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ACCELERATED COMMUNICATION

Abrogation of the Cell Death Response to Oxidative Stress by the c-Abl Tyrosine Kinase Inhibitor STI571

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ABSTRACT

Normal aerobic metabolism is associated with the production of reactive oxygen species (ROS) and, consequently, the induction of apoptosis and necrosis. The cell death response to oxidative stress is thought to contribute to aging, neurological degeneration, and other disorders. ROS-induced apoptosis and necrosis involves activation of the cytoplasmic c-Abl tyrosine kinase and thereby signaling to mitochondria. Herein, we show that STI571, an inhibitor of Bcr-Abl in chronic myelogenous leukemia, blocks activation of c-Abl in the response of mouse embryo fibroblasts and human U-937 myeloid leukemia cells to hydrogen peroxide (H_2O_2). Immunofluorescence microscopy and subcellular fractionation studies demonstrate that STI571 decreases H_2O_2-induced targeting of c-Abl to mitochondria in the two cell types by 59 to 85%. The results also show that STI571 attenuates H_2O_2-induced loss of the mitochondrial transmembrane potential. In concert with these effects, STI571 inhibits the death response to H_2O_2 exposure by 40 to 80% depending on the cell type. These findings indicate that inhibition of c-Abl signaling by STI571 attenuates mitochondrial dysfunction and cell death in the cellular response to oxidative stress.

The generation of H_2O_2 during normal cellular metabolism is associated with damage to DNA and proteins (Berlett and Stadtman, 1997; Croteau and Bohr, 1997) and, under certain circumstances, the induction of apoptosis (Jacobson et al., 1996; Manna et al., 1998). Few insights are available regarding the mechanisms responsible for ROS-induced cell death and how to abrogate aberrant regulation of this response when it contributes to disease. The $p66^{shc}$ adaptor protein (Migliaccio et al., 1999) and the p85 subunit of phosphatidylinositol 3-kinase (Yin et al., 1998) have been implicated in the apoptotic response to H_2O_2. Activation of topoisomerase II-mediated cleavage of chromosomal DNA by H_2O_2 also contributes to the induction of apoptosis (Li et al., 1999). Other studies have shown that H_2O_2-induced apoptosis is p53-dependent (Yin et al., 1998; Migliaccio et al., 1999) and that p53-induced apoptosis is mediated by ROS (Johnson et al., 1996; Polyak et al., 1997; Li et al., 1999).

The ubiquitously expressed c-Abl protein tyrosine kinase localizes to the nucleus and cytoplasm. Like other tyrosine and serine/threonine kinases (Khanna and Jackson, 2001; Skorski, 2002), c-Abl is activated in the cellular response to genotoxic stress (Kharbanda et al., 1995). c-Abl contributes to the induction of apoptosis by mechanisms dependent in part on p53 and its homolog p73 (Yuan et al., 1996, 1997, 1999; Agami et al., 1999; Gong et al., 1999). The cytoplasmic form of c-Abl is activated in response to oxidative stress by a mechanism dependent of protein kinase Cδ (PKCδ) (Sun et al., 2000a,b). Activation of c-Abl is attenuated by the PKCδ inhibitor, rottlerin, and by the overexpression of kinase-inactive PKCδ mutants. Rottlerin and overexpression of PKCδ(K-R) also block H_2O_2-induced targeting of c-Abl to mitochondria (Kumar et al., 2001). Targeting of c-Abl to mitochondria is dependent on the kinase function and is associated with the cell death response to oxidative stress (Kumar et al., 2001). These findings have collectively supported a signaling cascade in which PKCδ is an upstream effector of both c-Abl activation and localization to mitochondria in response to oxidative stress.

A small molecule inhibitor of c-Abl and the Bcr-Abl fusion

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ABBREVIATIONS: PKC, protein kinase C; MEF, mouse embryonic fibroblast; GST, glutathione *S*-transferase; DCF, dichlorofluorescein; ROS, reactive oxygen species; PBS, phosphate-buffered saline; PCNA, proliferating cell nuclear antigen; FITC, fluorescein isothiocyanate; $\Delta\psi m$, mitochondrial transmembrane potential.

protein (STI571) has been developed as an effective treatment for chronic myelogenous leukemia (Druker et al., 1996; Gorre et al., 2001). STI571 has exceptionally high affinity and selectivity for c-Abl (Zimmermann et al., 1997; Schindler et al., 2000). The effects of STI571 on the oxidative stress response are unknown. The present studies demonstrate that STI571 attenuates H_2O_2-induced mitochondrial targeting of c-Abl and loss of mitochondrial transmembrane potential. The results also demonstrate that STI571 blocks induction of cell death in the cellular response to oxidative stress.

