

# Fusion Tyrosine Kinases Induce Drug Resistance by Stimulation of Homology-Dependent Recombination Repair, Prolongation of G<sub>2</sub>/M Phase, and Protection from Apoptosis

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**Fusion tyrosine kinases (FTKs) such as BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFβR, TEL/TRKC(L), and NPM/ALK arise from reciprocal chromosomal translocations and cause acute and chronic leukemias and non-Hodgkin's lymphoma. FTK-transformed cells displayed drug resistance against the cytostatic drugs cisplatin and mitomycin C. These cells were not protected from drug-mediated DNA damage, implicating activation of the mechanisms preventing DNA damage-induced apoptosis. Various FTKs, except TEL/TRKC(L), can activate STAT5, which may be required to induce drug resistance. We show that STAT5 is essential for FTK-dependent upregulation of RAD51, which plays a central role in homology-dependent recombinational repair (HRR) of DNA double-strand breaks (DSBs). Elevated levels of Rad51 contributed to the induction of drug resistance and facilitation of the HRR in FTK-transformed cells. In addition, expression of antiapoptotic protein Bcl-xL was enhanced in cells transformed by the FTKs able to activate STAT5. Moreover, cells transformed by all examined FTKs displayed G<sub>2</sub>/M delay upon drug treatment. Individually, elevated levels of Rad51, Bcl-xL, or G<sub>2</sub>/M delay were responsible for induction of a modest drug resistance. Interestingly, combination of these three factors in nontransformed cells induced drug resistance of a magnitude similar to that observed in cells expressing FTKs activating STAT5. Thus, we postulate that RAD51-dependent facilitation of DSB repair, antiapoptotic activity of Bcl-xL, and delay in progression through the G<sub>2</sub>/M phase work in concert to induce drug resistance in FTK-positive leukemias and lymphomas.**

Chromosomal translocations are responsible for the appearance of oncogenes encoding fusion tyrosine kinases (FTKs) such as BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFβR, TEL/TRKC(L), and NPM/ALK (6, 35). BCR/ABL is derived from relocation of the portion of the *c-ABL* gene from chromosome 9 to the portion of the *BCR* gene locus on chromosome 22 [t(9;22)] and is present in most chronic myelogenous leukemia (CML) patients and a cohort of acute lymphocytic leukemia (ALL) patients (11, 19, 64). TEL/ABL results from a t(9;12) translocation reported in ALL, acute myelogenous leukemia (AML), and atypical CML (35) and consists of the amino-terminal fragment of the TEL domain fused in-frame with exon 2 of *ABL* (24). TEL/JAK2 was characterized as a product of a t(9;12) translocation which includes the TEL oligomerization domain and JAK2 catalytic domain (37) and was found in ALL (37, 51). TEL/PDGFβR is associated with a t(5;12) translocation which juxtaposes the amino-terminal region of TEL with the transmembrane and tyrosine kinase do-

main of the platelet-derived growth factor receptor β (23). TEL/PDGFβR was found in chronic myelomonocytic leukemia (35). The consequence of t(12;15) is expression of the TEL/TRKC fusion tyrosine kinase associated with AML, infantile fibrosarcoma, and congenital mesoblastic nephroma (41). The TEL/TRKC fusion in AML [TEL/TRKC(L)] includes exons 1 to 4 of the *TEL* gene fused in frame to the tyrosine kinase domain of TRKC lacking a 42-bp exon near the C terminus of the TRKC moiety. NPM/ALK, formed by the t(2;5) translocation, was implicated in the pathogenesis of anaplastic large cell lymphoma (38). The *NPM/ALK* fusion gene encodes a 75-kDa hybrid protein that contains the amino-terminal portion of the nucleolar phosphoprotein nucleophosmin (NPM) joined to the entire cytoplasmic portion of the receptor tyrosine kinase ALK (anaplastic lymphoma kinase) (44). These FTKs (BCR/ABL-related FTKs) show structural similarities, which include an amino-terminal oligomerization domain responsible for constitutive oligomerization and activation of the associated tyrosine kinase of the carboxy-terminal fusion partner.

FTKs and other oncogenic tyrosine kinases such as v-Src and HER-2/neu activate multiple signaling pathways responsible for protection from apoptosis, induction of growth factor-independent proliferation, transformation, and resistance to therapeutic drugs and to γ-radiation (25, 41, 43, 53, 54, 61, 85). Resistance to DNA-damaging agents is a cause for failure in the therapy of human cancer, including hematological malignancies. Several mechanisms of resistance to DNA damage

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have been suggested (18), including overexpression of the P-glycoprotein family of membrane transporters, which decrease the intracellular accumulation of the drugs (e.g., MDR1), changes in cellular proteins involved in detoxification (e.g., glutathione *S*-transferase) or activation of chemotherapeutic drugs (e.g., NADP), elevation of expression of antiapoptotic proteins regulating caspase-3 (e.g., Bcl-2 and Bcl-xL), transient cell cycle arrest, and modulation of DNA repair. Existing evidence indicates that the last three mechanisms may be engaged in drug resistance induced by BCR/ABL kinase (2, 4, 17, 46, 73).

BCR/ABL enhanced the expression and phosphorylation of the RAD51 protein (73). It is notable that RAD51 has been proposed to play a central role in homology-dependent recombinational repair (HRR) of DNA double-strand breaks (DSBs) (63, 75, 76). BCR/ABL-dependent stimulation of RAD51 facilitated HRR of DSBs and induced drug resistance in leukemic cells (73). DSBs are considered the principal lethal DNA damage resulting from treatment with radiomimetic and cross-linking drugs. DSBs can be generated when replication forks encounter the drug-induced DNA lesions (34). HRR mechanisms are known to resolve such replication fork-associated DSBs (3). It is of great importance that cells recognize DSBs and act upon them rapidly and efficiently, because cell death or impaired cell function can occur if these are left unrepaired or are repaired inaccurately.

