The BCR/ABL Tyrosine Kinase Induces Production of Reactive Oxygen Species in Hematopoietic Cells*

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Martin Sattler‡, Shalini Verma, Gautam Shrikhande, Christopher H. Byrne, Yuri B. Pride, Thomas Winkler, Edward A. Greenfield, Ravi Salgia, and James D. Griffin

From the Dana-Farber Cancer Institute, Department of Adult Oncology, Harvard Medical School, Boston, Massachusetts 02115

The BCR/ABL oncogene causes chronic myelogenous leukemia, a myeloproliferative disorder characterized by clonal expansion of hematopoietic progenitor cells and myeloid cells. It is shown here that transformation of the hematopoietic cell lines Ba/F3, 32Dcl3, and M07e with BCR/ABL results in an increase in reactive oxygen species (ROS) compared with quiescent, untransformed cells. The increase in ROS was directly due to BCR/ABL because it was blocked by the ABL-specific tyrosine kinase inhibitor STI571. Oxidative stress through ROS is believed to have many biochemical effects, including the potential ability to inhibit protein-tyrosine phosphatases (PTPases). To understand the significance of increased production of ROS, a model system was established in which hydrogen peroxide (H2O2) was added to untransformed cells to mimic the increase in ROS induced constitutively by BCR/ABL. H2O2 substantially reduced total cellular PTPase activity to a degree approximately equivalent to that of pervanadate, a well known PTPase inhibitor. Further, stimulation of untransformed cells with H2O2 or pervanadate increased tyrosine phosphorylation of each of the most prominent known substrates of BCR/ABL, including c-ABL, c-CBL, SHC, and SHP-2. Treatment of the BCR/ABL-expressing cell line M07/p210 with the reducing agents pyrrolidine dithiocarbamate or N-acetylcysteine reduced the accumulation of ROS and also decreased tyrosine phosphorylation of cellular proteins. Further, treatment of M07e cells with H2O2 or pervanadate increased the tyrosine kinase activity of c-ABL. Drugs that alter ROS metabolism or replete PTPases may antagonize BCR/ABL transformation.

Materials and Methods

Cell Culture—The human megakaryocytic cell line M07e was grown in Dulbecco’s modified Eagle’s medium with 20% (v/v) fetal calf serum and 10 ng/ml granulocyte macrophage-colony stimulating factor. The murine pre-B-cell line Ba/F3 and the murine myeloid cell line 32Dcl3 were grown in RPMI 1640 medium with 10% (v/v) fetal calf serum and 10% (v/v) WEHI-conditioned medium (as a source of interleukin-3). Cells transfected with a BCR/ABL cDNA (M07/p210, BaF3/p210, and 32D/p210) were grown in medium without growth factors. In some experiments M07e were deprived of growth factors for 18 h in Dulbecco’s modified Eagle’s medium containing 1% (v/v) bovine serum albumin or Ba/F3, and 32Dcl3 cells were deprived for the same period of time in RPMI 1640 medium containing 0.5% (v/v) bovine serum albumin. Viability of cells was determined by trypan blue exclusion.

Analysis of Intracellular ROS Levels—The relative levels of intracellular ROS were analyzed as described using the redox-sensitive fluorochrome 2′,7′-dichloro-fluorescin-diacetate (Acros Organics, Pittsburgh, PA) (5). In some experiments cells were pretreated with the ABL-specific tyrosine kinase inhibitor STI571 (Novartis), the antioxidants.
phorylated myelin basic protein was concentrated in Centricon 10 tubes (2.5 mCi of [γ-32P]ATP for 30 min. GST or GST-CRKL (2.5 μg) bound to glutathione beads was used as an in vitro substrate for the ABL kinase. The pGEX vector containing full-length CRKL was kindly provided by Dr. J. Groffen (Children's Hospital, UCLA, Los Angeles, CA), and GST or the GST-CRKL fusion protein were prepared as described (15). The immune complexes were subjected to SDS-polyacrylamide gel electrophoresis, transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA), and analyzed by autoradiography using BioMax film (Eastman Kodak Co.).

Viability Assays—The number of viable cells was determined by trypan blue exclusion or annexin V staining (Roche Molecular Biochemicals).