Materials and Methods

Cell Culture. Wild-type mouse embryo fibroblasts (MEFs) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Human U-937 myeloid leukemia cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. Cells were treated with 1 mM H_2O_2 (Sigma, St. Louis, MO) and/or STI571 (Novartis, Basel, Switzerland).

Analysis of Kinase Activity. Cell lysates were subjected to immunoprecipitation as described previously (Sun et al., 2000a) with anti-c-Abl (24-11; Santa Cruz Biotechnology, Santa Cruz, CA). The immunoprecipitates were resuspended in kinase buffer (20 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$) containing 2.5 μCi of [γ-^{32}P]ATP and GST-Crk(120–225) or GST-Crk(120–212) for 20 min at 30°C. The reaction products were analyzed by SDS-PAGE and autoradiography.

Measurement of ROS Levels. Cells were incubated with 10 μM DCF-diacetate (Sigma) for 15 min at 37°C to assess ROS-mediated oxidation to the fluorescent compound DCF (LeBel et al., 1992). Fluorescence of oxidated DCF was measured at an excitation wavelength of 480 nm and an emission wavelength of 525 nm using a flow cytometer (BD Biosciences, Lincoln Park, NJ).

Immunofluorescence Microscopy. Cells were plated onto poly-D-lysine coated glass coverslips 1 d before H_2O_2 treatment (1 h) and then fixed with 3.7% formaldehyde/PBS, pH 7.4, for 10 min. Cells were washed with PBS, permeabiliized with 0.2% Triton X-100 for 10 min, washed again and incubated for 30 min in complete medium. The coverslips were then incubated with 5 μg/ml of anti-c-Abl (K-12; Santa Cruz Biotechnology) for 1 h followed by Texas Red-goat anti-rabbit Ig (H+L) conjugate (Molecular Probes, Eugene, OR). Mitochondria were stained with 100 nM Mitotracker Green FM (Molecular Probes, Eugene, OR). Nuclei were stained with 4,6-diamino-2-phenylindole (1 μg/ml in PBS). Coverslips were mounted onto slides with 0.1 M Tris-HCl, pH 7.0, in 50% glycerol. Cells were visualized by digital confocal immunofluorescence microscopy as described previously (Monks et al., 1998). The images were captured with a cooled charge-coupled device camera mounted on a Zeiss Axioplan 2 microscope. Images were deconvolved using SlideBook software (Intelligent Imaging Innovations, Inc., Denver, CO).

Immunoblot Analysis. Lysates were subjected to immunoblotting with anti-c-Abl (K-12; Santa Cruz), anti-HSP60 (StressGen, Victoria, BC, Canada), anti-β-actin (Sigma), anti-PCNA (Calbiochem, San Diego, CA), anti-calreticulin (StressGen Biotechnologies, San Diego, CA), anti-calnexin (StressGen Biotechnologies) or anti-LAMP2 (Calbiochem). The antigen-antibody complexes were visualized by enhanced chemiluminescence (enhanced chemiluminescence; Amersham Biosciences).

Isolation of Mitochondria. Mitochondria were purified as described previously (Kumar et al., 2001). Briefly, cells (3×10^6) were washed twice with PBS, homogenized in buffer A (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.4) and 110 μg/ml digitonin in a glass homogenizer (Pyrex 7727-07) and centrifuged at 500*g* for 20 min. Pellets were resuspended in buffer A, homogenized in a small glass homogenizer (Pyrex 7726) and centrifuged at 2000*g* for 5 min. The supernatant was collected and centrifuged at 11,000*g* for 10 min to pellet the mitochondria. The mitochondrial pellet was resuspended in buffer A and loaded onto a continuous Percoll gradient consisted of 30% (v/v) Percoll. After centrifugation at 55,000*g* for 45 min, the mitochondria were collected with a pipette. The mitochondrial fraction was diluted with buffer A and centrifuged at 11,000*g* for 10 min. The mitochondrial pellets were disrupted in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 10 mg/ml leupeptin and aprotinin) at 4°C and then centrifuged at 15,000*g* for 15 min. Protein concentration was determined using a protein estimation kit (Bio-Rad, Hercules, CA).