In addition to DNA repair mechanisms, cell cycle checkpoint activation processes are initiated in response to DNA damage (14). More specifically, DNA damage signals to arrest cell cycle progression, giving the cell more time to repair what might otherwise be a fatal lesion. Numerous studies have implicated a G<sub>2</sub>/M checkpoint, because its disruption sensitized tumor cells to genotoxic agents (8, 9). Delay in the G<sub>2</sub>/M transition appears to be necessary for resistance to DNA damage in BCR/ABL-positive cells (4, 46, 77).

Mechanisms preventing mitochondrion-dependent activation of caspase-3 were also implicated in drug resistance of BCR/ABL cells (2, 17). BCR/ABL can inhibit apoptosis by modulation of Bcl-2 family members (1, 59, 60), which regulate activation of caspase-3 (26). Some FTKs can enhance expression of antiapoptotic members of the family, such as Bcl-xL (1, 33) and Bcl-2 (67), and inhibit the proapoptotic function of Bad (59). These events may prevent the release of cytochrome *c* from the mitochondria and activation of caspase-3 (2, 17, 31).

Taken together, previous reports implicated DNA repair, activation of a cell cycle checkpoint, and dysregulation of Bcl-2 family members in resistance to DNA damage (2, 4, 17, 46, 73). In this work we demonstrate that these mechanisms can work in concert to induce drug resistance in FTK-transformed cells.

#### MATERIALS AND METHODS

**Plasmids.** The pSR $\alpha$ -BCR/ABL and pSR $\alpha$ -NPM/ALK retroviral constructs have been described (45, 72). The MigR1-BCR/ABL retroviral construct was obtained from Warren Pear (University of Pennsylvania, Philadelphia, Pa.). The cDNAs for TEL/ABL, TEL/JAK2, and TEL/TRK(L) were obtained from D. Gary Gilliland (Brigham and Women's Hospital, Boston, Mass.) and subcloned into the pMSCV retroviral plasmid. The pSR $\alpha$ -TEL/PDGFR $\beta$  retroviral construct was received from Martin Carroll (University of Pennsylvania, Philadelphia, Pa.). Human *RAD51* cDNA in the sense (S) and antisense (AS) orientations was cloned as *RAD51*-internal ribosome entry site (IRES)-green fluorescent protein (GFP) into the retroviral vector MigR1 (73). In addition,

*RAD51*(S) and *RAD51*(AS) cDNAs were cloned into the pLXSP-puro and pMX-puro retroviral constructs. The pGL3-Basic vector containing the *RAD51* promoter region and the pMX retroviral construct carrying the *STAT5* dominant-negative mutant (*STAT5*-DNM,  $\Delta$ *STAT5B*) have been described (45, 73).

**Cells.** The murine growth factor-dependent myeloid cell line 32Dcl3 and BCR/ABL-expressing clones have been described (45). The murine growth factor-dependent pro-B lymphoid cell line BaF3 and the BCR/ABL-transformed clone were obtained from Richard Van Etten (Harvard Medical School, Boston, Mass.). BaF3 cell lines transfected with NPM/ALK or with empty virus (BaF3-*neo*) were obtained from Steven Morris (St. Jude Children's Research Hospital, Memphis, Tenn.). BaF3 cells expressing TEL/JAK2, TEL/ABL, and TEL/TRK(L) were obtained from D. Gary Gilliland, and BaF3 cells expressing TEL/PDGFR $\beta$  were obtained from Martin Carroll. 32Dcl3 cells and BCR/ABL-positive cells expressing ectopic *RAD51* were obtained after retroviral infection with the pMX-*RAD51* retroviral construct and selection of the clones in puromycin. Overexpression of *RAD51* protein was confirmed by Western analysis. 32Dcl3 cells overexpressing Bcl-xL were obtained from Daniel Link (Washington University School of Medicine, St. Louis, Mo.) and electroporated with the pMX-*RAD51* plasmid. Clones overexpressing both Bcl-xL and *RAD51* were obtained after selection in puromycin. Western analysis confirmed the elevated expression of Bcl-xL and *RAD51*.

Cell lines were maintained in Iscove's Modified Dulbecco medium supplemented with 10% fetal bovine serum (FBS) and 15% WEHI-conditioned medium. Bone marrow mononuclear cells (BMCs) from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, Maine) were obtained 6 days after treatment with 5-fluorouracil (67, 71). BMCs from CML blast crisis (CML-BC) patients were obtained after informed consent was given, and CD34<sup>+</sup> cells were isolated (69). Primary hematopoietic cells were maintained in the presence of recombinant human interleukin-3 (IL-3) and stem cell factor as described (67, 69, 71). Draa-40 cells (52) were cultured in alpha minimal essential medium ( $\alpha$ -MEM) supplemented with 10% FBS. Tk<sup>-</sup>ts13 hamster fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

**Drug resistance assays.** Cisplatin (Platinol-AQ; Bristol-Myers Squibb Co., Princeton, N.J.) or mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added to cells growing in semisolid medium (10<sup>3</sup>/ml) (MethoCult H4230; StemCell Technologies Inc., Vancouver, Canada) supplemented with IL-3. Colonies were scored after 7 days. Results are represented as the percentage of colony-forming cells after drug treatment in comparison to the untreated control group. Viable cells in suspension cultures were detected by trypan blue dye exclusion after 48 h of incubation with the drug. Results represent the percentage of drug-treated cells excluding trypan blue in comparison to the untreated cells.

**Western analysis.** Cells were solubilized in lysis buffer (10 mM HEPES [pH 7.5], 150 mM NaCl, 1% NP-40, 10% glycerol, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10  $\mu$ g each of aprotinin and leupeptin/ml). Cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and examined by Western analysis with the following antibodies: anti-*RAD51* (3C10; Upstate Biotechnology, Lake Placid, N.Y.), antiphosphotyrosine (PY20 from Oncogene Research Products, Cambridge, Mass., and 4G10 from Upstate Biotechnology, Lake Placid, N.Y.), antiactin (C11; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), anti-Bcl-2 (N-17; Santa Cruz), and anti-Bcl-x (Transduction Laboratories, Lexington, Ky.).

