BCR/ABL translocates to the nucleus and disrupts an ATR-dependent intra-S phase checkpoint

Jamil Dierov, Raia Dierova, and Martin Carroll*

Division of Hematology and Oncology, University of Pennsylvania, Philadelphia, Pennsylvania 19104
*Correspondence: carroll2@mail.med.upenn.edu

Summary

Chronic myelogeneous leukemia (CML) is a two-stage disease associated with expression of the BCR/ABL tyrosine kinase protein. However, whether BCR/ABL expression directly causes blast crisis, and if so by what mechanism, is unknown. We have found that BCR/ABL translocates from the cytoplasm to the nucleus after genotoxic stress. Furthermore, BCR/ABL increases DNA double-strand damage after etoposide treatment and leads to a defect in an intra-S phase checkpoint, causing a radioresistant DNA synthesis (RDS) phenotype. In the nucleus, BCR/ABL associates with the ataxia-telangiectasia and rad 3-related protein (ATR) and disrupts ATR-dependent signal transduction. Overexpression of ATR in a BCR/ABL-expressing cell line corrects the DNA damage phenotype. These results demonstrate a nuclear role for BCR/ABL in altering the cellular response to DNA damage.

Introduction

Chronic phase CML is a clonal, hyperproliferative disease with an overproduction of white blood cells that retain the capacity to differentiate. However, all patients with CML will advance in 3–5 years to blast crisis, which is characterized by a block in differentiation and resistance to chemotherapy. Blast crisis is associated with the acquisition of additional genetic damage including chromosomal translocations and deletions (Ahuja et al., 1989; Griesshammer et al., 1997). Whereas recent work has established that the BCR/ABL oncogene is sufficient to cause chronic phase CML (Daley et al., 1990; Druker et al., 2001; Pear et al., 1998), there is relatively little information about why CML cells inevitably evolve to blast crisis. BCR/ABL is a well-studied tyrosine kinase oncogene that stimulates dysregulated cellular proliferation (Salesse and Verfaillie, 2002). The translocation leads to fusion of an oligomerization domain of BCR with the kinase and signaling domains of the c-ABL protein. Work from many groups has shown that BCR/ABL is a constitutively oligomerized and activated tyrosine kinase that activates multiple signal transduction pathways including STAT, Ras, and PI3 kinase (Gishizky and Witte, 1992; Konopka et al., 1984). However, activation of cytoplasmic signaling proteins does not appear to explain the progression to blast crisis by itself.

Previous work on the role of BCR/ABL in inducing blast crisis has been limited because of the absence of a reproducible animal model of blast crisis. It has been proposed that the increased growth rate of CML stem cells would lead to an increase in the accumulation of DNA damage; however, the increase in the growth rate of CML versus normal hematopoietic stem cells is minimal, suggesting that this explanation is inadequate. Deutsch et al. (2001, 2003) recently suggested that stable expression of BCR/ABL alters the expression of the DNA repair enzyme, DNA-PKcs, a PIKK family kinase. Interestingly, the authors found that the human cell line, UT-7, expressing transduced BCR/ABL, had increased chromosomal aberrations compared to nontransduced cells after γ-irradiation. This result is consistent with the hypothesis that BCR/ABL induces gross chromosomal instability, but a mechanism whereby BCR/ABL would affect DNA-PKcs expression was not proposed. Another group, using a yeast two-hybrid approach, found that BCR/ABL can associate with the xeroderma pigmentosum group B protein (XPB), but XPB deficiency leads to a nucleotide excision repair defect that would lead to the formation of point mutations, not chromosomal translocations (Takeda et al., 1999). Interestingly, Slupaniek et al. have studied whether BCR/ABL directly leads to the chemoresistance seen in blast crisis. They have shown that BCR/ABL upregulates STAT5 signaling, leading to increased expression of Rad51 and a modification of homologous recombination (HR) repair (Slupianek et al., 2001). The authors have proposed that BCR/ABL-expressing cells repair their DNA damage more rapidly than control cells and that this results in chemoresistance. It is unclear if the described increased rate of homologous recombination repair is "error prone," leading

SIGNIFICANCE

CML is a two-stage disease in which the initial hyperproliferative phase is followed by a second lethal phase designated blast crisis. Extensive work has demonstrated that the BCR/ABL oncogene directly causes increased proliferation of cells and leads to myeloid hyperproliferation. However, it has never been clear why all patients with CML progress to blast crisis. Here, we show that the BCR/ABL oncogene has a second previously undescribed mechanism. After DNA damage, BCR/ABL moves from the cytoplasm to the nucleus. In the nucleus, BCR/ABL binds to a critical cell cycle checkpoint regulator, ATR, and blocks its function, leading to increased DNA damage in the BCR/ABL-expressing cells. This impaired DNA repair may cause BCR/ABL-expressing cells to accumulate further genetic damage, leading to blast crisis.

to genomic instability. Thus, although several suggestions have been made about how BCR/ABL could induce chromosomal instability, it is still unclear if BCR/ABL itself is responsible for chromosomal instability leading to blast crisis.

An alternative approach to understanding the oncogenic role of BCR/ABL has been proposed based on the cellular homolog of BCR/ABL, c-ABL. c-ABL is a tyrosine kinase that usually resides in the nucleus but can translocate to the cytoplasm where it may regulate cell adhesion (Taagepera et al., 1998; Van Etten et al., 1989; Wetzler et al., 1993). In the nucleus, c-ABL is activated in response to DNA damage. After DNA damage, c-ABL binds to the ataxia-telangiectasia mutant (ATM) protein as well as to the p53 homolog, p73 (Agami et al., 1999; Baskaran et al., 1997; Gong et al., 1999; Kharbanda et al., 1998; Shafman et al., 1997; Wang, 2000; White and Prives, 1999). c-ABL can phosphorylate p73 and promote apoptosis after DNA damage, although how essential a role this plays in the DNA damage response is unclear. It has been postulated that BCR/ABL may usurp or disrupt some of the functions of c-ABL but this has never been documented. BCR/ABL, which is normally localized in the cytoplasm (Wetzler et al., 1993), can be found in the nucleus after treatment of cells with the ABL kinase inhibitor, STI571, and the nuclear export inhibitor, leptomycin B (LMB) (Vigneri and Wang, 2001), but the physiologic significance of these conditions is unclear. In particular, there has been no previous demonstration of a direct role of BCR/ABL in modifying nuclear signal transduction pathways.

In order to study the mechanism whereby BCR/ABL could affect DNA damage and repair, we have used the tetracycline-inducible BCR/ABL-expressing cell line Ba/F3 pTET-ON p210 (Klucher et al., 1998). The use of a tetracycline-inducible system allows for comparison of isogenic cells that do or do not express BCR/ABL. We have examined cells primarily after genotoxic stress induced by the topoisomerase 2 inhibitor etoposide in order to amplify possibly small differences in the response to DNA damage. Etoposide has been recently found to induce an ataxia-telangiectasia and rad 3-related (ATR) dependent DNA repair process (Costanzo et al., 2003). Here we report that BCR/ABL-expressing cells have an increase in DNA double-strand breaks and a radioresistant DNA synthesis (RDS) phenotype that results from disruption of the intra-S phase checkpoint after treatment with etoposide. Furthermore, BCR/ABL translocates rapidly to the nucleus after etoposide-induced DNA damage. Translocation is completely reversible after removal of the DNA-damaging agent. In the presence of etoposide, BCR/ABL, like c-ABL, associates with ATM but does not disrupt the function of ATM. In contrast, BCR/ABL disrupts the function of the ATM homolog, ATR, as characterized by the lack of phosphorylation of Chk1 and by the inability of BCR/ABL-expressing cells to inactivate the cdc7/Dbf4 regulatory kinase after treatment with etoposide. Overexpression of ATR in a BCR/ABL-expressing cell line leads to the appropriate activation of Chk1 after DNA damage and a block in the DNA damage phenotype induced by BCR/ABL. Taken together, these results show that BCR/ABL expression inhibits activation of ATR after DNA damage and alters the cellular response to DNA damage.