Analysis of Mitochondrial Membrane Potential. Cells were incubated with 50 ng/ml Rhodamine 123 (Molecular Probes) for 15 min at 37°C. After washing with PBS, samples were analyzed by flow cytometry using 488 nm excitation and measurement of emission through a 575/26 (ethidium) bandpass filter.

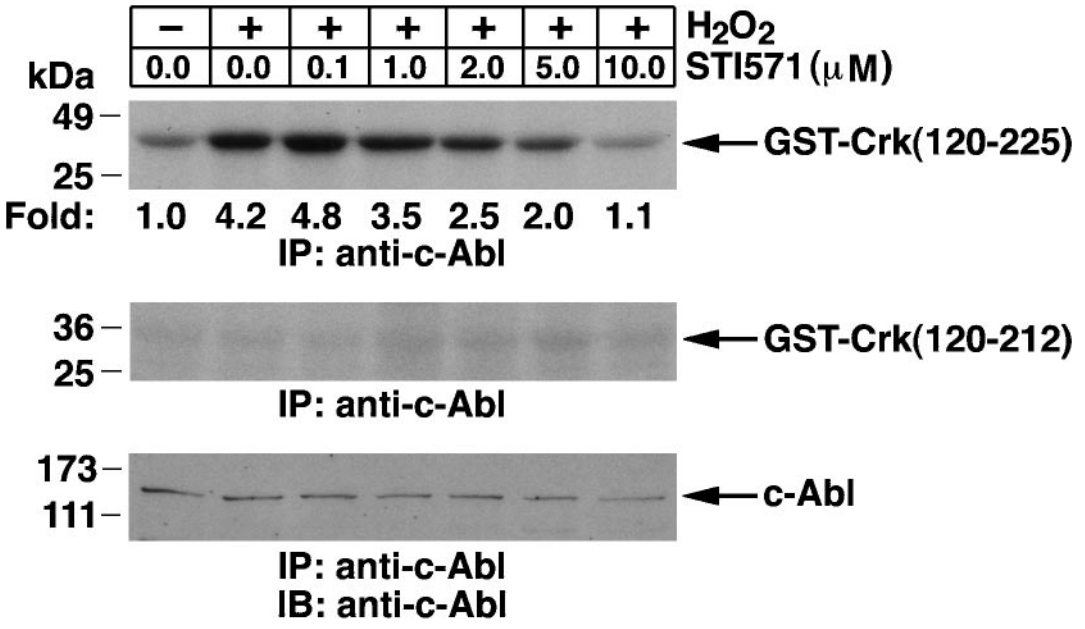

Fig. 1. STI571 blocks c-Abl activation by H_2O_2. MEFs were pretreated with the indicated concentrations of STI571 for 24 h and then with 1 mM H_2O_2 for 15 min. Cell lysates were subjected to immunoprecipitation (IP) with anti–c-Abl. The immunoprecipitates were incubated with [γ-^{32}P]ATP and GST-Crk(120–225) (top) or GST-Crk(120–212) (middle). The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The immunoprecipitates were also subjected to immunoblotting (IB) with anti–c-Abl (bottom). The intensity of the signals in the top were determined by densitometric scanning and the results are presented as fold-change relative to control. Similar results were obtained in three separate experiments.