**Luciferase assay.** The *RAD51* promoter transactivation assay was performed as described (73) with modifications. Tk<sup>-</sup>ts13 cells were transiently transfected by calcium phosphate (67, 68) with plasmids encoding FTKs or empty plasmid, *STAT5*-DNM or empty plasmid, as well as the *RAD51* reporter plasmid containing the fused CpG-rich region of the human *RAD51* promoter, the simian virus 40 (SV40) promoter, and the luciferase gene (*RAD51*-SV40-luc). The negative control plasmid encoded the SV40 promoter fused to the luciferase gene only (SV40-luc). A  $\beta$ -galactosidase plasmid was also transfected into the Tk<sup>-</sup>ts13 cells as a transfection efficiency control. A total of 20  $\mu$ g of cDNA/10-cm plate was used (FTK:DNM ratio, 1:4). At 48 h after transfection, serum-free medium containing 0.1% bovine serum albumin was added to the cells, and 12 h later luciferase was quantified by the luciferase assay system (Promega, Madison, Wis.). Transfection efficiency was normalized by measuring  $\beta$ -galactosidase activity. Transactivation units were calculated as a ratio of the counts from *RAD51*-SV40-luc to the counts from SV40-luc in particular groups.

**GFP-positive cells.** Cells were infected with retrovirus-based particles encoding GFP as described (45), except that ecotropic Bosc23 (obtained from Warren Pear) and amphotropic Phoenix (American Type Culture Collection [ATCC], Manassas, Va.) packaging cells were used to infect murine and human cells, respectively. Transduced cells were collected after 48 to 72 h of cocultivation with

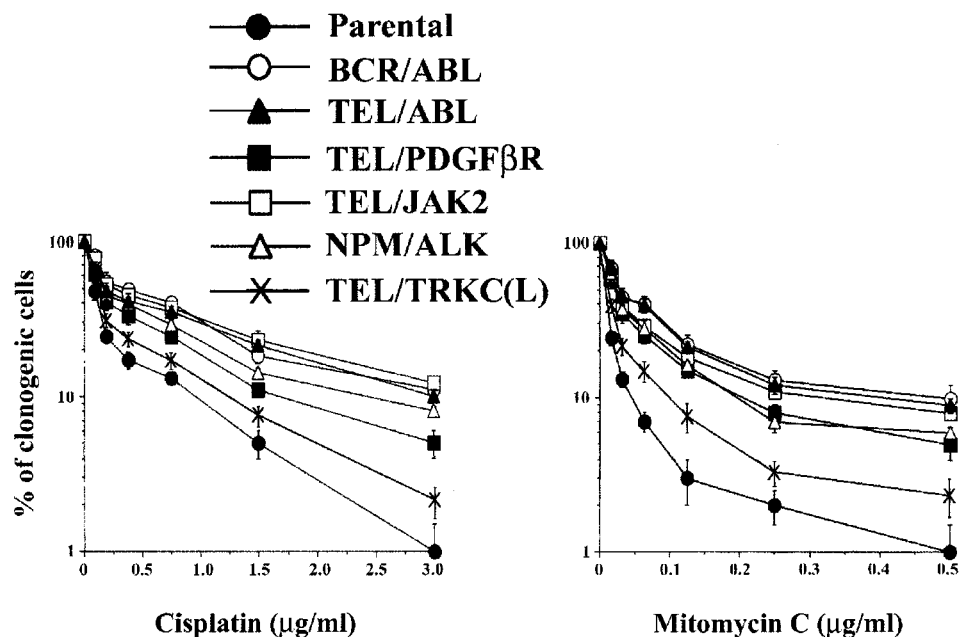


FIG. 1. FTK-induced drug resistance. Parental BaF3 cells and cells expressing BCR/ABL-related FTKs were exposed to cisplatin or mitomycin C and plated in methylcellulose in the presence of IL-3. Cell viability was assessed after 7 days by the clonogenic assay. Results represent the mean  $\pm$  standard deviation (SD) from three independent experiments.

the packaging cell line. GFP-positive cells were isolated by fluorescence-activated cell sorting (Becton Dickinson).

**Inhibition of BCR/ABL kinase.** ABL kinase inhibitor STI571 (16) was obtained from Novartis Pharma AG (Basel, Switzerland). Cells ( $10^6$ /ml) were incubated for 24 h with 1  $\mu$ M STI571 in the presence of IL-3, then washed, and used for experiments.

**HRR assay.** Draa-40 cells (generously provided by M. Jasin, Sloan-Kettering Cancer Center, New York, N.Y.) have integrated one or two copies of the modified gene for GFP (ScGFP) as a recombination reporter and a fragment of the *GFP* gene as a donor for homologous repair (52). ScGFP has an inactivating insertion containing the restriction site for the rare-cutting *I-SceI* endonuclease. When *I-SceI* is expressed in vivo, a DSB results. An HRR event with a donor *GFP* gene fragment restores functional GFP expression, readily detected by flow cytometry. Cells were electroporated as described (52) with 100  $\mu$ g of the *I-SceI* plasmid, 40  $\mu$ g of the plasmid carrying FTK or not (control), and 40  $\mu$ g of the *RAD51* antisense plasmid or empty plasmid, and 48 h later  $5 \times 10^4$  cells were analyzed by flow cytometry for the expression of GFP.

**Modifications of  $G_2/M$  cell cycle.**  $G_2/M$  delay was induced by nocodazole (46). Briefly, 15 ng of nocodazole (Sigma, St. Louis, Mo.) per ml was added to the cell culture 4 h before the drug. Twenty-four hours later, nocodazole and the drugs were removed by washing and replacing the culture medium. This protocol caused transient accumulation of cells in  $G_2/M$  cell cycle detectable after 12 h and 24 h but not after 48 h of nocodazole treatment (data not shown). DNA damage-dependent  $G_2/M$  delay was abolished by treatment with caffeine (84). Briefly, cisplatin or mitomycin C was added at 0 h, followed by addition of 2 mM caffeine at 12 h.