RESULTS

BCR/ABL Is Associated with an Increased Intracellular Oxidative State—We and others have previously shown that activated growth factors increase the intracellular levels of ROS in hematopoietic cells (3–5). In Ba/F3, 32Dc13, and MO7e cells transfected with BCR/ABL the relative ROS levels are also increased, compared with the untransformed cells that were deprived of growth factors. ROS were measured by the fluorochrome 2',7'-dichloro-fluorescin-diacetate (Fig. 1A, upper panel). BCR/ABL-transfected and untransformed cells have equal levels of autofluorescence (Fig. 1A, bottom panel).

To determine if the intracellular ROS levels require BCR/ABL kinase activity, BCR/ABL-transformed Ba/F3, 32Dc13, and MO7e cells were transfected with the ABL kinase inhibitor STI571 (formerly CGP57148B) (1 μM) (16) or solvent alone. STI571 reduced the relative levels of intracellular ROS in all three cell lines compared with Me2SO-treated cells (Fig. 1B, top panel). Although experiments using the ABL kinase inhibitor STI571 support our observation of increased ROS levels in BCR/ABL-transformed cells, it is possible that this inhibitor may have additional unsuspected mechanisms of action. STI571 treatment did not alter the viability or the autofluorescence of the cells during the assay (Fig. 1B, bottom panel).

To demonstrate that the intracellular ROS levels can be manipulated by oxidizing or reducing agents, MO7/p210 cells were treated with the reducing agents PDTC (1 mM) for 3 h or NAC (4 mM) for 30 min and untransformed MO7e cells were treated with the oxidizing reagent H2O2 (1 mM) for 1 h. In each case, there was no loss of cell viability at the end of the experiment. PDTC and NAC reduced the ROS levels of MO7/p210 cells (Fig. 1C, left panel and middle panel), and H2O2 increased the intracellular levels of ROS in MO7e cells (Fig. 1C, top panel) compared with untreated cells. PDTC, NAC, or H2O2 treatment did not alter the autofluorescence of the cells (Fig. 1B, bottom panel).

Because mitochondria are an important source of ROS in many cells, we examined the effects of rotenone, an inhibitor of the electron transfer from complex I to ubiquinone (17). ROS levels were measured 1 h after treatment of MO7e or MO7/p210 cells with 0.1 μM rotenone or with the solvent Me2SO. The relative ROS levels were reduced by an average of 22% in MO7/p210 cells but did not change significantly in MO7e cells (Fig. 1D). These data suggest that increased levels of ROS in MO7/p210 cells, at least in part, depend on the mitochondrial respiratory chain.

The Cellular PTPase Activity Is Decreased by H2O2 and BCR/ABL—ROS such as H2O2 have been implicated in the regulation of PTPase activities (7). Because changes in the level of PTPase activity would be expected to have a significant impact on BCR/ABL transformation (18), we measured cellular PTPase activity in normal and BCR/ABL-transformed cells. Starved MO7e cells were left untreated or treated with H2O2 or pervanadate. The cellular PTPase activity was determined in whole cell lysates of MO7e cells using 32P-labeled myelin basic protein as a substrate and compared with cells transformed with the BCR/ABL oncogene (Fig. 2). Both H2O2 and pervanadate significantly decreased the PTPase activity to 22% and 16% of untreated cells, respectively. BCR/ABL-transformed MO7e cells also showed a significant decrease in PTPase activity to 59% of that of untransformed cells (Fig. 2). In Ba/F3 cells, BCR/ABL transformation was associated with only an 18% decrease in PTPase activity (from 475 fmol of 32P release/mg protein/min in Ba/F3 cells to 391 fmol of 32P release/mg protein/min in BCR/ABL transformed Ba/F3 cells).

ROS Increase Tyrosine Phosphorylation of Cellular Proteins in MO7e Cells—Transformation by BCR/ABL requires constitutive activation of its ABL tyrosine kinase activity (16, 19). Biological effects activated by BCR/ABL are therefore likely to be stimulated through tyrosine phosphorylation of cellular proteins, which in turn are regulated by PTPases. Because BCR/ABL-transformed cells have increased levels of ROS and decreased PTPase activity, we measured the changes in cellular tyrosine phosphorylation induced by ROS, pervanadate, or BCR/ABL. Pervanadate (20 μM vanadate and 500 μM H2O2) increased tyrosine phosphorylation of cellular proteins in growth factor-deprived MO7e cells (Fig. 3A, right panel). At the concentration used to generate pervanadate, neither vanadate nor peroxide increased tyrosine phosphorylation when used alone (data not shown). When MO7e cells were exposed to 5 mM H2O2, the tyrosine phosphorylation pattern was similar to that of cells treated with pervanadate and also to that of BCR/ABL-transformed cells (Fig. 3A, left panel).