Results

BCR/ABL increases DNA double-strand breaks

To determine how BCR/ABL would affect the induction of DNA damage and the regulation of DNA repair, we have used Ba/F3 pTET-ON p210 cells (a kind gift of Dr. George Daley, MIT, Cambridge, MA) (Klucher et al., 1998). We confirmed (Figure 1A) that exposure of cells to doxycycline induces expression of p210 BCR/ABL over 24 hr and that cells grew at nearly equivalent rates in the presence of interleukin 3 (IL3) or doxycycline (Figure 1B). Initially, we used cell survival as a surrogate for DNA damage. In general, in cells with equivalent apoptotic responses, increased DNA damage leads to increased apoptosis. However, cells grown in IL3 or in doxycycline for at least 24 hr showed only minor differences in cytotoxicity after treatment with several chemotherapeutic agents (Figure 1C and data not shown). With etoposide, as shown, there is a statistically significant enhancement of toxicity in BCR/ABL-expressing cells, but it is unclear if this difference is of major biologic significance, as it changes the level at which 50% of the cells are killed (ED50) by less than 1 μM. Cells deprived of IL3 and doxycycline showed increased cell death as expected (Figure 1B, open boxes). In order to examine DNA damage directly, we next used the comet assay (Singh et al., 1988). The comet assay is a single cell gel electrophoresis assay. After treatment with a DNA-damaging agent, cells are implanted in agarose and lysed in alkaline lysis buffer that both disrupts the cell membrane and allows damaged DNA to uncoil. Cells are then placed in an electric field and damaged DNA migrates, forming a "tail." The undamaged DNA does not migrate, forming the comet "head." The length and intensity of the comet tail is proportional to the amount of DNA damage (Figure 1D). Cells were incubated with etoposide for varying periods of time as shown (Figure 1E). At indicated time points, cells were harvested and processed for analysis using the comet assay. Olive tail moment (% DNA in comet tail $\times$ distance of center of gravity of DNA from the head) was calculated for 50–100 cells and results averaged. Ba/F3 pTET-ON p210 cells growing in IL3 alone (white bars) had a time-dependent increase in DNA damage (Figure 1E) while in the presence of etoposide. After a 2 hr exposure to etoposide, cells were washed and replated in media free of drug for 90 min. The majority of the DNA damage was repaired within 90 min (Figure 1E, repair), and all DNA double-strand breaks were resolved by 24 hr (data not shown). When cells were treated with doxycycline to induce BCR/ABL in the absence (striped bars) or presence (black bars) of IL3, the amount of DNA double-strand breaks was increased at each time point. Curiously, despite this increased DNA damage, BCR/ABL-expressing cells were able to repair DNA damage quickly and completely (Figure 1E, repair), suggesting that the role of BCR/ABL in altering DNA repair is reversible. Overall, this experiment demonstrates that BCR/ABL expression leads to increased DNA damage after treatment of cells with etoposide.

To compare BCR/ABL-induced effects on DNA double-strand breaks to known mutants with an effect on DNA repair, we have used fibroblast cell lines (Figure 1F). DNA double-strand breaks were again induced by etoposide. We compared NIH 3T3 cells to 3T3 cells stably expressing BCR/ABL (3T3 BCR/ABL) and to ATM-deficient fibroblasts. As expected, etoposide induced DSB in NIH 3T3 cells and these breaks were rapidly repaired (Figure 1F, white bars). ATM-deficient fibroblasts suffered increased DNA damage from the same dose of etoposide, as expected (Figure 1F, gray bars). DNA repair was still rapid in ATM-deficient cells, and all of the DNA damage was repaired by 24 hr after this short treatment with etoposide. Consistent with the results above, BCR/ABL-expressing cells incurred sig-

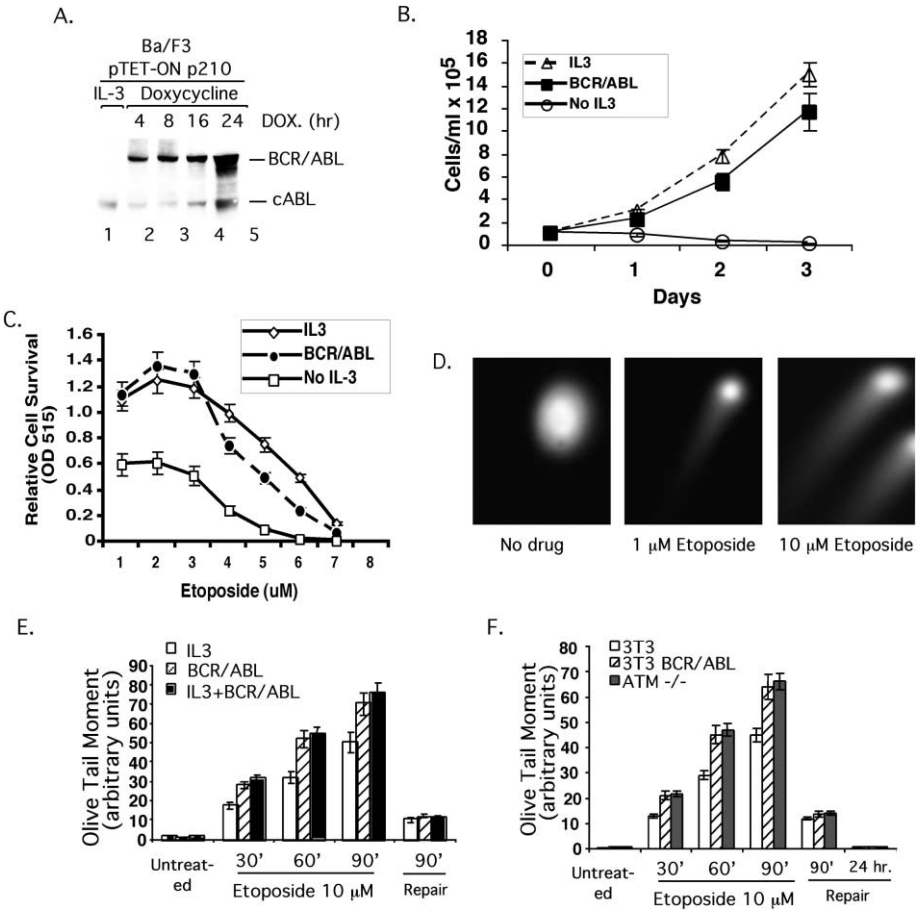

Figure 1. BCR/ABL increases DNA double-strand breaks

A: Ba/F3 pTET-ON p210 cells were grown in IL3 alone (lane 1) or washed free of IL3 and grown in doxycycline alone for the indicated times. Cells were lysed and lysates analyzed by Western blot for the presence of BCR/ABL.

B: Cells were again grown in IL3 (open triangle), in doxycycline to induce BCR/ABL (closed squares), or in the absence of IL3 and doxycycline (open circles). Cell number was determined by trypan blue exclusion.

C: Ba/F3 pTET-ON p210 cells were incubated in the presence of IL3, the presence of doxycycline (BCR/ABL) or in the absence of IL3 and doxycycline. Cells were treated with varying concentrations of etoposide for 48 hr and relative cell survival assayed using the XTT assay. Data in **B** and **C** is from 3 separate experiments.

D: Ba/F3pTET-ON p210 cells were treated for 1 hr with no drug or etoposide at indicated concentration and analyzed as described in Experimental Procedures. Photomicrographs of individual cells stained with propidium iodide are shown.

E: Ba/F3 pTET-ON p210 cells were grown under indicated conditions (BCR/ABL), treated with etoposide, and analyzed using the comet assay. For each condition, 50–100 cells were analyzed and mean ± standard deviation calculated.

F: 3T3 cells, 3T3 p210, or ATM-deficient cells were treated with etoposide for indicated periods of time and analyzed by the comet assay. Olive tail moment was measured by Komet 7 software (Kinetic Imaging, Germany) for **E** and **F**. The arbitrary units are the same for both **E** and **F**.

nificantly more DNA damage than 3T3 cells (Figure 1F, striped bars). Interestingly, the amount of DNA damage incurred was comparable to ATM-deficient cells. These results demonstrate that the increase in DNA double-strand breaks induced by BCR/ABL is not restricted to hematopoietic cells and that the extent of the phenotype is comparable to cells with a deficiency in ATM, a known tumor suppressor.