**Cell cycle analysis.** Cells ( $10^6$ ) were fixed in 70% ethanol for 15 min at 4°C, washed, and incubated in 1 ml of phosphate-buffered saline (PBS) containing 0.1% NP-40 and 1 mg of DNase-free RNase (Boehringer Mannheim Co., Indianapolis, Ind.) per ml for 10 min at room temperature. DNA was stained by propidium iodide. Cells were analyzed with a FACSCalibur (Becton Dickinson) using the CellQuest program (70).

**Immunofluorescence.** RAD51 was detected by immunofluorescence as described (73). Briefly, cytospin slides were prepared from GFP<sup>+</sup> CML-BC cells infected with the IRES-GFP or RAD51(AS)-IRES-GFP retrovirus. Cells were fixed with PBS containing 0.02% Triton X-100 and 4% formaldehyde. RAD51 was detected by mouse anti-RAD51 monoclonal antibody (Upstate Biotechnology), followed by tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse immunoglobulin secondary antibody (Molecular Probes Inc., Eugene, Oreg.). Negative controls were performed without primary antibodies.

DNA was counterstained with the DNA fluorochrome 4',6'-diamidino-2-phenylindole (DAPI). Cells were visualized with a Nikon Eclipse E300 fluorescence microscope equipped with a digital camera. Images were prepared with Adobe Photoshop.

## RESULTS

**FTK-transformed cells displayed drug-induced DNA damage but remained drug resistant.** BaF3 murine hematopoietic cells (parental), cells expressing a neomycin phosphotransferase resistance gene (BaF3-*neo*), and cells expressing the BCR/ABL-related FTKs were treated with different concentrations of the DNA cross-linking drug cisplatin or the radiomimetic drug mitomycin C. Cells expressing FTKs displayed resistance to these drugs in comparison to normal cells, as evaluated by the clonogenic assay (Fig. 1). FTK-transformed cells formed more colonies than parental cells after treatment with cisplatin or mitomycin C. Colonies from FTK-positive cells arose even at the drug concentrations that eliminated all colonies from parental cells. Results from the trypan blue dye exclusion test were concordant with those from the clonogenic assay. FTK-transformed cells survived the genotoxic stress better than parental cells (data not shown). TEL/TRKC(L)-positive cells displayed the lowest drug resistance among the cells transformed by various FTKs.

To determine if FTK-induced drug resistance is attributable simply to the protection from drug-induced DNA damage, the comet assay was performed to measure the extent of DNA strand breaks (66). Similar or even higher levels of DNA damage could be detected in FTK-transformed cells in comparison to parental cells or BaF3-*neo* cells (data not shown). Therefore, FTKs did not protect cells from the drug-induced DNA damage.