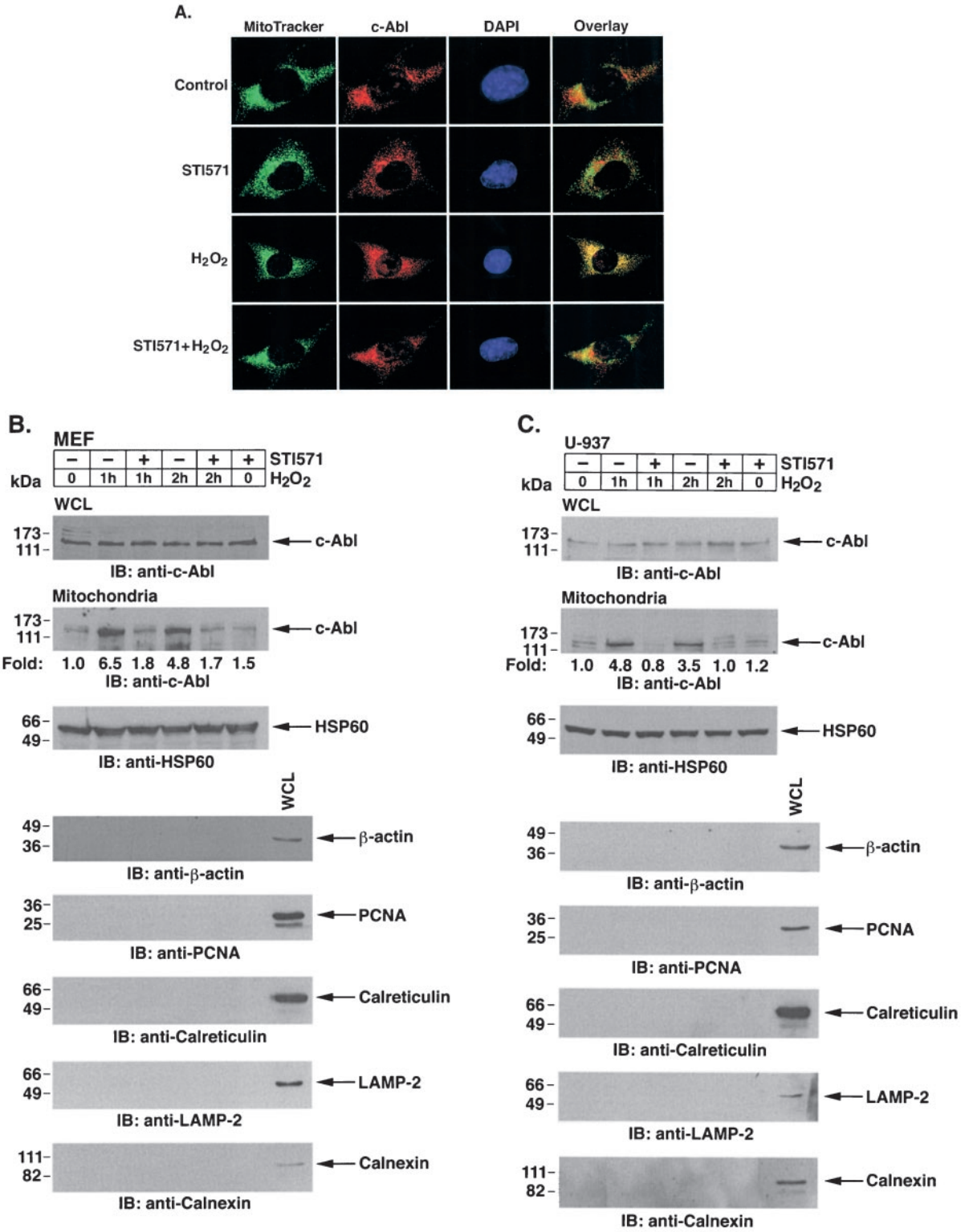

Fig. 2. STI571 blocks mitochondrial targeting of c-Abl in response to H_2O_2 treatment. A, MEFs were treated with 10 μM STI571 for 24 h and then exposed to 1 mM H_2O_2 for 1 h. After washing, the cells were fixed and incubated with anti–c-Abl (K-12; Santa Cruz) followed by Texas Red-conjugated goat anti-rabbit IgG. Mitochondria were stained with Mitotracker Green. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). Cells were visualized by digital confocal immunofluorescence. B, MEFs were treated with 10 μM STI571 for 24 h before adding 1 mM H_2O_2 for the indicated times. Mitochondrial fractions were subjected to immunoblotting with anti–c-Abl, anti-HSP60, anti–β-actin, anti-PCNA, anti-calreticulin, anti-calnexin, and anti-LAMP-2. Whole cell lysate (WCL) was included as controls for expression of the c-Abl and the markers to assess purity of the mitochondrial fraction. Intensity of the signals was determined by densitometric scanning. The results of three separate experiments demonstrate that STI571 decreases H_2O_2-induced mitochondrial targeting of c-Abl by 58.7 $\pm$ 16.2% (mean $\pm$ S.D.). C, human U-937 myeloid leukemia cells were treated with 10 μM STI571 and then 1 mM H_2O_2 for the indicated times. Mitochondrial lysates were analyzed by immunoblotting with the indicated antibodies. Intensity of the signals was determined by densitometric scanning. The results of three separate experiments demonstrate that STI571 decreases H_2O_2-induced mitochondrial targeting of c-Abl by 85.3 $\pm$ 25.4% (mean $\pm$ S.D.).

Apoptosis Assays. DNA content was assessed by staining ethanol fixed cells with propidium iodide and monitoring by FACScan (BD Biosciences).

Annexin V Binding and Nuclear Staining Assays. Annexin V binding and propidium iodide staining was performed using the Annexin-V–FITC apoptosis detection kit (Oncogene Research Products) according to the manufacturer's instructions. The cells were visualized by microscopy.