BCR/ABL disrupts an intra-S phase checkpoint and causes a RDS phenotype

To understand the mechanism whereby BCR/ABL could disrupt DNA repair, we examined the effect of BCR/ABL expression on cell cycle regulation after treatment of cells with etoposide. Ba/F3 pTET-ON p210 cells were again grown in IL3 or treated with doxycycline to induce BCR/ABL. Cells were treated with a continuous exposure to etoposide for varying lengths of time and analyzed for cell cycle distribution (Figure 2A). Both IL3-stimulated cells and BCR/ABL-expressing cells demonstrated a G2/M cell cycle arrest between 6 and 18 hr after treatment with etoposide (Figure 2A), consistent with an intact G2/M cell cycle checkpoint. However, this arrest was seen several hours earlier in BCR/ABL-expressing cells (compare closed circles to open circles), suggesting the possibility of a defective S phase checkpoint function in BCR/ABL-expressing cells, allowing cells to pass through S phase more quickly than control cells. To determine if BCR/ABL-expressing cells had a defect in an intra-S phase checkpoint, we studied fibroblasts and hematopoietic cells using the radioresistant DNA synthesis (RDS) assay. The RDS assay was originally described as an assay for disruption of the ataxia-telangiectasia mutant (ATM) protein but has recently been found to be an assay for disruption of an intra-S phase checkpoint (Painter and Young, 1980; Sorensen et al., 2003). Cells were initially labeled with ^{14}C thymidine to provide a control for DNA content. Cells were rested, treated with a genotoxic agent, and then labeled with tritiated (^{3}H) thymidine to determine the amount of ongoing DNA synthesis after DNA damage. Results are displayed as the ratio of $^{3}H/^{14}C$ compared to untreated cells, which are arbitrarily defined as 100% (Figure 2B, black bars). As shown in Figure 2B, Ba/F3 pTET-ON p210 cells growing in IL3 show decreased DNA synthesis after treatment with γ-irradiation (white bars) or etoposide (striped bars), consistent with an intact S phase checkpoint. However, when BCR/ABL is induced, the same cells show continued DNA synthesis after DNA damage, showing that BCR/ABL expression disrupts the intra-S phase checkpoint leading to a RDS phenotype (Figure 2B, right side). To compare this phenotype to cells deficient in ATM, which have a known RDS phenotype, we studied fibroblast cells (Figure 2C). NIH 3T3 fibroblasts showed decreased DNA synthesis after treatment with γ-irradiation. In contrast, neither 3T3 cells expressing BCR/ABL nor ATM-deficient fibroblasts showed an appropriate decrease in DNA synthesis, demonstrating that BCR/ABL induces a phenotype similar to ATM-deficient cells. These results demonstrate that BCR/ABL disrupts an intra-S phase checkpoint.

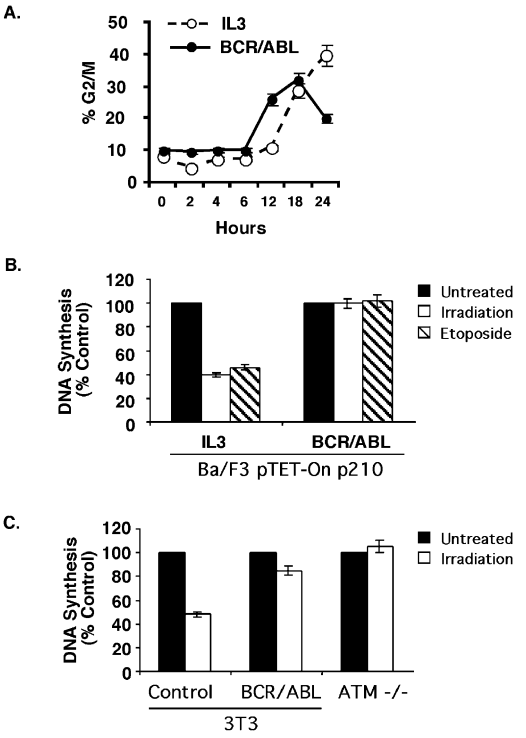

Figure 2. BCR/ABL induces radioresistant DNA synthesis

A: Ba/F3 pTET-ON p210 cells growing in IL3 (open circles) or doxycycline to induce BCR/ABL (closed circles) were plated in fresh media containing 10 μM etoposide for indicated periods of time. Cells were fixed and stained with propidium iodide and percent of cells in phases of cell cycle determined on a FACStar using the ModFit software.

B: RDS assay was performed as described in methods on Ba/F3 pTET-ON p210 cells growing in IL3 or doxycycline (BCR/ABL). Cells were either untreated (black bars) or treated with 20 Gy γ-irradiation (white bars) or 10 μM etoposide (striped bars).

C: RDS assay was performed as described in methods on NIH 3T3 cells, NIH 3T3 cells stably expressing p210 BCR/ABL (BCR/ABL), or ATM-deficient cells, GM02052 ($ATM^{-/-}$). Untreated cells (black bars) are compared to cells treated with 10 Gy γ-irradiation.

BCR/ABL translocates to the nucleus after DNA damage

A critical question raised by the experiments above was how BCR/ABL could affect DNA repair or cell cycle regulation when it is sequestered in the cytoplasm. Either BCR/ABL could act indirectly through affecting signal transduction proteins that move to the nucleus (such as STAT proteins), or BCR/ABL could act directly by translocating to the nucleus after DNA damage. Recent results suggested that BCR/ABL could move to the nucleus (Vigneri and Wang, 2001); therefore, we examined localization of BCR/ABL after treatment of cells with etoposide (Figure 3). Analysis of cells by immunofluorescence, 1–4 hr after treatment with 10 μM etoposide, showed consistent and complete translocation of BCR/ABL from the cytoplasm to the nucleus (compare Figures 3A and 3D). To eliminate background staining from endogeneous c-ABL, we then examined $ABL^{-/-}$ fibroblasts (Figures 3B, 3C, 3E, and 3F). Analysis of untransfected cells confirmed the absence of c-ABL (Figure 3C). Cells were transiently transfected with BCR/ABL and treated with etoposide. Staining of untreated cells confirmed the cytoplasmic

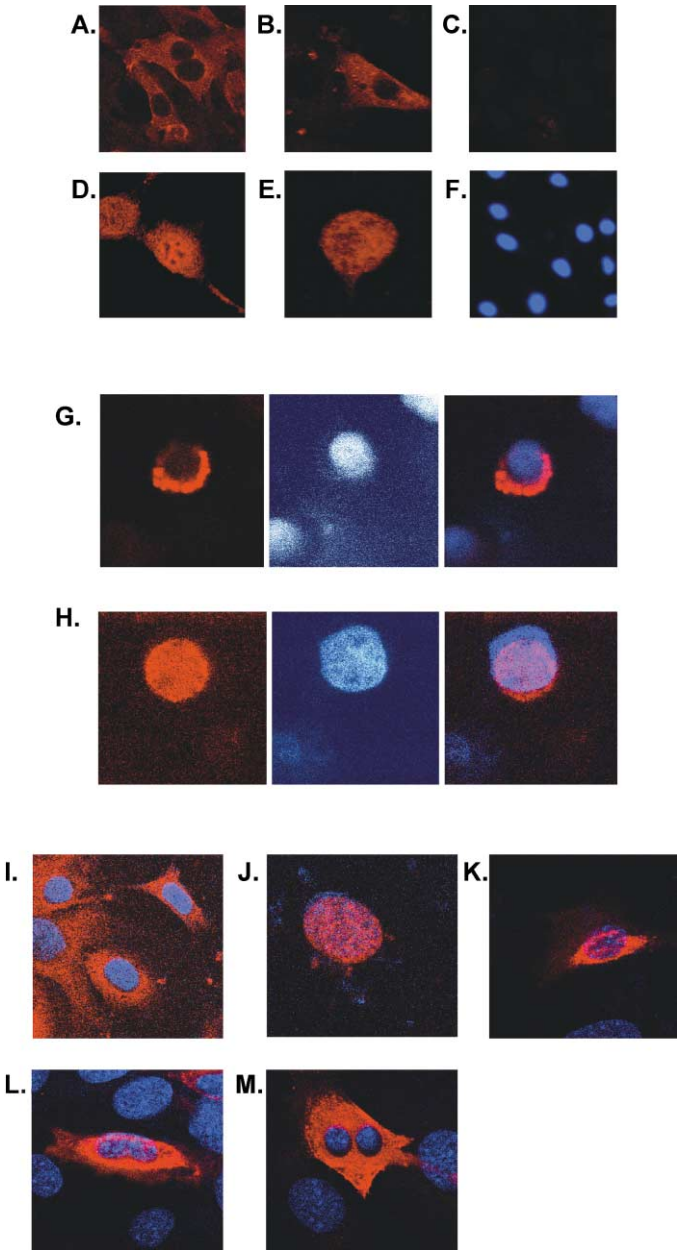

Figure 3. BCR/ABL translocates to the nucleus after DNA damage

A–F: NIH 3T3 cells stably expressing p210 BCR/ABL (**A and D**) or $ABL^{-/-}$ fibroblasts transfected with p210 BCR/ABL (**B and E**) were either left untreated (**A and B**) or treated with 10 μM etoposide for 1 hr (**D and E**) and then stained with antibody against ABL. As control for background staining, untransfected $ABL^{-/-}$ cells were stained with anti-ABL antibody (**C**) and counterstained with DAPI (**F**).