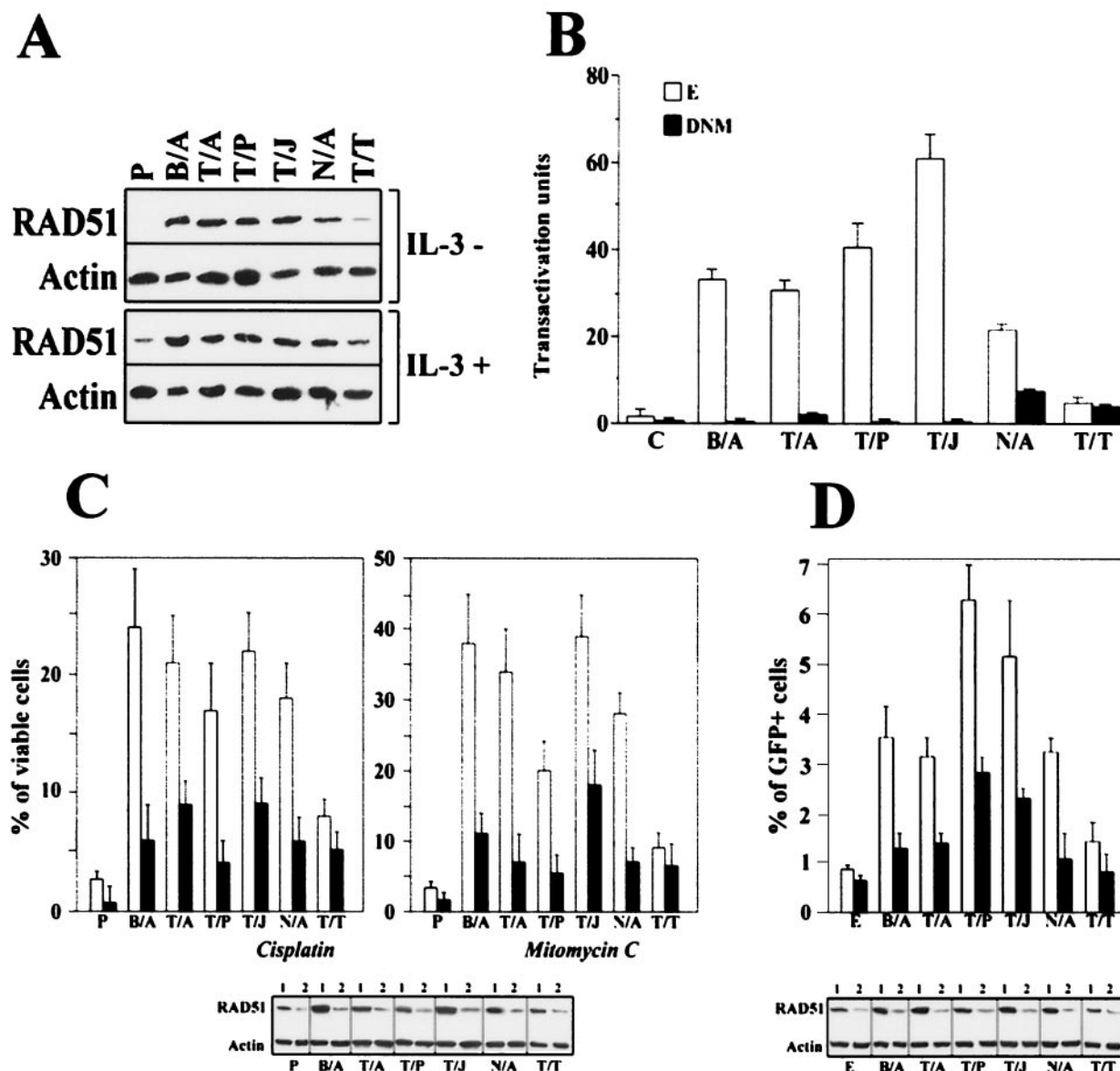


FIG. 2. Role of RAD51 in FTK-mediated drug resistance. (A) Expression of RAD51 in FTK-transformed cells. Total cell lysates were obtained from BaF3 cells (P) and cells transformed by the indicated FTK (BCR/ABL [B/A], TEL/ABL [T/A], TEL/JAK2 [T/J], TEL/PDGFR [T/P], NPM/ALK [N/A], and TEL/TRKC[L] [T/T]) cultured in the presence (+) or absence (-) of IL-3. RAD51 protein expression was assessed by Western analysis. Actin was detected as a loading control. Results are representative of two experiments. (B) Role of STAT5 in FTK-stimulated transactivation of the *RAD51* promoter. The luciferase assay was performed in  $Tk^{-}$  ts13 cells transiently transfected with the plasmid containing the indicated FTK or the empty plasmid (control, C) along with the plasmid carrying STAT5-DNM (DNM) or empty plasmid (E) and the plasmid encoding the luciferase reporter gene driven by the *RAD51* promoter. Luciferase activity is expressed in arbitrary units. (C) Role of RAD51 in FTK-mediated inhibition of drug-induced apoptosis. BaF3 cells (P) and cells stably transfected with FTKs were infected with a retroviral construct containing RAD51(AS)-IRES-GFP (black bars) or with empty IRES-GFP virus (white bars). GFP<sup>+</sup> cells were sorted and cultured in the presence of IL-3 with 1.5  $\mu$ g of cisplatin per ml or 0.06125  $\mu$ g of mitomycin C per ml for 48 h. Cell viability was assessed by the trypan blue dye exclusion test (upper panels). RAD51 expression in GFP<sup>+</sup> cells transfected with IRES-GFP (lanes 1) or RAD51(AS)-IRES-GFP (lanes 2) was determined by Western analysis (lower panel). (D) RAD51-mediated HRR in FTK-positive cells. Draa-40 recombination-reporter cells were cotransfected with plasmids encoding FTKs or empty plasmid (E), *I-SceI* (to induce a DSB within one of the heterozygous GFP alleles), and either an empty plasmid (white bars) or a plasmid containing *RAD51*(AS) cDNA (black bars). GFP<sup>+</sup> cells were counted after 48 h by flow cytometry. RAD51 expression in cells transfected with an empty plasmid (lanes 1) or *RAD51*(AS) cDNA (lanes 2) was determined by Western analysis (lower panel). Results in sections B, C, and D represent the mean  $\pm$  SD from three independent experiments.

**RAD51 plays an essential role in FTK-induced drug resistance.** Our previous findings implicated RAD51 in resistance to radiomimetic and cross-linking drugs in BCR/ABL cells (73). Western analysis showed that cells transformed by vari-

ous FTKs, except TEL/TRKC(L), expressed five to eight times more RAD51 than parental cells growing in the presence of IL-3 (Fig. 2A, panel IL-3+). High levels of RAD51 were also detected in these cells growing in the absence of IL-3 (Fig. 2A,

panel IL-3-). STAT5-mediated transactivation of the *RAD51* promoter and inhibition of RAD51 cleavage by caspase-3 might contribute to elevated expression of RAD51 in BCR/ABL-transformed cells (73). Interestingly, cells expressing TEL/TRKC(L), which did not activate STAT5 (41), also did not elevate RAD51 protein (Fig. 2A).

To obtain more evidence about the role of STAT5 in FTK-dependent overexpression of RAD51, transactivation of the *RAD51* promoter was examined (73). Robust transactivation of the *RAD51* promoter was detected in cells transfected with BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR, and NPM/ALK, but not TEL/TRKC(L) (Fig. 2B). The STAT5-DNM inhibited this effect.

Because RAD51 overexpression appeared to be directly associated with drug resistance in BCR/ABL-transformed cells (73), we sought to determine if RAD51 plays a more general role in drug resistance of the cells transformed by FTKs able to activate STAT5. Genetic studies of mammalian RAD51 are complicated by the fact that it appears to be intimately involved in cellular proliferation (75) and the mouse gene knockout model displays both an embryo- and cell-lethal phenotype (40, 78). To circumvent these problems, we developed a derivative of retroviral construct MigR1 (50) to efficiently transduce isogenic cell lines and transiently affect RAD51 expression. The MigR1 derivative encodes a variant of the murine myeloproliferative sarcoma virus long terminal repeat (LTR) promoter upstream of the *RAD51* antisense [*RAD51*(AS)] gene, an IRES, and the *GFP* gene.