Results and Discussion

To determine whether STI571 affects ROS-induced signaling, MEFs were pretreated with 0.1 to 10 μM STI571 and then exposed to hydrogen peroxide (H_2O_2). Cell lysates were subjected to immunoprecipitation with anti–c-Abl. The immunoprecipitates were analyzed for phosphorylation of the c-Abl substrate, GST-Crk(120–225). As shown previously (Sun et al., 2000a), the results demonstrate that c-Abl is activated in response to H_2O_2 treatment (Fig. 1, top). Pretreatment with 0.1 and 1.0 μM STI571 had little effect on H_2O_2-induced c-Abl activity, whereas 2 and 5 μM partially blocked c-Abl activation (Fig. 1, top). By contrast, pretreatment with 10 μM STI571 was associated with less c-Abl activity than that found constitutively (Fig. 1, top). As a control, there was no detectable phosphorylation when the anti–c-Abl immunoprecipitates were incubated with GST-Crk(120–212) which lacks the c-Abl phosphorylation site at Tyr-221 (Fig. 1, middle). Immunoblot analysis of the immunoprecipitates with anti–c-Abl demonstrated equal amounts of c-Abl protein (Fig. 1, bottom). Whereas certain drugs can exhibit antioxidant properties (Kagan et al., 2001), the effects of STI571 on intracellular ROS levels was assessed with the fluorochrome DCF-diacetate. The results demonstrate that STI571 has no detectable antioxidant effect on control or H_2O_2-treated cells (data not shown). These findings collectively demonstrate that activation of c-Abl in the oxidative stress response is completely inhibited by 10 μM STI571.

To determine whether mitochondrial targeting of c-Abl in the ROS response is modulated by STI571, we investigated