G and H: Cells from a patient with CML in blast crisis were thawed and plated onto fibronectin-coated coverslips for 1 hr and then either not treated (**G**) or treated with etoposide (10 μM) for 1 hr (**H**). Cells were stained with anti-ABL antibody (left) and counterstained with DAPI (center). Images were overlayed (right).

I–M: In a separate experiment, $ABL^{-/-}$ fibroblasts were transfected with pMSCV p210 BCR/ABL and transferred to fibronectin-coated coverslips. Cells were either untreated (**I**), treated with etoposide for 10 min (**J**), or treated with etoposide for 10 min, washed, and allowed to recover for 30, 60, or 120 min (**K–M**), respectively. Cells were fixed, permeabilized, and stained with anti-ABL antibody.

localization of BCR/ABL (Figure 3B) and again BCR/ABL translocated to the nucleus after treatment with etoposide (Figure 3E). In order to demonstrate that BCR/ABL translocation was not an artifact of high-level expression in fibroblast cells, we next examined primary cells from patients with CML (Figures 3G and 3H). Mononuclear cells from peripheral blood were stored as viable cells in DMSO. Cells were thawed, plated onto fibronectin-coated coverslips in media, and examined on the day of thawing. Analysis of cells that were not treated with etoposide again confirmed the cytoplasmic localization of BCR/ABL (Figure 3G). After 1 hr of treatment with etoposide (10 μM), BCR/ABL again translocates to the nucleus (Figure 3H). Previous work in CML has demonstrated that BCR/ABL is overexpressed relative to endogeneous c-ABL and therefore most of the staining in Figure 3 represents primarily BCR/ABL (Wetzler et al., 1993). Similar results were seen in a total of 3 patient samples, one from chronic phase and two in blast crisis. In contrast, $CD34^+$ hematopoietic stem cells and cells from patients with acute myeloid leukemia (data not shown) demonstrated only c-ABL nuclear staining without or with etoposide. Thus, BCR/ABL translocation from the cytoplasm to the nucleus occurs after genotoxic stress in both fibroblasts and primary patient cells.

Previous reports and our own data (cf. Figure 1B) have demonstrated that BCR/ABL expression in cytokine-dependent cells provides protection against apoptosis that may be dependent on activation of cytoplasmic signaling proteins including Akt and BAD (Bedi et al., 1994; Neshat et al., 2000). However, if BCR/ABL translocates to the nucleus after DNA damage, it may not have access to appropriate substrates. c-ABL is known to shuttle between the cytoplasm and the nucleus, although regulation of this shuttling is poorly understood (Taagepera et al., 1998). In order to determine if BCR/ABL might shuttle in a similar fashion, we performed a time course analysis of BCR/ABL localization after DNA damage and repair in $ABL^{-/-}$ fibroblasts. Initial results demonstrated that BCR/ABL translocates to the nucleus within 10 min of addition of etoposide (Figure 3J). Earlier time points were not examined. Cells were then washed free of etoposide and localization of BCR/ABL followed over time. BCR/ABL can be found in the cytoplasm within 30 min of removal of etoposide and is completely cytoplasmic by 2 hr after removal of etoposide (Figures 3K–3M). Thus, BCR/ABL translocation is rapid and reversible.

BCR/ABL associates with ATM after DNA damage but does not affect ATM function

The translocation of BCR/ABL to the nucleus suggested that BCR/ABL may disrupt DNA repair and cell cycle regulation directly. The nuclear protein c-ABL is known to associate with ATM after DNA damage (Baskaran et al., 1997; Shafman et al., 1997), and disruption of ATM can cause a RDS phenotype. We therefore examined the association of BCR/ABL with ATM in Ba/F3 pTET-ON p210 cells after treatment with etoposide. Cells were either grown without etoposide, treated with etoposide (10 μM) for 90 min, or treated for 90 min, washed free of drug, and allowed to recover for 90 min (Figure 4). Examination of uninduced pTET-ON p210 cells confirmed that DNA damage induced association of ATM and c-ABL (Figure 4A, lanes 2 and 8). In BCR/ABL-expressing cells, immunoprecipitation with antibody against ATM and immunoblotting with an antibody against ABL that recognizes both c-ABL and BCR/ABL revealed

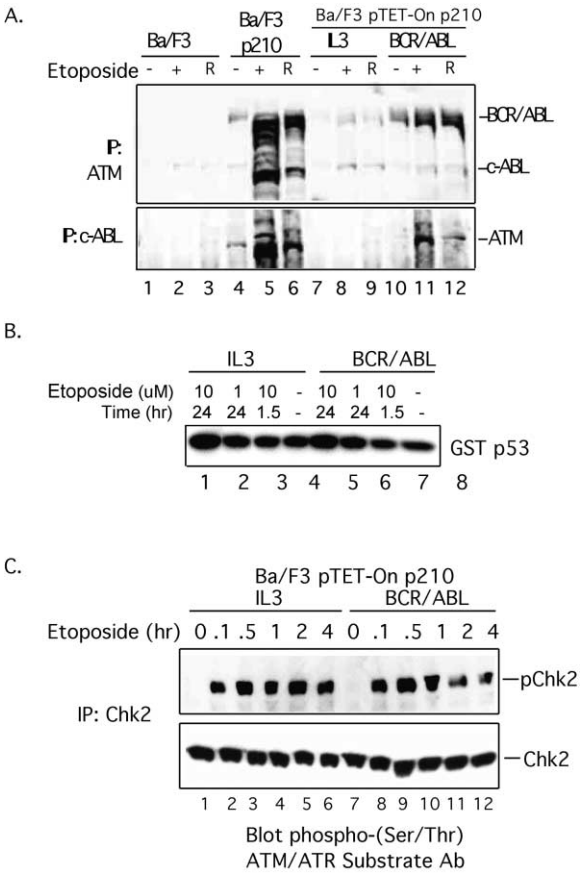

Figure 4. BCR/ABL associates with ATM and alters signal transduction after DNA damage

A: Parental Ba/F3 cells (lanes 1–3), Ba/F3 cells stably transfected with p210 BCR/ABL (lanes 4–6), Ba/F3 pTET-ON p210 cells growing in IL3 (lanes 7–9), or washed free of IL3 and treated with doxycycline (lanes 10–12) were either growing in log phase (lanes 1, 4, 7, and 10), treated with 10 μM etoposide for 90 min (lanes 2, 5, 8, and 11), or treated with etoposide for 90 min, washed, and incubated for an additional 90 min to allow for DNA repair (lanes 3, 6, 9, and 12). Cells were lysed and indicated proteins immunoprecipitated. Immunoprecipitated proteins were separated by SDS-PAGE and blotted using anti-ABL antibody (top) or anti-ATM antibody (bottom).

B: Ba/F3 pTET-ON p210 cells growing in IL3 (lanes 1–4) or doxycycline (lanes 5–8) were treated with etoposide at different doses and times. ATM kinase activity was assayed as described in the Experimental Procedures.

