouse IgG, as a control, or anti-c-Abl antibody. The precipitates were assayed for phosphorylation of a GST-Crk (120–225) fusion protein (27, 28). There was no detectable phosphorylation of GST-Crk (120–225) with the control immunoprecipitates (Fig. 1a, left). A low level of GST-Crk (120–225) phosphorylation was detectable when assaying anti-c-Abl immunoprecipitates from control cells, whereas exposure to H2O2 resulted in stimulation (4–5-fold) of the Crk kinase activity (Fig. 1a, right, first 2 lanes). By contrast, there was no detectable H2O2-induced phosphorylation of a GST-Crk (120–212) fusion protein that lacks the c-Abl phosphorylation site at

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‡ The abbreviations used are: ROS, reactive oxygen species; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen activated protein kinase; PKC, protein kinase C; NAC, N-acetyl-t-cysteine; ERK, extracellular signal-regulated kinase; MEF, mouse embry fibroblasts; SAPK/JNK, stress-activated protein kinase; e-Jun NH2-terminal kinase; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
The results also show that H2O2 treatment is not associated with increases in the level of c-Abl protein (Fig. 1a, right). To confirm involvement of ROS in c-Abl activation, cells were treated with NAC, a scavenger of reactive oxygen intermediates and precursor of glutathione (29, 30). NAC treatment inhibited H2O2-induced phosphorylation of GST-Crk (120–225) by c-Abl (Fig. 1b). The induction of c-Abl activity was dependent on H2O2 concentration, with 5-fold increases upon exposure to 1 mM H2O2 (Fig. 1c, left). In addition, maximal induction of c-Abl activity was observed at 30–60 min (Fig. 1c, right). The finding that human DLD1 cells respond to H2O2 with activation of c-Abl further indicated that the results are not restricted to certain cell types (Fig. 1d).

To extend the analysis of H2O2-induced activation of c-Abl to other pathways involved in the ROS response, we studied mouse embryo fibroblasts that are null for c-Abl expression (c-Abl−/− MEFs) (21). There was no detectable c-Abl activity in control or H2O2-treated c-Abl−/− cells (Fig. 2a). By contrast, wild-type MEFs responded to H2O2 with induction of c-Abl activity. Recent studies have demonstrated that c-Abl interacts with PKCδ in the response to oxidative stress (20). To determine whether c-Abl is required for activation of PKCδ, we assayed anti-PKCδ immunoprecipitates from c-Abl−/− and wild-type MEFs. The results demonstrate that, whereas PKCδ is required for activation of c-Abl (20), c-Abl is dispensable for activation of PKCδ in the ROS response (Fig. 2b). Other studies have demonstrated that ERK1 is activated in cells exposed to H2O2 (31). Analysis of anti-ERK1 immunoprecipitates from H2O2-treated c-Abl−/− and wild-type MEFs demonstrated activation of ERK1 by a c-Abl-independent mechanism (Fig. 2c).

These findings demonstrate that activation of c-Abl in the ROS response is not functional in the induction of PKCδ or ERK1 activities.

As nuclear c-Abl is activated in the stress response to DNA

**FIG. 1. Activation of c-Abl by H2O2.** a, COS7 cells were treated with 1 mM H2O2 and harvested at 15 min. Cell lysates were subjected to immunoprecipitation with mouse IgG or anti-c-Abl. In vitro immune complex kinase assays were performed with GST-Crk (120–225) fusion protein as substrate (left and right panel, first 2 lanes). GST-Crk (120–212), which lacks the critical Tyr-221, was used as a negative control (right, last 2 lanes). The immunoprecipitates (IP) were also analyzed by immunoblotting (IB) with anti-c-Abl (bottom panel). b, COS7 cells were exposed to 1 mM H2O2 or 30 mM NAC as indicated and harvested at 15 min. Anti-c-Abl immunoprecipitates were analyzed for phosphorylation of GST-Crk (120–225) (upper panel) and by immunoblotting with anti-c-Abl (lower panel). c, COS7 cells were treated with the indicated concentrations of H2O2 for 15 min (left panel) or with 1 mM H2O2 for the indicated times (right panel). Anti-c-Abl immunoprecipitates were analyzed for c-Abl activity (upper panels) and c-Abl protein (lower panels). d, DLD1 cells were treated with 1 mM H2O2 for 15 min. Anti-c-Abl immunoprecipitates were analyzed for phosphorylation of GST-Crk (120–225).

**FIG. 2. H2O2-induced activation of PKCδ and MAPK is mediated by a c-Abl-independent mechanism.** a, c-Abl−/− and wild-type MEFs were treated with 1 mM H2O2 for 15 min. Anti-c-Abl immunoprecipitates were analyzed for phosphorylation of GST-Crk (120–225). b, c-Abl−/− and wild-type MEFs were transfected to express Flag-PKCδ (20) and then treated with 1 mM H2O2 for 15 min. Anti-Flag immunoprecipitates (IP) were analyzed for phosphorylation of histone H1. c, Abl−/− and wild-type MEFs were treated with 1 mM H2O2 for 30 min. Anti-ERK1 immunoprecipitates were analyzed for phosphorylation of myelin basic protein (MBP).
COS7 cells were treated with 1 mM H2O2 for 15 min. Cell lysates were separated into cytoplasmic (Cyto) and nuclear (Nuc) fractions. Anti-c-Abl immunoprecipitates (IP) were analyzed for phosphorylation of GST-Crk (120–225). IB, immunoblotting. b, cytoplasts prepared from COS7 cells were treated with 1 mM H2O2 for 15 min. Lysates were subjected to immunoprecipitation with anti-c-Abl, and the precipitates were analyzed for c-Abl kinase activity.