The apparent similarities in tyrosine phosphorylation patterns of H2O2, pervanadate, and BCR/ABL were further explored by specifically looking at tyrosine phosphorylation of several known BCR/ABL substrates. H2O2 and pervanadate induced tyrosine phosphorylation of c-CBL, SHC, SHP-2, and...
c-ABL, although c-CBL was partially tyrosine-phosphorylated in unstimulated cells (Fig. 3B).

The Antioxidants PDTC and NAC Decrease Tyrosine Phosphorylation of Cellular Proteins in MO7/p210 Cells—ROS-dependent tyrosine phosphorylation of cellular proteins can be decreased by reducing agents such as PDTC and NAC (5, 9, 20). PDTC treatment of MO7/p210 cells gradually reduced tyrosine phosphorylation of cellular proteins over a 60-min time period (Fig. 4A, left panel). A significant reduction in tyrosine phosphorylation was observed for several, but not all, proteins with an apparent molecular mass between 210 and 55 kDa. PDTC also reduced tyrosine phosphorylation of cellular proteins in the Ph positive cell line K562, but had no effect on the Ph positive cell line BV173 under these conditions (data not shown). We next tested if NAC also decreases cellular tyrosine phosphorylation. MO7/p210 cells were left untreated or treated with NAC for 10 min. NAC was not cytotoxic under these conditions, and the cells were fully viable at the end of the experiment. Treatment of MO7/p210 cells with 4 mM NAC significantly reduced cellular tyrosine phosphorylation but had little effect at lower concentrations (Fig. 4A, right panel). The effect of PDTC on known BCR/ABL substrates that were shown before to be increased tyrosine phosphorylated by H2O2 and pervanate (Fig. 3B) was examined in MO7/p210 cells. PDTC decreased tyrosine phosphorylation of the BCR/ABL substrates c-CBL, SHP-2, and SHC (Fig. 4B). It is also possible that in addition to BCR/ABL, other tyrosine kinases, previously activated by BCR/ABL, are also reduced in their activity. This could be either a result of decreased BCR/ABL activity or a result of direct inhibition by PDTC and NAC. Overall, these data suggest that reducing agents like NAC and PDTC can decrease intracellular tyrosine kinase activity in BCR/ABL transformed cells.

BCR/ABL in Vitro Tyrosine Kinase Activity Is Reduced in PDTC-treated MO7/p210 Cells—Reduced tyrosine phosphorylation of BCR/ABL substrates is consistent with activation of cellular PTPases, but could also be because of reduced BCR/ABL kinase activity. To address this issue, an in vitro kinase assay was performed using anti-ABL immune complexes from cell lysates of MO7/p210 cells that were left untreated, PDTC- (1 mM) treated, and PDTC-treated with subsequent pervanate treatment. These immune complexes specifically phosphorylate the substrate GST-CRKL but not GST using lysate from untreated MO7/p210 cells (Fig. 5, top left). The in vitro kinase activity in anti-ABL immunoprecipitates and the BCR/ABL phosphorylation were reduced after PDTC treatment (Fig. 5, top right), and this correlated with the reduced phosphorylation of cellular proteins shown in Fig. 4B. It is possible that the
detected reduction in ABL kinase activity was also in part due to reduced c-ABL kinase activity. However, in the absence of ionizing radiation, the level of c-ABL kinase activity is thought to be extremely low (21).