C: Ba/F3 pTET-ON p210 cells growing in IL3 (lanes 1–7) or doxycycline (lanes 8–14) were treated with etoposide (10 μM) for indicated times. Cells were lysed and protein lysates were separated by SDS-PAGE and blotted onto nitrocellulose. Blots were probed with antibody to phospho-Chk2 (top), then stripped and reprobed for total Chk2 (bottom).

both the 145 kDa c-ABL band and the 210 kDa BCR/ABL protein (Figure 4A, lanes 5 and 11). Association persisted but was decreased 90 min after withdrawal of etoposide (Figure 4A, lanes 6 and 12). Thus, association of BCR/ABL with ATM is inducible by DNA damage and has a time course similar to the translocation of BCR/ABL to the nucleus described above. Similar results were seen comparing parental Ba/F3 cells (Figure 4A, lanes 1–3) with Ba/F3 cells stably transfected with BCR/ABL (Figure 4A, lanes 4–6) as well as in one patient sample (data not shown). However, when we examined ATM kinase activity, which is increased in cells after DNA damage, we saw no difference in

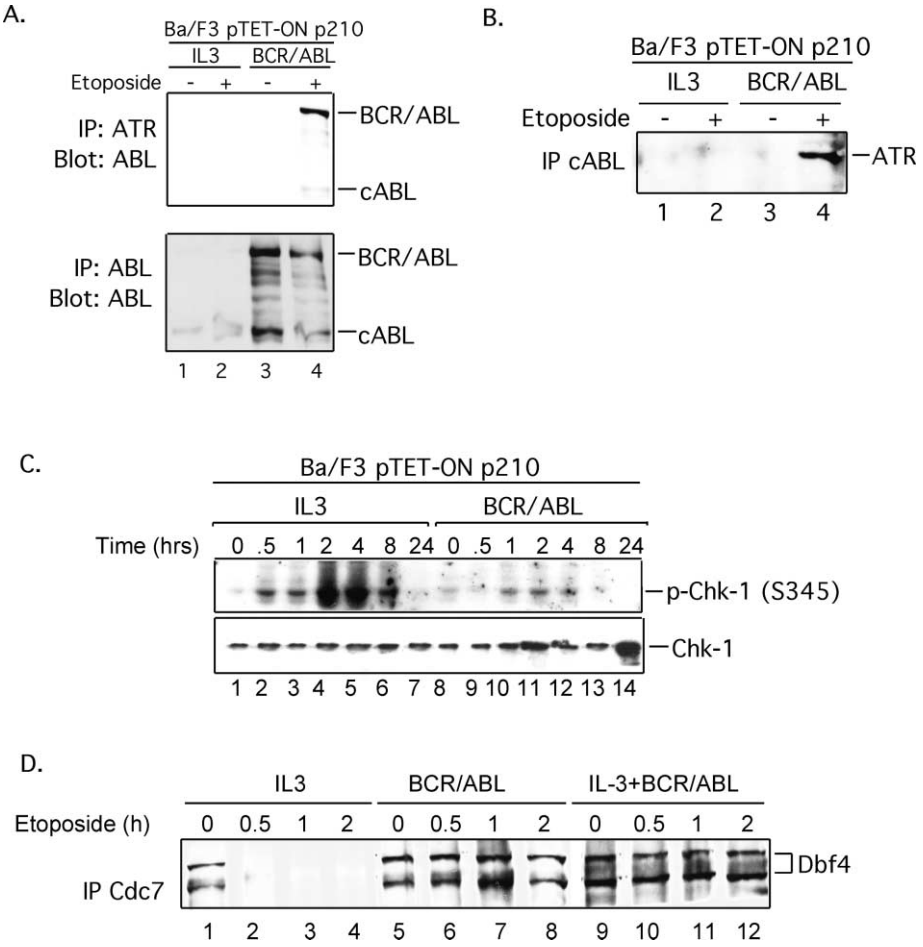

Figure 5. BCR/ABL associates with ATR and disrupts ATR-dependent signaling

A and B: Ba/F3 pTET-ON p210 cells were incubated in IL3 (lanes 1 and 2) or doxycycline to induce BCR/ABL expression (lanes 3 and 4). Cells were either not treated (lanes 1 and 3) or treated with 10 μM etoposide for 2 hr. Cells were lysed and lysates immunoprecipitated with indicated antibodies. Immunoprecipitates were separated by SDS-PAGE and analyzed by Western blot.

C: Ba/F3 pTET-ON p210 cells were grown in either IL3 or doxycycline to induce BCR/ABL expression. Cells were either not treated (lanes 1 and 8) or treated with etoposide 10 μM for the indicated periods of time. Cells were lysed and analyzed by Western blot using an antibody specific for the phosphorylated form of Chk1. Blot was stripped and reprobed for total Chk1 expression.

D: Cells were treated as above. In addition, some cells were maintained in both IL3 and doxycycline (lanes 9–12). Cells were not treated (lanes 1, 5, and 9) or treated with etoposide for the indicated periods of time and then lysed and analyzed by immunoprecipitation using anti-cdc7 antibody followed by Western blotting for Dbf4.

ATM kinase activity in cells growing in IL3 or expressing BCR/ABL (Figure 4B) using a GST-p53 1-101 fusion protein as substrate. Likewise, we saw no difference in phosphorylation of the ATM substrate, Chk2, in cells under either set of conditions (Figure 4C). Thus, although BCR/ABL, like c-ABL, can associate with ATM after DNA damage, it does not appear to alter ATM function.

BCR/ABL associates with ATR and disrupts ATR-dependent signal transduction events

Although first described in association with ATM deficiency, the RDS phenotype has recently been described in cells deficient in Chk1, a critical regulatory substrate of the ataxia telangiectasia and rad 3-related (ATR) protein. ATR is a homolog of ATM with a critical role in regulating chromosomal stability (Brown and Baltimore, 2000, 2003; Heffernan et al., 2002). We therefore examined cells for an etoposide inducible association of BCR/ABL and ATR (Figures 5A and 5B). Ba/F3 pTET-ON p210 cells were grown in IL3 (Figure 5A, lanes 1 and 2) or in doxycycline to induce expression of BCR/ABL (Figure 5A, lanes 3 and 4). Cells were either untreated or treated with etoposide and then lysed. Immunoprecipitation of lysates with an anti-ATR antibody and subsequent blotting with an anti-ABL antibody demonstrated an association of ATR and BCR/ABL only after treatment of cells with etoposide (Figure 5A, lane 4). Induction of BCR/ABL was confirmed in the same lysates (Figure 5A, lower panel) and the immunoprecipitation with anti-ABL antibody and blotting with anti-ATR antibody demonstrated the same result (Figure 5B). Thus, BCR/ABL associates with ATR after treatment of cells with etoposide.

To determine if BCR/ABL affects Chk1 activity, we examined phosphorylation of Chk1 after DNA damage (Figure 5C). Chk1 is a serine threonine kinase activated by ATR after DNA damage (Liu et al., 2000). Cells were induced to express BCR/ABL, and control cells and BCR/ABL-expressing cells were treated with etoposide for varying periods of time as indicated. Phosphorylation of Chk1 was examined by Western blotting using an antibody specific for Chk1 phosphorylated on serine 345, the site where ATR phosphorylates the protein. Phosphorylation of Chk1 was induced normally in the IL3-stimulated cells (Figure 5C, lanes 1–7). However, in the BCR/ABL-expressing cells, only minimal phosphorylation of Chk1 is seen (Figure 5, lanes 8–14). This experiment was repeated multiple times, and no substantial phosphorylation of Chk1 was detected at any time point. This result suggested to us that the RDS phenotype seen in BCR/ABL-expressing cells may represent a defect in ATR signaling, rather than a defect in ATM signaling.

A recent report has described activation of ATR by etoposide-induced DNA damage (Costanzo et al., 2003). The authors describe an ATR-dependent "etoposide checkpoint" that is mediated through regulation of the cdc7/Dbf4 complex. To examine regulation of this complex, we examined Ba/F3 pTET-ON