Damage (9), studies were performed to define the subcellular localization of ROS-induced c-Abl activation. Cells were treated with H2O2 before preparation of nuclear and cytoplasmic fractions. Analysis of cytoplasmic anti-c-Abl immunoprecipitates demonstrated increased phosphorylation of GST-Crk (120–225) (Fig. 3a). By contrast, there was no detectable activation of c-Abl in the nuclear fraction (Fig. 3a). These results indicate that cytoplasmic c-Abl is activated in the response to oxidative stress by a mechanism independent of nuclear signals.

The cellular response to genotoxic stress includes release of mitochondrial cytochrome c and the induction of apoptosis (34). To determine whether oxidative stress induces cytochrome c release, cytoplasmic lysates from wild-type and c-Abl−/− cells treated with H2O2 were subjected to immunoblotting with anti-cytochrome c. The results demonstrate that H2O2 treatment of wild-type MEFs is associated with increased levels of cytochrome c (Fig. 4a). By contrast, cytochrome c release was not detectable in c-Abl−/− MEFs treated with H2O2 (Fig. 4a). To determine whether c-Abl contributes to the induction of apoptosis by oxidative stress, H2O2-treated MEFs were assayed for the appearance of sub-G1 DNA. The results demonstrate that, compared with wild-type MEFs, the c-Abl−/− MEFs exhibit an attenuated apoptotic response to H2O2 exposure (Fig. 4b). Analysis at 3 to 24 h of H2O2 exposure confirmed that cells deficient in c-Abl expression exhibit a defective apoptotic response (Fig. 4c). The finding that H2O2-induced release of cytochrome c is completely abrogated in c-Abl−/− cells indicates that the attenuated induction of apoptosis in response to H2O2 is mediated by a cytochrome c-independent pathway (Fig. 4b). These results collectively demonstrate that cytoplasmic H2O2 induces cytochrome c release and apoptosis by a c-Abl-dependant mechanism.

Oxidative cellular damage contributes to aging (5) and, in the presence of acute ROS exposure, the induction of apoptosis (35). Previous work has shown that the nuclear c-Abl kinase is activated in the apoptotic response of cells to genotoxic stress (12–14, 18). Conversely, the present studies demonstrate that cytoplasmic, and not nuclear, c-Abl is activated in the apoptotic response to oxidative stress. Whereas DNA damage-induced apoptosis is mediated by activation of c-Abl and the release of mitochondrial cytochrome c (34), less is known about involvement of mitochondrial signals in H2O2-induced cell death. The present results demonstrate that cytochrome c release is also induced in response to oxidative stress and that this event is c-Abl-dependent. These findings support a model in which c-Abl functions in determining cell fate by conferring stress-induced signals to the release of cytochrome c and thereby apoptosis. The findings further indicate that the subcellular distribution of c-Abl determines localization of the specific response to apparently diverse environmental stresses. However, ROS induce damage to DNA (1), as well as other cellular components (2), and thus it is conceivable that cells have evolved with conservation of a similar response to both genotoxic and oxidative stress. The present findings demonstrate...
that, analogous to activation of nuclear c-Abl by DNA-damaging agents (9), cytoplasmic c-Abl is activated by ROS-induced stress.

REFERENCES
1. Croteau, D., and Bohr, V. (1997) J. Biol. Chem. 272, 25409–25412
2. Berlett, S., and Stadtman, E. (1997) J. Biol. Chem. 272, 20313–20316
3. Li, T., Chen, A., Yu, C., Mao, Y., Wang, H., and Liu, L. (1999) Genes Dev. 13, 1553–1560
4. Yin, Y., Terauchi, Y., Solomon, G., Aizawa, S., Rangarajan, P., Yazaki, Y., Kadowaki, T., and Barrett, J. (1998) Nature 391, 707–710
5. Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P. P., Lanfrancone, L., and Pelicci, P. G. (1999) Nature 402, 309–313
6. Johnson, T., Yu, Z., Ferrans, V., Lowenstein, R., and Finkel, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11848–11852
7. Polyak, K., Xia, Y., Zweier, J. L., Kinzler, K. W., and Vogelstein, B. (1999) Nature 399, 300–305
8. Li, P., Dietz, R., and Harsdorf, R. (1999) EMBO J. 18, 6027–6036
9. Imlay, J. A., and Linn, S. (1988) Science 240, 1302–1309
10. Roederer, M., Staal, F. J., Raju, P. A., Ela, S. W., Herzenberg, L. A., and Herzenberg, L. A. (1990) Proc. Natl. Acad. Sci. (U. S. A.) 87, 9943–9947