Parental and FTK-transformed cells were infected with the MigR1 (control) or MigR1-*RAD51*(AS) plasmid, respectively, and GFP<sup>+</sup> cells were isolated by fluorescence-activated cell sorter. RAD51 protein expression in FTK (BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR, and NPM/ALK)-positive GFP<sup>+</sup> cells transfected with *RAD51* antisense cDNA was downregulated to the levels detectable in GFP<sup>+</sup> parental cells transfected with empty plasmid (Fig. 2C, lower panel). Expression of RAD51 in GFP<sup>+</sup> TEL/TRKC(L) cells was similar to that in parental cells. GFP<sup>+</sup> cells were incubated with cisplatin or mitomycin C, and viable cells were detected by using the trypan blue exclusion test. The clonogenic assay could not be applied at this time because downregulation of RAD51 may have an adverse effect on hematopoietic cell proliferation after 5 to 7 days of culture (data not shown).

Both tests, the clonogenic assay and trypan blue exclusion assay, demonstrated concordance for determining drug resistance and survival following drug treatment (73). Inhibition of RAD51 expression reduced the resistance to cisplatin and mitomycin C in FTK (BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR, and NPM/ALK)-transformed cells (Fig. 2C, upper panels). Diminished levels of RAD51 did not significantly alter the sensitivity of parental cells and TEL/TRKC(L)-positive cells to these drugs. Taken together, these observations and previous findings (73) clearly implicate RAD51 in the drug resistance of FTK-positive hematopoietic malignancies overexpressing RAD51.

Drug resistance induced by the BCR/ABL→RAD51 pathway was associated with enhanced HRR of DSBs (73). Thus, we examined if other FTKs are also able to stimulate HRR. The frequency of DSB repair in cells can be quantitated by using a stable cell line which contains heteroallelic nonfunc-

tional fragments of the GFP protein (52). In this system, recombination is induced by transfection of the *I-SceI* restriction enzyme, which results in a DSB within one of the GFP parental alleles. HRR of this DSB can result in the expression of a functional GFP protein, which may be identified as GFP-positive cells (52). We observed a significant ( $P < 0.05$ ) increase in the percentage of GFP<sup>+</sup> cells after cotransfection with FTKs (BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR, NPM/ALK) and *I-SceI* compared to empty vector and *I-SceI* (Fig. 2D). Moreover, cotransfection of the *RAD51*(AS) with the *I-SceI* resulted in a significant ( $P < 0.05$ ) reduction of GFP repair induced by these FTKs (Fig. 2D). Interestingly, TEL/TRKC(L), which did not elevate RAD51 expression, exhibited a reduced ability to stimulate GFP repair (Fig. 2D). Taken together, these results link RAD51-dependent recombination repair with the drug resistance of FTK-transfected cells overexpressing RAD51.

**Elevated level of RAD51 is not the only factor contributing to drug resistance in FTK-transformed cells.** To determine if RAD51 is solely responsible for the drug resistance of FTK-transformed cells, we took advantage of the BCR/ABL-selective inhibitor STI571 (16). Inhibition of BCR/ABL kinase activity by STI571 (Fig. 3A, lower panel, compare lanes 3 and 4) was associated with abrogation of drug resistance (Fig. 3B) and downregulation of RAD51 protein (Fig. 3A, upper panel, compare lanes 3 and 4). This observation, however, did not quantify the contribution of RAD51 to the drug resistance phenotype because other genes and mechanisms are also dysregulated after inhibition of the BCR/ABL kinase (21, 48).

To estimate the importance of RAD51 for drug resistance in BCR/ABL cells, high expression of RAD51 following STI571 treatment was rescued by an ectopic *RAD51* gene driven by the LTR promoter. A mixture of clones expressing BCR/ABL and ectopic RAD51 was exposed to STI571 to inhibit the BCR/ABL kinase and its downstream effectors excluding the RAD51 protein (Fig. 3A, upper panel, compare lanes 5 and 6). STI571-treated cells expressing BCR/ABL plus ectopic RAD51 maintained their drug resistance phenotype, although not to the same extent as the untreated BCR/ABL cells (Fig. 3B). Moreover, parental 32Dcl3 cells displaying elevated levels of RAD51 (Fig. 3A, upper panel, compare lanes 1 and 2) acquired modest drug resistance, similar to that observed in STI571-treated BCR/ABL plus ectopic RAD51 cells (Fig. 3B). This suggests that another mechanism(s), in addition to that dependent on RAD51, contributes to the drug resistance in FTK-transformed cells.

**Collaboration between RAD51, G<sub>2</sub>/M delay, and Bcl-xL in drug resistance.** Previous findings indicated that BCR/ABL stimulates RAD51, Bcl-xL (but not Bcl-2), and G<sub>2</sub>/M delay, which contributes to the resistance to genotoxic stress (1, 4, 73). We investigated if these processes are regulated by other FTKs. Western analysis and the DSB repair assay demonstrated that RAD51 is stimulated by the FTK→STAT5 pathway (Fig. 2A and 2B). The levels of Bcl-xL but not Bcl-2 protein were also upregulated by FTKs able to activate STAT5 (Fig. 4A). Parental BaF3 and FTK (BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR, and NPM/ALK)-positive cells growing in the presence of IL-3 accumulated in G<sub>2</sub>/M phase 12 h after drug treatment, but only the latter cells remained accumulated in G<sub>2</sub>/M after 16 h (percentages: parental, 12% ±