Because both H$_2$O$_2$ and pervanadate increase the tyrosine phosphorylation of c-ABL in intact MO7e cells, we also measured c-ABL kinase activity in vitro in cellular lysates from untransformed cells. The in vitro kinase activity toward GST-CRKL in ABL immune complexes was found to be elevated by H2O2 as well as pervanadate treatment of MO7e cells (Fig. 5, bottom panel). These results suggest that inactivation of PT-Pases alone is sufficient to activate ABL kinase activity. In addition, we used a recombinant ABL kinase domain and measured its phosphorylation in the presence or absence of 1 mM PDTC and 5 mM H2O2. We did not find significant changes in the phosphorylation of ABL (data not shown), suggesting that PDTC and H$_2$O$_2$ are unlikely to directly modulate ABL kinase activity. Thus, these data favor a mechanism whereby cellular ROS levels alter activity of one or more PTPases, which then regulate the kinase activity of both BCR/ABL and c-ABL.

**DISCUSSION**

BCR/ABL mimics some signaling events induced by activated growth factor receptors (1). Recent evidence suggests that activation of growth factor receptors coincides with an increase of intracellular ROS levels, including the receptor for platelet-derived growth factor (3), transforming growth factor-β (4), or granule macrophage-colony stimulating factor (5). In addition, antioxidants or antioxidant enzymes have been shown to reduce tyrosine phosphorylation events in cells stimulated with these growth factors. ROS are thought to signal, at least in part, through the inactivation of PTPases (7). We have demonstrated that BCR/ABL is associated with increased levels of ROS in three different hematopoietic cell lines compared with their nontransformed parental cell lines. In these un-
Fig. 5. Oxidative stress regulates ABL kinase activity. BCR/ABL and c-ABL in vitro kinase activity was measured in ABL immunoprecipitates using GST-CRKL as a substrate or GST alone as a control. Anti-ABL immunoprecipitates of MO7/p210 cells \((20 \times 10^6)\) were used for in vitro kinase assays. Lysates were prepared from untreated cells (top left panel) or untreated and PDTC treated cells (top right panel). Also, anti-ABL immunoprecipitates of MO7e cells \((20 \times 10^6)\) that were left untreated (CTRL) or treated with \(\mathrm{H}_2\mathrm{O}_2\) (PEROX) or with pervanadate (PERVER) were used for in vitro kinase assays (bottom panel). Phosphorylation of the in vitro substrate GST-CRKL was visualized by autoradiography.

Transformed cell lines, increasing ROS using exogenous \(\mathrm{H}_2\mathrm{O}_2\) inhibited PTPase activity and dramatically increased tyrosine phosphorylation of cellular proteins in MO7e cells in a pattern similar to that of the known PTPase inhibitor pervanadate. Consistent with a potential role of ROS in BCR/ABL signaling, we also found reduced PTPase activity in MO7/p210 cells. Further, BCR/ABL-induced tyrosine phosphorylation was inhibited by the addition of a reducing agent, PDTC. Finally, ABL kinase activity was shown to be regulated by ROS, suggesting that ROS play an important role in BCR/ABL-induced transformation. Thus, these data suggest a model in which BCR/ABL signaling is amplified by simultaneous reduction in the activity of one or more PTPases.

It is not known how BCR/ABL modulates the levels of ROS. We have demonstrated that inhibition of the mitochondrial respiratory chain by rotenone significantly decreased intracellular ROS levels. This suggests that mitochondria are an important source for ROS in BCR/ABL-transformed cells. In addition, it is also possible that BCR/ABL affects the protein levels or enzymatic activities of one or more enzymes that regulate ROS. There is considerable precedent for BCR/ABL affecting the level of intracellular enzymes. For example, BCR/ABL is known to markedly reduce the expression of leukocyte alkaline phosphatase (22), increase the level of PTP1B (23), and decrease the polyinositol 5-phosphatase SHIP (24). One obvious candidate would be catalase, but in experiments not shown BCR/ABL did not alter catalase levels in MO7e cells as measured by immunoblotting.