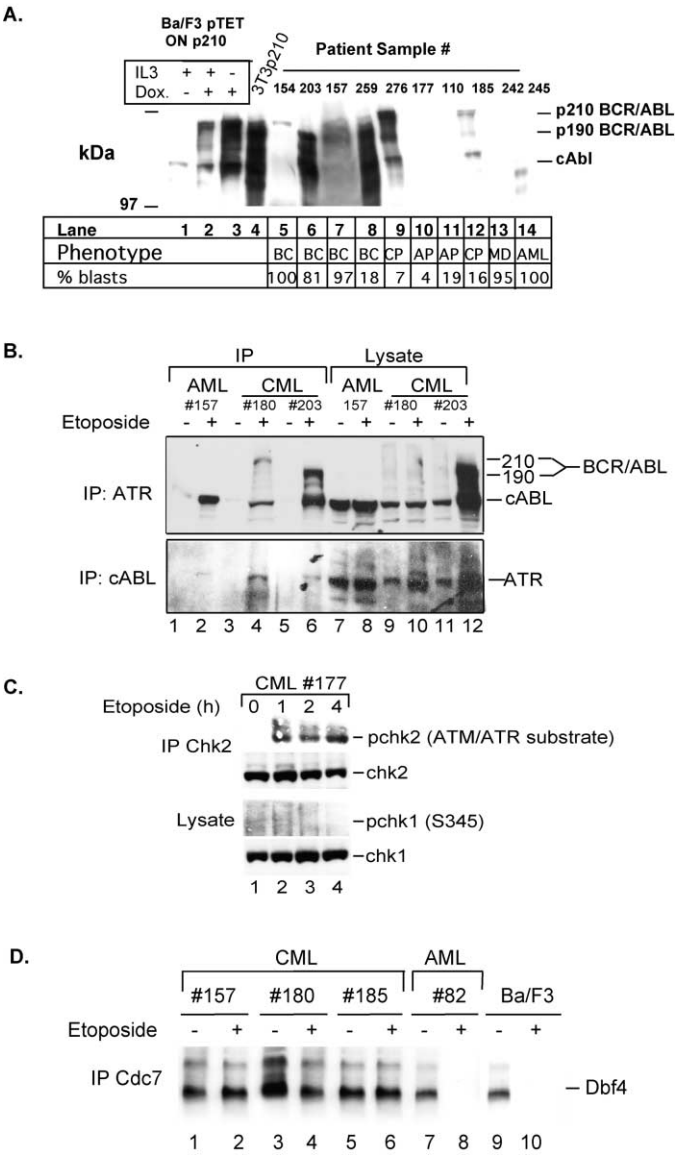

Figure 6. BCR/ABL associates with ATR and disrupts ATR signaling in primary CML cells

Patient samples were rapidly thawed and plated in media for 1 hr prior to initiating experiments.

A: Ba/F3 pTET-ON p210 cells (lanes 1–3) were grown in the presence of IL3 and doxycycline for 24 hr as indicated. 3T3p210 cells were harvested from subconfluent plates. Patient samples were thawed and immediately lysed. Lysates were immunoprecipitated with antibody against c-ABL (8E9); immunoprecipitated proteins were separated by SDS-PAGE and blotted with the same anti-ABL antibody. Abbreviations: BC, CML blast crisis; CP, CML chronic phase; AP, CML accelerated phase; MD, myelodysplastic syndrome; AML, acute myeloid leukemia. Percentage blasts is based on values obtained from clinical specimens analyzed by the Hospital of the University of Pennsylvania Pathology Department.

B: Cells were either not treated or treated with etoposide (10 μM) for 1 hr. Cells were lysed and indicated proteins immunoprecipitated. Immunoprecipitated proteins were analyzed by Western blotting.

C: Cells were treated with etoposide (10 μM) for the indicated periods of time and then harvested and lysed. For analysis of Chk2 phosphorylation, total Chk2 protein was immunoprecipitated and analyzed using the anti-phospho-ATM/ATR substrate antibody (Cell Signaling). Blot was stripped and reprobed with antibody against total Chk2. For analysis of Chk1 phosphorylation, protein lysate was analyzed by Western blotting using an antibody specific for the phosphorylated form of Chk1. Blot was again stripped and reprobed for expression of total Chk1.

p210 cells again after treatment of cells with etoposide. Cells growing in IL3 without etoposide showed activation of the complex as shown by co-immunoprecipitation of cdc7 and Dbf4 (Figure 5D, lane 1). After treatment with etoposide, however, the complex is disrupted, demonstrating an intact ATR-dependent signaling response to etoposide in the cells growing in IL3. On the other hand, when cells are induced to express BCR/ABL, the complex is not dissociated after treatment with etoposide (Figure 5D, lanes 5–8). To ensure that it is the induction of BCR/ABL that disrupts the ATR checkpoint and not withdrawal of IL3, we also examined cells in which BCR/ABL expression is induced but IL3 was not withdrawn (Figure 5D, lanes 9–12). These cells showed the same lack of ability to disrupt the cdc7-Dbf4 complex, showing that it is the presence of BCR/ABL and not the absence of IL3 that disrupts ATR.

BCR/ABL Disrupts ATR Signaling in Primary Patient Cells

To ensure that the disruption of ATR signaling in BCR/ABL-expressing cells is not an artifact of overexpression in cell lines, we again examined primary patient cells (Figure 6A). Initially, in order to determine the relative levels of expression of BCR/ABL in cell lines compared to primary cells, we prepared lysates from both cell lines and primary patient samples. Patient samples (Figure 6A, lanes 5–14) were obtained from CML patients with different stages of disease and with different percentage blasts as indicated. Expression of BCR/ABL in hematopoietic cell lines was again induced by addition of doxycycline in the presence or absence of interleukin 3 (Figure 6A, lanes 1–3). Expression in the absence of IL3 and the presence of doxycycline was highest and equivalent to expression in stable fibroblast cell lines (Figure 6A, lanes 3 and 4). Expression in CML blast crisis samples was variable and ranged from equivalent to expression in cell lines (cf. sample 203, which expresses p190 BCR/ABL, and sample 276) to 10-fold less (sample 154, Figure 6A, lane 5). Proteolysis during preparation of protein lysates from primary myeloid cells is a common problem, and the lower molecular weight bands seen in multiple samples probably represent a combination of c-ABL staining and degradation of BCR/ABL. Overall, this experiment demonstrates that expression of BCR/ABL in these cell lines is equivalent to or greater than expression in patient samples.

To determine the effect of BCR/ABL on ATR in primary cells, we examined cells from patients with AML or CML for association of BCR/ABL and ATR. In two separate patient samples (Figure 6B, lanes 3–6), immunoprecipitation of ATR and blotting with anti-ABL antibody demonstrated an etoposide-induced association of the two proteins. Interestingly, this association was seen both in a patient expressing the p210 isoform of BCR/ABL studied in our cell line experiments and in a patient with the p190 isoform (pt. 203, Figure 6B, lanes 5 and 6). Of note, in these human cells, the anti-ATR immunoprecipitate also contained c-ABL, which was not seen in our experiments in the murine Ba/F3 cells (compare Figure 6B, lane 2, to Figure 5A,

D: Patient samples and parental Ba/F3 cells were incubated in appropriate media and either not treated or treated with etoposide (10 μM) for 1 hr. Cells were lysed and anti-cdc7 immunoprecipitates performed and association with Dbf4 analyzed by Western blotting.

lane 2). Whether this represents a difference in species or between the cell line and primary cells is not clear. These results were confirmed by immunoprecipitating with anti-ABL antibody and blotting with anti-ATR antibody (Figure 6B, lower panel). Uniform expression of ATR was also demonstrated in the lysate fractions of this blot (Figure 6B, lower panel, lanes 7–12). Thus, this experiment confirms that ATR associates with BCR/ABL in primary patient cells. To determine if ATR is functionally active in CML patient cells, we examined signal transduction downstream of ATR. Cells from a patient with CML were thawed and allowed to recover. Cells were then either not treated (Figure 6C, lane 1) or treated with etoposide (10 μM) for the indicated amounts of time. Chk2 phosphorylation was again examined by immunoprecipitating and blotting for the phosphorylated form of the protein as in Figure 5 above. Consistent with the results above, and suggesting an intact ATM pathway, Chk2 is phosphorylated in these primary cells after treatment with etoposide. In contrast, no phosphorylation of Chk1 was seen at any of the time points examined (Figure 6C, lower panels). Finally, we examined CML and AML patient samples for the ability to inactivate cdc7 after treatment with etoposide. AML cells (Figure 6D, lanes 7 and 8) showed association of cdc7 and Dbf4 in the absence of etoposide, which was not present after treatment of cells. In contrast, three CML patient samples (Figure 6D, lanes 1–6) showed association of cdc7 and Dbf4 in the absence or presence of etoposide. This result confirms that BCR/ABL inhibits ATR signaling in response to etoposide in primary CML patient samples.