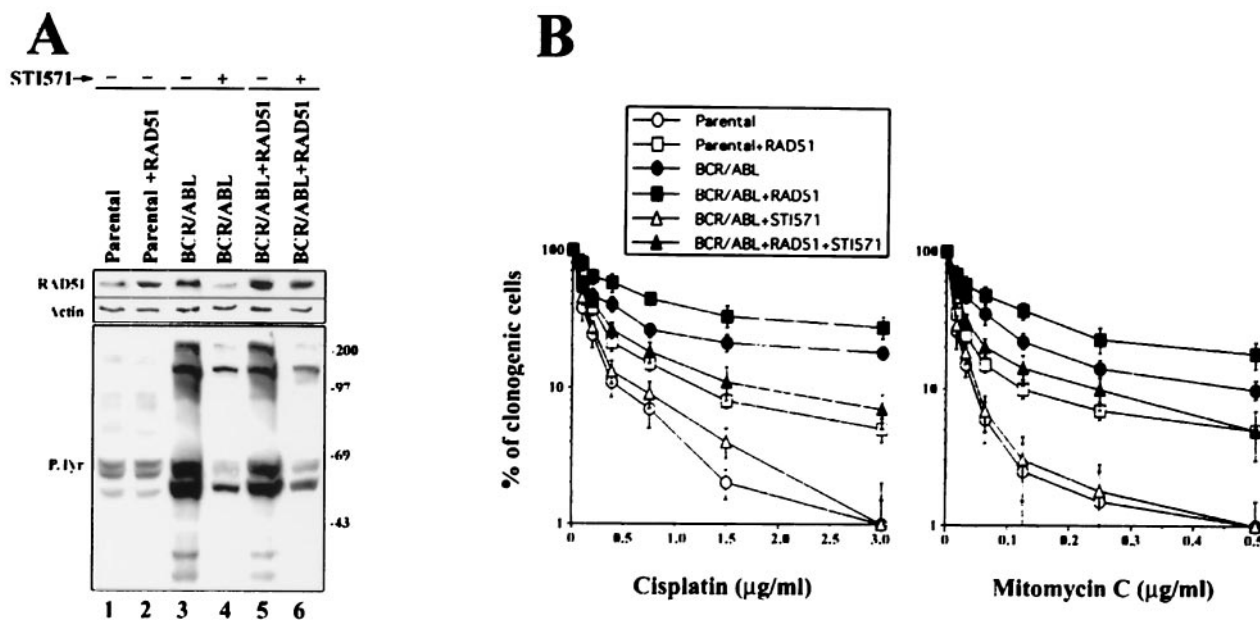


FIG. 3. Overexpression of RAD51 only partially restored therapeutic drug resistance of BCR/ABL cells treated with STI571. 32Dcl3 cells (Parental) and BCR/ABL-expressing cells were infected with a retrovirus encoding RAD51-IRES-GFP (Parental+RAD51 and BCR/ABL+RAD51 cells, respectively) or IRES-GFP (Parental and BCR/ABL cells, respectively). GFP-positive cells were isolated by fluorescence-activated cell sorting, and the ABL kinase-selective inhibitor STI571 (1  $\mu$ M) was added (+) or not (-) for 24 h in the presence of IL-3. (A) Expression of RAD51 and tyrosine phosphorylation of cellular proteins were examined by immunoblotting with anti-RAD51 antibody and antiphosphotyrosine antibody, respectively. Results are representative of two independent experiments. (B) Cells were plated in methylcellulose in the presence of IL-3 with the indicated concentrations of cisplatin or mitomycin C. Colonies were counted 7 days later. Results show the percentage of clonogenic cells (mean  $\pm$  SD) from three independent experiments.

2%, versus BCR/ABL, 39%  $\pm$  5%; TEL/ABL, 33%  $\pm$  4%; TEL/PDGFR $\beta$ , 37%  $\pm$  4%; TEL/JAK2, 44%  $\pm$  4%; and NPM/ALK, 40%  $\pm$  3%;  $P < 0.05$ ) and 24 h (percentages: parental, 10%  $\pm$  3%, versus BCR/ABL, 29%  $\pm$  4%; TEL/ABL, 27%  $\pm$  4%; TEL/PDGFR $\beta$ , 25%  $\pm$  5%; TEL/JAK2, 32%  $\pm$  4%; and NPM/ALK, 28%  $\pm$  3%;  $P < 0.05$ ) (Fig. 4B). Similar G<sub>2</sub>/M delay was induced in BCR/ABL-transformed 32Dcl3 cells after treatment with cisplatin and mitomycin C (data not shown). TEL/TRKC(L)-transformed cells exhibited accumulation in S phase at 12 h after the treatment, but at 16 h and 24 h a significant proportion of these cells was also accumulated in G<sub>2</sub>/M (28%  $\pm$  4% and 31%  $\pm$  3%, respectively;  $P < 0.05$  in comparison to parental cells) (Fig. 4B). At 48 h after treatment, FTK-positive cells did not display G<sub>2</sub>/M accumulation (data not shown). Thus, all FTK-positive cells examined displayed G<sub>2</sub>/M delay upon DNA damage.

Lack of delayed accumulation in G<sub>2</sub>/M phase was accompanied by an increased percentage of apoptotic cells containing subdiploid amounts of DNA after 16 h (percentages: parental, 28%  $\pm$  4%, versus BCR/ABL, 2%  $\pm$  1%; TEL/ABL, 0.5%  $\pm$  0.3%; TEL/PDGFR $\beta$ , 1%  $\pm$  1%; TEL/JAK2, 2.5%  $\pm$  1%; NPM/ALK, 2%  $\pm$  1.5%;  $P < 0.05$ ) and 24 h (percentages: parental, 56%  $\pm$  7%, versus BCR/ABL, 6%  $\pm$  3%; TEL/ABL, 7%  $\pm$  2.5%; TEL/PDGFR $\beta$ , 7.5%  $\pm$  4%; TEL/JAK2, 5.5%  $\pm$  2%; NPM/ALK, 5%  $\pm$  3.5%;  $P < 0.05$ ). Fewer TEL/TRKC(L)-positive cells than parental cells displayed subdiploid amounts of DNA at 16 h and 24 h after treatment (10%  $\pm$  3% and 24.5%  $\pm$  5% versus 28%  $\pm$  4% and 56%  $\pm$  7%, respectively;  $P < 0.05$ ), but more than cells expressing other

FTKs (BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR $\beta$ , and NPM/ALK).

These processes (RAD51, Bcl-xL, and G<sub>2</sub>/M delay) are regulated independently, because stimulation of a single factor did not affect the others. For example, overexpression of RAD51 did not influence Bcl-xL or G<sub>2</sub>/M, overexpression of Bcl-xL did not affect RAD51 or G<sub>2</sub>/M, and G<sub>2</sub>/M delay had no effect on RAD51 or Bcl-xL (Fig. 5A and B). Individually, elevated expression of RAD51 (Fig. 5A, lane 3) or Bcl-xL (Fig. 5A, lane 4) and G<sub>2</sub>/M delay (Fig. 5B, panel 5) caused only moderate drug resistance in parental cells (Fig. 5C).