The growth promoting effect of ROS and the growth-inhibiting effect of antioxidants are of interest. The available data suggest that the mechanism involves enhanced tyrosine phosphorylation, possibly by inhibiting one or more PTPases. ROS can regulate protein function in part through oxidation of redox-sensitive cysteine residues in some proteins. For example, oxidation of Cys\(^{118}\) in RAS is known to activate its GTPase activity (25). Also, PTPases contain a redox-sensitive cysteine residue in their active site that must be in the reduced state for full enzyme activity (7). Recent work implies that ROS can modulate the function of protein kinases as well as PTPases. For example, the phosphorylation of cellular proteins can be increased by increasing kinase activities, decreasing PTPase activities, or both. This is of special interest because inhibition of PTPases through redox modulation would augment transformation. ROS have been demonstrated to activate signaling pathways by inducing tyrosine phosphorylation of cellular proteins, including FAK (26), SHC (27), or LCK (28). Suh et al. (29) have recently demonstrated that increased production of ROS can lead to a transforming phenotype. Overexpression of the superoxide-generating NADPH oxidase Mox1 in NIH3T3 fibroblasts increases cell growth and induces tumors in athymic mice (29). However, the exact mechanism of ROS action has not been entirely elucidated.

We have demonstrated that the PTPase activity in untransformed cells is decreased by \(\mathrm{H}_2\mathrm{O}_2\) and upon BCR/ABL transformation. We have also shown that PDTC inhibits BCR/ABL autokinase activity and reduces the total cellular tyrosine phosphorylation. It is possible that this effect is mediated in part by activation of PTPases. It will therefore be of interest to determine if BCR/ABL is not only involved in generating ROS but is also regulated by redox-sensitive PTPases itself. Likely candidates that would regulate BCR/ABL function are PTPases that are known to interact with the BCR/ABL kinase such as SHP-1 (30), SHP-2 (31), or PTP1B (23). The high level of tyrosine phosphorylation of SHP-1 (30) and SHP-2 (31) in BCR/ABL-expressing cells demonstrates an imbalance between kinase and PTPase activities.

Our data demonstrating a decrease in PTPase activity in BCR/ABL-transformed MO7e cells compared with untransformed cells are consistent with the known reduced alkaline phosphatase activity in CML cells (32). Both in vitro activities overlap because PTPase activity as well as alkaline phosphatase activity can be measured with the same substrates, including tyrosine-phosphorylated peptides and \(p\)-nitrophenyl-phosphate (22, 33, 34). It will be important to identify the specific PTPases that contribute to the observed reduction in PTPase activity and evaluate their response to changes in the cellular redox status. In contrast to the data presented here, LaMontagne et al. (18) have reported an increase in PTPase activity in BCR/ABL-transformed RAT1 fibroblasts compared with untransformed cells. The increase in PTPase activity was solely contributed to a severalfold increase in PTP1B protein expression (23). However, the increase in PTP1B expression was not maintained when subclones of BCR/ABL expressing RAT1 fibroblasts were examined (18). We did not detect significant BCR/ABL-dependent PTP1B induction in three different hematopoietic cell lines, including MO7e, Ba/F3, and 32Dc13 cells, or a Ba/F3 cell line with BCR/ABL expression under the control of a doxycycline inducible promoter (data not shown). It is possible that the measured differences in the amount of PTPase activity are because of the different PTPase substrates used for analysis.

Of particular relevance to human CML is the fact that an increase in ROS could also have long term consequences for genetic stability. ROS levels are not only quenched by enzymes, antioxidants, sulfydryl groups, but also by reacting with DNA bases (35). ROS can modify the DNA bases adenine, guanine, thymidine, or cytosine and lead to derivatives such as 5-formamido-4,6-diamino-pyrimidine, 8-hydroxy-guanine, thy-
mine glycol, and 5-hydroxy-caffeine, respectively (36). Although these modifications can be efficiently removed by DNA repair mechanisms, a persistent increase in ROS could lead to accumulation of mutations, and ROS have previously been linked to tumor induction as a result of tobacco smoke (37–39). In the CML stable phase, ROS therefore have the potential to contribute to the progression of CML.

In any case, it is likely that further characterization of redox-sensitive PTPases and other proteins will be helpful in understanding the signaling of BCR/ABL and in particular its mechanism of transformation. Our studies, using antioxidants to reduce the kinase activity of BCR/ABL and reducing the cell growth of BCR/ABL expressing cells point at new targets of drug treatment of CML. Ideally, drugs that would be efficient in CML treatment would lower ROS through targeting directly the ROS-producing enzymes or would supplement the antioxidant potential of cells. Such drugs could be used in combination with BCR/ABL kinase inhibitors such as STI571 to block BCR/ABL signaling and thus progression of the disease.

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