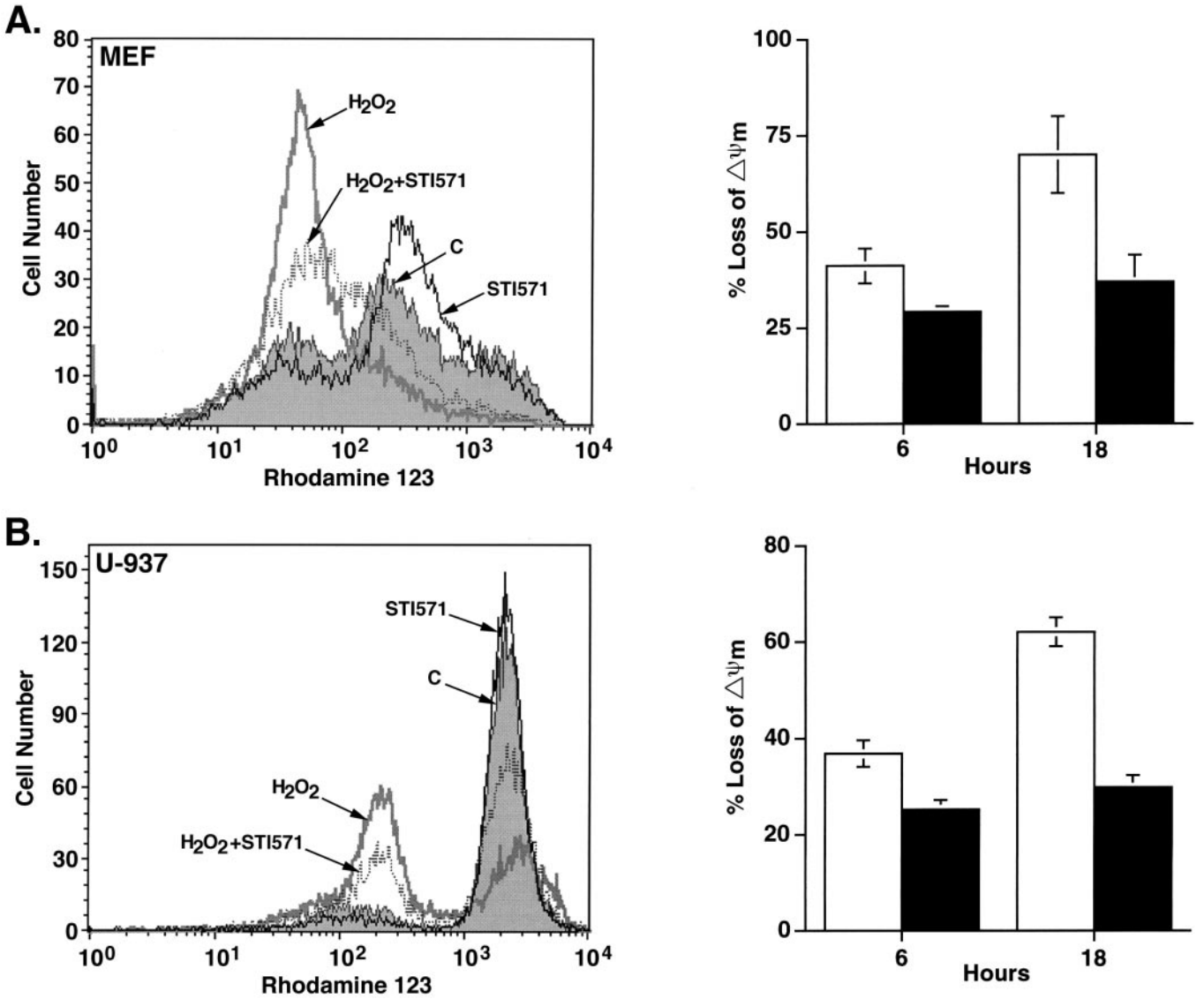

Fig. 3. STI571 attenuates loss of mitochondrial transmembrane potential ($\Delta\psi$m) in reponse to H_2O_2. MEFs (A) and U-937 cells (B) were treated with 10 μM STI571 for 24 h and then exposed to 1 mM H_2O_2 for 6 or 18 h. The cells were stained with Rhodamine 123. Samples were analyzed by flow cytometry (left). Percentage (mean ± S.E.) of control $\Delta\psi$m obtained after treatment with STI571 for 24 h and then H_2O_2 for 6 or 18 h as determined from three separate experiments (right). C, control.