Overexpression of ATR in BCR/ABL-expressing cells reverts the DNA damage phenotype

In order to determine if inhibition of ATR function causes the DNA damage phenotype described above, we hypothesized that the interaction of BCR/ABL with ATR is stochiometric and that overexpression of ATR would lead to free ATR, which would be able to activate the signaling pathway after DNA damage. To test this hypothesis, we transfected NIH 3T3 cells, which stably express p210 BCR/ABL with a FLAG-tagged ATR cDNA and examined cells for the response to DNA damage (Figure 7). Expression of the FLAG-tagged ATR was confirmed by Western blotting (Figure 7A, lanes 5 and 6). In these fibroblast cell lines, we confirmed that treatment of wild-type fibroblasts with etoposide induces the phosphorylation of Chk1 (Figure 7A, lane 2). In the stably expressing BCR/ABL lines, Chk1 is not phosphorylated after treatment with the drug as expected (Figure 7A, lane 4). However, when ATR is overexpressed and the cells are treated with etoposide, Chk1 phosphorylation is again seen (Figure 7A, lane 6), demonstrating that overexpression of ATR reconstitutes ATR signaling in response to DNA damage in BCR/ABL-expressing cells. To determine how this would affect DNA damage, we again performed the comet assay (Figure 7B). Untreated cells showed no differences in spontaneous DNA damage whether they did or did not express BCR/ABL or ATR. BCR/ABL-expressing cells again show an increase in DNA damage after 90 min treatment with etoposide (Figure 7B, striped bars). However, when ATR is overexpressed, little DNA damage is seen at this time point, demonstrating that ATR overexpression blocks the effect of BCR/ABL to increase DNA damage seen in BCR/ABL-expressing cells. This experiment demonstrates that the ability of BCR/ABL to increase DNA damage is dependent on blocking the function of ATR.

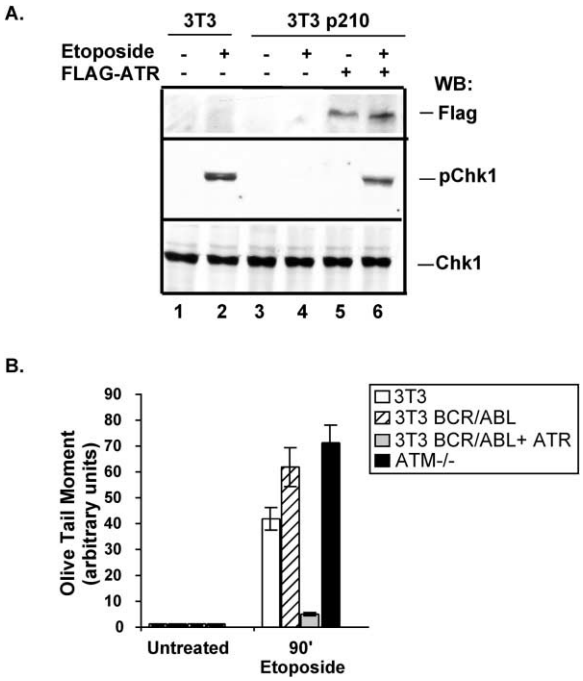

Figure 7. Overexpression of ATR in BCR/ABL-expressing cells reverts the DNA damage phenotype

A: 3T3 cells stably expressing p210 BCR/ABL were transfected with FLAG-tagged ATR construct. 48 hr after transfection, control 3T3 cells (lanes 1 and 2), nontransfected 3T3 p210 cells (lanes 3 and 4), or transfected cells (lanes 5 and 6) were either not treated (lanes 1, 3, and 5) or treated with etoposide (10 μM) for 90 min (lanes 2, 4, and 6). Cells were lysed and analyzed for expression of FLAG-tagged ATR (top), phosphorylated Chk1 (middle), or total Chk1 (lower).

B: Cells prepared as in **A** were analyzed using the comet assay. ATM-deficient cells were used as a control for the assay.

Discussion

A role for BCR/ABL in altering the cellular response to DNA damage has been previously hypothesized (Deutsch et al., 2001). We demonstrate that BCR/ABL expression leads to an increase in the amount of DNA double-strand breaks after treatment of cells with etoposide and a disruption of an intra-S phase checkpoint. Our results demonstrate that these are direct effects of BCR/ABL as BCR/ABL translocates to the nucleus after DNA damage, placing the protein in the appropriate cellular compartment for disrupting DNA repair processes. Translocation of BCR/ABL is rapid and reversible and occurs both in fibroblasts and primary patient cells. In addition, we demonstrate that BCR/ABL associates with ATR after treatment of cells with etoposide and disrupts ATR-dependent signal transduction. Disruption of ATR is demonstrated by the decrease in the phosphorylation of Chk1 and the lack of inactivation of the cdc7/Dbf4 complex, which is required for the replication arrest induced by etoposide. This is an entirely novel mechanism of oncogenesis by BCR/ABL.

There has been controversy as to whether BCR/ABL increases or decreases DNA damage and repair processes. Skorski and colleagues have proposed that BCR/ABL increases the expression of Rad51 and leads to an increase in homologous recombination repair that leads to chemotherapy resistance (Majsterek et al., 2002; Slupianek et al., 2001). Neither we (data

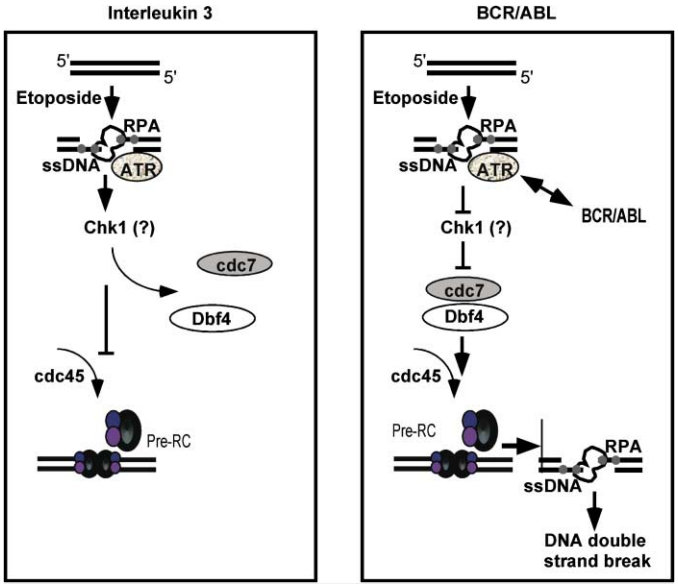

Figure 8. Effect of BCR/ABL on an ATR-dependent cell cycle checkpoint

Left: Model is based on that proposed by Costanza et al. (2003). In cells growing in normal cytokines, etoposide-induced DNA damage triggers a DNA double-strand break, which is processed to a single strand end. This triggers binding of both RPA and ATR and activation of ATR. ATR phosphorylates Chk1, which inactivates the cdc7/Dbf4 complex, leading to decreased loading of cdc45 in the pre-RC.

Right: BCR/ABL binds to ATR. Double-headed arrow indicates that binding may happen on or off DNA. Such binding prevents ATR from activating Chk1. The cdc7/Dbf4 complex remains active and replication proceeds inappropriately. Bottom of figure indicates that collision of the replication complex with the topoisomerase II/etoposide complex on DNA may lead to further DNA double-strand breaks.

not shown) nor others have reproduced the increase in Rad 51 expression described by Slupaniek, and we do not see a significant alteration in chemotherapy resistance comparing cells growing in IL3 alone versus BCR/ABL-expressing cells in the absence of IL3 (Deutsch et al., 2003). However, experimental conditions, particularly the presence or absence of IL3 in the culture, the use of different cell lines, and the type of DNA-damaging agent may alter the results of the two experiments. On the other hand, Deutsch and colleagues have demonstrated that BCR/ABL-expressing cells have an increase in sister chromatid exchanges and chromosomal translocations after treatment with IR. Although we have not examined SCE or translocations directly, these would be predicted to be increased in cells with disruption of ATR function and an increase in DNA double-strand breaks. In fact, the results of Deutsch and colleagues are entirely consistent with the model we propose (cf. Figure 8 and below), although we describe a different mechanism of DNA damage. Overall, our results support a model that BCR/ABL has a direct role in increasing DNA damage, which may eventually lead to CML blast crisis.

Another significant difference in our experiments compared to other work involves the use of etoposide. Costanzo et al. (2003) demonstrate that the early response to etoposide-induced DNA damage is dependent on ATR. ATR acts to inactivate Cdc7, a cell cycle kinase necessary for the formation of the prereplication complex (pre-RC). Thus, ATR acts in response to etoposide to check DNA replication (Figure 8). Applied to our model, these results suggest that the phenotype we have described may be restricted to the cellular response to etoposide-induced DNA damage only, to agents that leave single strand intermediates, or through effects that lead to stalled replication forks that may be repaired through a similar mechanism. In this regard, however, the RDS phenotype is present in BCR/ABL-expressing fibroblasts after treatment with IR and IR also leads to increased DSB, so the results are not exclusive to etoposide alone (Figure 2). Future studies will need to address whether BCR/ABL disrupts the response to DNA damage only through this pathway or affects the response to other agents as well.