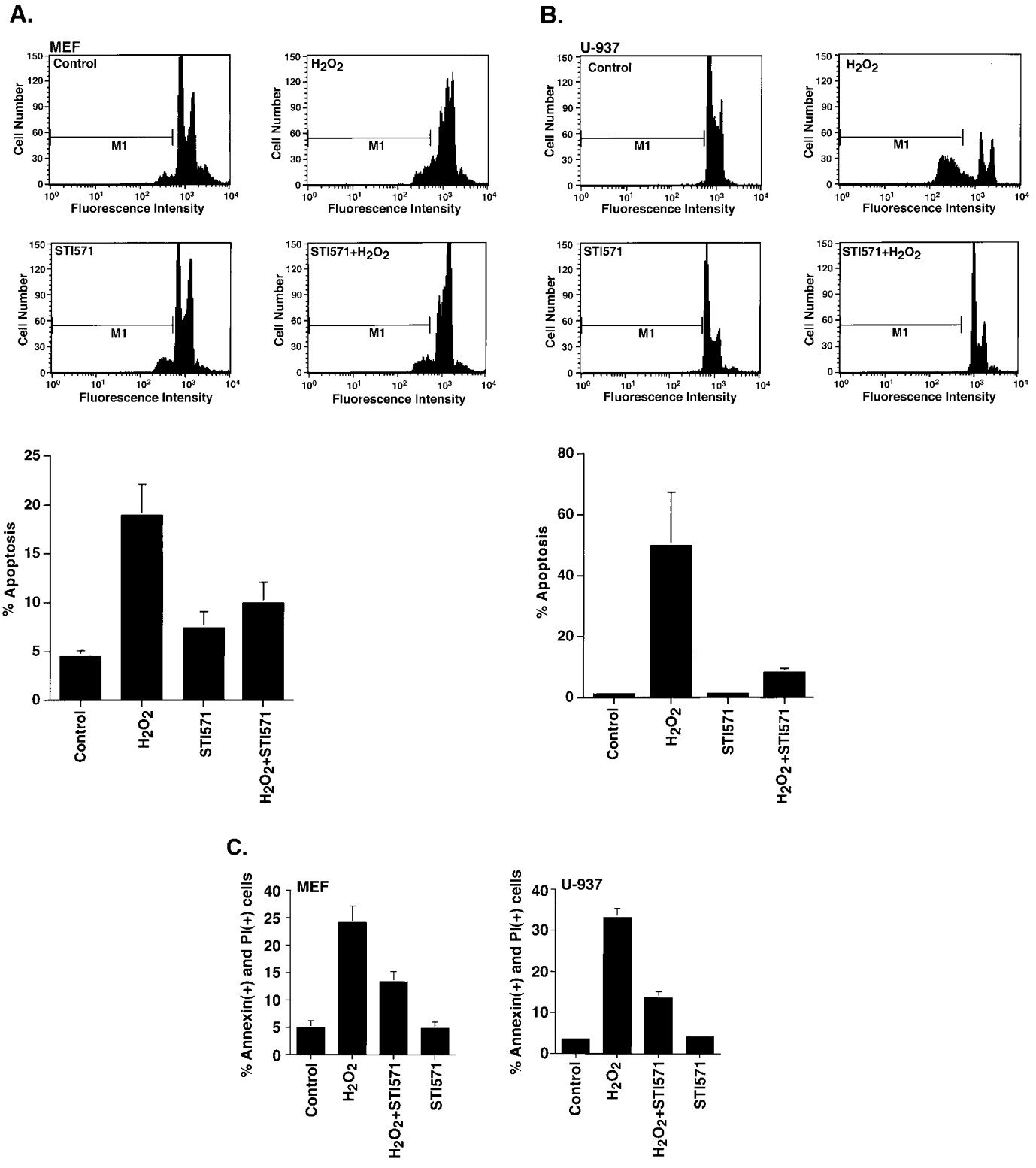

Fig. 4. STI571 inhibits the cell death response to oxidative stress. MEFs (A) and U-937 cells (B) were treated with 10 μM STI571 for 24 h and then exposed to 1 mM H_2O_2 for 18 h. Ethanol-fixed cells were stained with propidium iodide and monitored for sub-G_1 DNA by FACScan (BD Biosciences) (left). Percentage (mean ± S.E.) of cells with sub-G_1 DNA as determined from three separate experiments (right). C, MEFs and U-937 cells were treated with 10 μM STI571 for 24 h and then exposed to 1 mM H_2O_2 for 6 h. The cells were stained with annexin-V–FITC and propidium iodide. The cells were visualized by microscopy. Percentage (mean ± S.E.) of annexin-positive and propidium iodide-positive MEFs (left) and U-937 cells (right) after treatment with STI571 and/or H_2O_2 was determined from three separate experiments.

the subcellular localization of c-Abl by immunofluorescence microscopy. Fluorescence markers obtained with control MEFs showed distinct patterns for c-Abl (red signal) and the mitochondrion-selective dye (MitoTracker; green signal) (Fig. 2A). In concert with constitutive localization of c-Abl to mitochondria (Kumar et al., 2001), the control cells exhibited a low but detectable colocalization of the fluorescence signals (red + green-> yellow/orange) (Fig. 2A). Exposure to H_2O_2 resulted in increased mitochondrial localization of c-Abl that was evident throughout the cell population (Fig. 2A). By contrast, the results show that pretreatment with STI571 blocks H_2O_2-induced mitochondrial targeting of c-Abl (Fig. 2A). To quantitate the effects of STI571, whole-cell lysates and mitochondrial fractions from the treated MEFs were analyzed by immunoblotting with anti-c-Abl. Densitometric scanning of the signals demonstrated a 5- to 6-fold increase in mitochondrial c-Abl protein at 1 and 2 h of H_2O_2 exposure (Fig. 2B). The results further demonstrate that STI571 blocks the H_2O_2-induced targeting of c-Abl to mitochondria and that these agents have no effect on c-Abl levels (Fig. 2B and legend). Immunoblotting for the mitochondrial HSP60 protein demonstrated equal loading of the lanes (Fig. 2B). Purity of the mitochondrial fractions was confirmed by reprobing the blots with antibodies against the cytoplasmic (β-actin) nuclear (PCNA), endoplasmic reticulum (calreticulin and calnexin), and the lysosomal (LAMP2) proteins (Fig. 2B). The demonstration that mitochondrial targeting of c-Abl is blocked by STI571 in H_2O_2-treated U-937 cells (Fig. 2C and legend) and human neuroblastoma cells (data not shown) indicated that this inhibitory effect occurs in diverse cell types.

ROS-induced targeting of c-Abl is associated with loss of mitochondrial transmembrane potential ($\Delta\psi$m) (Kumar et al., 2001). To assess involvement of c-Abl in ROS-induced decreases in $\Delta\psi$m, STI571-pretreated cells were exposed to H_2O_2 and then stained with rhodamine 123. Analysis by flow cytometry demonstrated that H_2O_2 induces loss of $\Delta\psi$m (Fig. 3A, left). Treatment with STI571 alone had no apparent effect on $\Delta\psi$m (Fig. 3A). Moreover, STI571 substantially inhibited the H_2O_2-induced decreases in $\Delta\psi$m (Fig. 3A). The inhibitory effects of STI571 were also detectable at longer periods of H_2O_2 exposure (Fig. 3A). Similar findings were obtained when U-937 cells were pretreated with STI571 and then exposed to H_2O_2 (Fig. 3B).

To assess the effects of STI571 on H_2O_2-induced apoptosis, MEFs were assayed for the appearance of subG1 DNA. The results demonstrate that MEFs respond to H_2O_2 with induction of apoptosis and that treatment with STI571 alone has no apparent effect (Fig. 4A). Pretreatment with STI571 was associated with a substantial block of H_2O_2-induced apoptosis (Fig. 4A). The results show that STI571 decreases H_2O_2-induced apoptosis by over 50% (Fig. 4A). STI571 pretreatment also blocked the apoptotic response of U-937 cells to H_2O_2 (Fig. 4B). In concert with the results in MEFs, STI571 inhibited H_2O_2-induced apoptosis of U-937 cells by 60% (Fig. 4B). One of the early events in apoptosis is the externalization of phosphatidylserine to the outer leaflet of the plasma membrane (Martin and Green, 1995). We have used a phosphatidylserine-binding protein (Annexin-V–FITC) as a specific probe to detect apoptosis-dependent redistribution of this phospholipid. Cells were also counter-stained with propidium iodide (red) to distinguish apoptosis from necrosis based on loss of plasma membrane integrity. The finding that H_2O_2-treated U-937 cells and MEFs were both positive for both annexin-V and propidium iodide indicated that they were in a late stage of apoptosis or necrosis (Fig. 4C). Moreover, pretreatment of both U-937 cells and MEFs with STI571 was associated with a substantial block of H_2O_2-induced apoptosis and necrosis (Fig. 4C).

STI571 (also known as CGP57148B and Gleevec) inhibits Bcr-Abl and c-Abl, and although inactive against other tyrosine and serine/threonine kinases, exceptions are the platelet-derived growth factor receptor and c-kit (Druker et al., 1996; Carroll et al., 1997). The present results demonstrate that STI571 blocks ROS-induced activation of c-Abl. These findings with STI571 were not attributable to an antioxidant effect, as is observed when cells are treated with *N*-acetylcysteine (Roederer et al., 1990; Staal et al., 1990) or pyrrolidine dithiocarbamate (Shi et al., 2000). STI571 exhibits a K_i of ~40 nM for unphosphorylated Abl and a K_i of 7 μM after tyrosine phosphorylation of the Abl activation loop (Schindler et al., 2000; Zimmermann et al., 1997). In the oxidative stress response, c-Abl is activated by a PKCδ-dependent mechanism and phosphorylates PKCδ on tyrosine (Sun et al., 2000a,b). These findings are in concert with the demonstration that 10 μM STI571 is required to completely inhibit H_2O_2-induced c-Abl activation and targeting of c-Abl to mitochondria. The results also demonstrate that STI571 inhibits ROS-induced mitochondrial dysfunction and cell death. Localization of c-Abl to mitochondria is associated with loss of $\Delta\psi$m (Kumar et al., 2001), an event known to precede apoptosis and necrosis. How c-Abl contributes to loss of $\Delta\psi$m is not clear, but should be better understood with identification of the downstream effectors of c-Abl in mitochondria.

The fact that the cell death response to oxidative stress is not completely abrogated by STI571 may be explained by the findings that PKCδ is targeted to mitochondria in c-Abl–deficient cells and that inhibition of PKCδ activation attenuates H_2O_2-induced apoptosis (Majumder et al., 2001). In addition, involvement of topoisomerase II (Li et al., 1999), $p66^{shc}$ (Migliaccio et al., 1999) and the p85 subunit of phosphatidylinositol 3-kinase in the death response to H_2O_2 may be mediated by c-Abl–independent mechanisms. Other studies have demonstrated that STI571 induces apoptosis of cells expressing the constitutively activated Bcr-Abl fusion protein (Druker et al., 1996, 2001). Whereas Bcr-Abl exhibits an antiapoptotic function, inhibition of Bcr-Abl activity by STI571 might be expected to induce apoptosis in this setting. By contrast, c-Abl transduces prodeath signals in response to oxidative stress and the present results demonstrate that inhibition of H_2O_2-induced c-Abl activation by STI571 attenuates this response.

Acknowledgments

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