The demonstration that BCR/ABL disrupts the ATR signaling pathway and causes an RDS phenotype may have profound implications for chromosomal stability in chronic myeloid leukemia. ATR is a member of a family of proteins that includes ATM and DNA PKcs (Abraham, 2001). Several reports confirm the critical importance of the intra-S phase checkpoint in maintaining genomic stability (Kolodner et al., 2002; Ye et al., 2003). Thus, disruption of ATR signaling, even transiently at critical moments when cells contain DNA double-strand breaks, may predispose cells to the formation of chromosomal translocations or deletions. A novel aspect of our results is the transient inhibition of ATR function associated with a DNA damage-induced redistribution of BCR/ABL from the cytoplasm to the nucleus. Although the mechanism of this translocation is unclear, this finding is consistent with the results for c-ABL and BCR/ABL previously described (Taagepera et al., 1998; Vigneri and Wang, 2001). Given the lethality of an ATR null phenotype, it is perhaps not surprising that BCR/ABL does not constitutively associate with ATR. Future experiments will be required to fully define the consequences of the DNA damage-induced association of ATR and BCR/ABL.

In conclusion, we have found that BCR/ABL translocates from the cytoplasm to the nucleus after genotoxic stress. Furthermore, BCR/ABL expression increases DNA double-strand damage after etoposide and leads to a defect in an intra-S phase checkpoint, causing a radioresistant DNA synthesis (RDS) phenotype. This represents the first detailed description of disruption of a cell cycle checkpoint by BCR/ABL. Finally, BCR/ABL disrupts ATR-dependent signal transduction after treatment with etoposide. Future experiments will determine if BCR/ABL expression leads to genomic instability, which may lead to CML blast crisis.

Experimental procedures

Cells and constructs

Ba/F3 pTET-ON p210 cells have been previously described (Klucher et al., 1998). $ABL^{-/-}$ 3T3 cells were a kind gift of Dr. Warren Pear (University of Pennsylvania) and were derived from $ABL^{-/-}$ murine embryo fibroblasts (MEFs). GM02025D cells are a human fibroblast line derived from a patient deficient in ATM and were obtained from the Coriell Cell repository (Camden, NJ). Cells from patients with CML and AML were obtained from the Stem Cell and Leukemia Core Facility at the University of Pennsylvania Cancer Center. Pathologic diagnosis including stage of CML was obtained from the bone marrow biopsy reports as performed by the Hospital of the University of Pennsylvania. pMSCV GFP and pMSCV p210 BCR/ABL IRES GFP were a kind gift of Dr. Warren Pear. Nucleotides 1–101 of p53 were amplified by PCR and cloned into the EcoRI and Bam HI sites of GST expression vector pGEX-2T (Amersham Biosciences) for ATM kinase assays. FLAG-tagged ATR cDNA was a kind gift of Stuart Schreiber and Karlene Cimprich and has been previously described (Cliby et al., 1998).

Immunofluorescence

Where indicated, cells were transfected using either FuGene 2000 or Lipofectamine reagent ($ABL^{-/-}$ cells) according to the manufacturer's directions. Constructs were either pMSCV-GFP control or pMSCV BCR/ABL p210/GFP. Cells were transfected and incubated for 24 hr. Cells were then trypsinized and replated on fibronectin-coated coverslips. Cells were incubated in appropriate media for an additional 24 hr on coverslips and then treated as indicated in each figure. Cells were fixed with paraformaldehyde, permeabilized, and stained with antibodies against ABL at 1:200 dilution (8E9, Pharmingen) and anti-mouse Cy 5 at 1:1000 (Molecular Probes), and then counterstained with the DNA dye, DAPI (10 nM). Cells were visualized using a confocal microscope fitted with two lasers. Data was analyzed using Confocal Assistant software provided by Bio-RAD. Primary cell samples were thawed and plated directly onto fibronectin-coated coverslips for 1 hr prior to incubation with etoposide.

Western blotting and immunoprecipitations

Cells were lysed in TTN buffer (50 mM Tris HCl [pH 7.6], 1% Triton X-100, 150 mM NaCl with protease and phosphatase inhibitors). Protein was quantified using a Bradford reagent. 1000 μg of protein lysate were used per IP and 100 μg per lane for Western blots. Antibodies were: α-ABL 8E9 (Pharmingen), α-ATM Ab-1, α-ATM Ab-3 (CalBiochem), α-ATM B-12, cdc7, Dbf4 (Santa Cruz Biotech), p-Chk1, Chk1, p53, phospho-p53 (serine 15) (Cell Signaling, Beverly, MA), and α-ATR (Serotec, London).

ATM kinase assay

Cells were harvested and lysed through sonication in TTN buffer. 1000 μg lysate was precleared with mouse IgG and protein A/G Sepharose and then immunoprecipitated with anti-ATM antibody (B12, Santa Cruz) and protein A/G Sepharose. Immunoprecipitates were washed twice with TTN buffer and twice with kinase buffer (10 mM HEPES [pH 7.6], 50 mM NaCl, 50 mM glycerophosphate, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DDT, and protease inhibitors). Washed beads were resuspended in 30 μl kinase buffer containing 10 μCi [γ-^{32}P]ATP and 1 μg GST p53 and incubated for 30 min at 30°C. Kinase reactions were stopped by the addition of an equal volume of 2× SDS sample buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose, and incorporated radioactivity was analyzed with a phospho-imager (Molecular Dynamics). GST p53 1-101 fusion protein was expressed in bacteria and purified on glutathione-conjugated agarose beads.

Radioresistant DNA synthesis assay

Assay was performed with a modification of the original protocol. Cells were grown in appropriate media and labeled with 20 nCi of ^{14}C thymidine per ml (specific activity, 50 mCi/mmol) for 24 hr. Cells were washed free of label, rested for 12 hr in nonradioactive medium, and replated at 5×10^4 cells/ml. The cells were treated with 0 Gy (control), 5 Gy (for fibroblast cells), or 20 Gy (Ba/F3 cells) of IR, or 0 μm (control) or 10 μm etoposide incubated at 37°C for 30 min and then labeled (30 min) with ^{3}H thymidine (2.5 mCi/ml). The cells were harvested by centrifuge, washed, and transferred to 96-well plates for analysis. Cells were harvested using a "Tomtec Harvester 96" onto a glass fiber filter and the filter air dried overnight. The 96 filtered samples were manually cut off and radioactivity measured using the liquid scintillation counter. The resulting ratios of ^{3}H:^{14}C were expressed as a percentage of control values. Control experiments showed minimal channel interference between two isotopes.

Comet assay

A modified comet assay was performed as described (Singh et al., 1988). Exponentially growing cells were treated with the desired concentrations of etoposide or irradiated. The medium was replaced with fresh complete medium and incubated for the required postincubation time. Cells were diluted to a density of 2.5×10^4 cells/ml and kept on ice. Microscope slides were precoated with 1% (wt/vol) type-IA agarose, and 0.5 ml of cells was mixed with 1 ml of 1% (wt/vol) type-VII agarose and spread over a precoated slide in duplicate. A coverslip was added, and the agarose was allowed to solidify. Coverslips were removed, and slides were placed in lysis solution (100 mM disodium EDTA, 2.5 M NaCl, 10 mM Tris-HCl [pH 10.5]) containing 1% Triton X-100 at 4°C and incubated for 1 hr in the dark. Slides were subsequently washed with ice-cold water for 15 min, and this was repeated three times. The slides were then transferred to an electrophoresis tank containing ice-cold alkaline solution (50 mM NaOH, 1 mM disodium EDTA [pH 12.5]) and incubated for 45 min in the dark. Electrophoresis was carried out for 25 min at 18 V (0.6 V/cm) and 250 mA in the dark. Slides were removed, and 1 ml of neutralizing solution (0.5 M Tris-HCl [pH 7.5]) was added and incubated for 10 min. Each slide was rinsed twice with 1 ml of phosphate-buffered saline (PBS) and allowed to dry overnight at room temperature. Slides were stained with propidium iodide (2.5 μg/ml), and comets were analyzed using a Nikon DIAPHOT TDM inverted epifluorescent microscope (consisting of a high-pressure mercury vapor light source, a 580 nm dichromic mirror, 510 to 560 nm excitation filter, and 590 nm barrier filter) at 20× magnification. 50–100 cells were analyzed per slide using Komet Assay software (Kinetic Imaging, Liverpool, UK) for olive tail moment, and mean and standard deviation for each experiment were calculated using Microsoft Excel.

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