Elevated expression of RAD51 and Bcl-xL and/or G<sub>2</sub>/M delay may work in concert to protect FTK-transformed cells from drug-induced cytotoxicity. This hypothesis was tested by using three different approaches: (i) cells overexpressing RAD51 were treated with nocodazole to induce transient G<sub>2</sub>/M delay (Fig. 5B, panel 7); (ii) cells overexpressing Bcl-xL were transfected with RAD51 cDNA to enhance its expression (Fig. 5A, lane 6); and (iii) cells overexpressing RAD51 and Bcl-xL were treated with nocodazole (Fig. 5B, panel 8). Although elevated expression of Bcl-xL and RAD51 or elevated expression of RAD51 and transient G<sub>2</sub>/M delay enhanced drug resistance, its magnitude was below that observed in cells transformed with BCR/ABL (Fig. 5C). However, induction of G<sub>2</sub>/M delay (Fig. 5B, panel 8) in cells overexpressing RAD51 and Bcl-xL (Fig. 5A, lane 8) caused drug resistance similar to that observed in BCR/ABL-transformed cells (Fig. 5C). Thus, these three mechanisms can work in a synergistic or additive fashion.

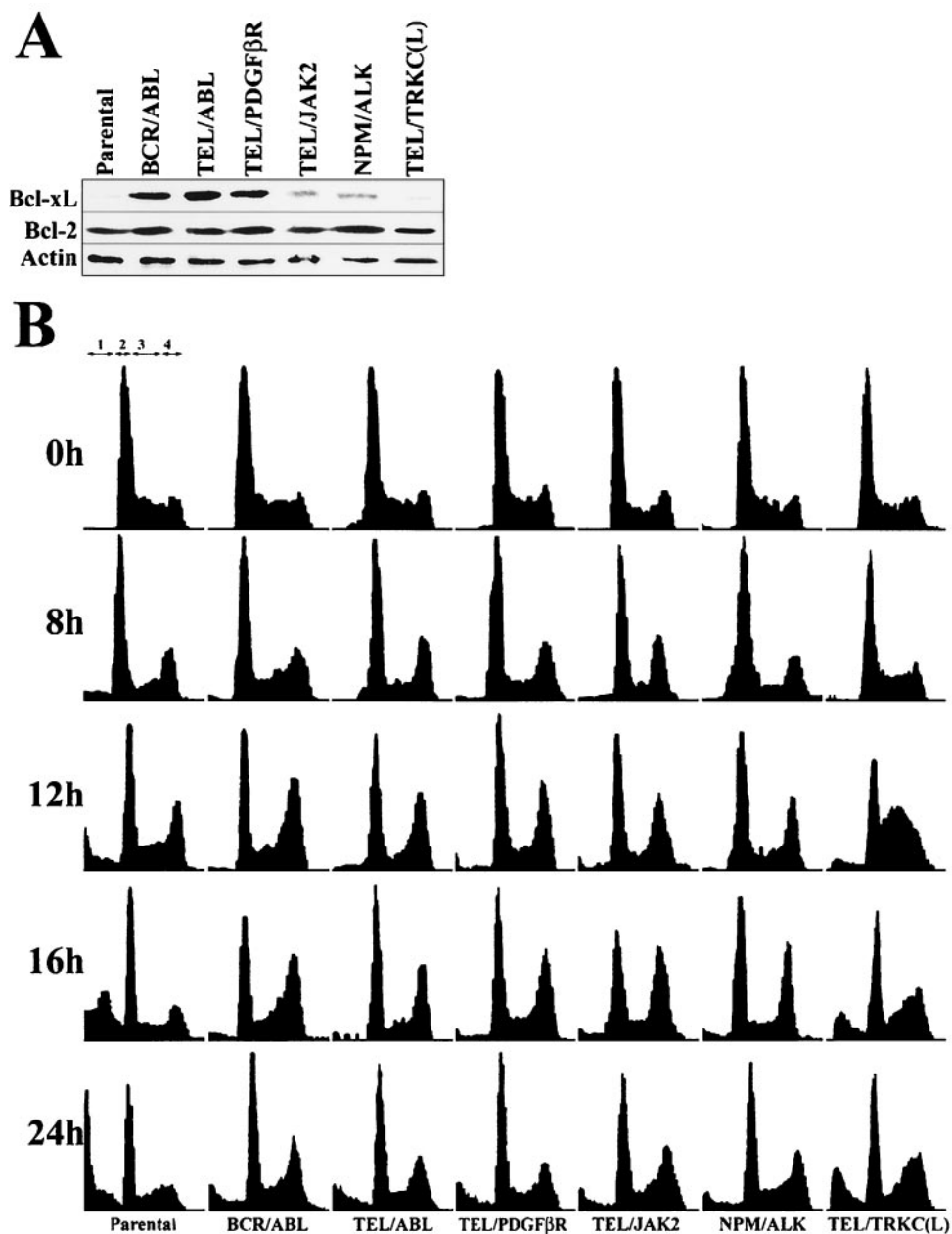


FIG. 4. Expression of Bcl-xL and induction of DNA damage-dependent  $G_2/M$  delay in FTK-transformed cells. (A) Western analysis of the expression of Bcl-xL and Bcl-2 in BaF3 parental cells (Parental) and in cells transformed with the indicated FTK and cultured in the presence of IL-3. Actin was detected to show the protein load. (B) The same cells were treated with  $1 \mu\text{g}$  of cisplatin per ml, and cell cycle distribution was analyzed 0, 8, 12, 16, and 24 h later by flow cytometry after staining of DNA with propidium iodide (cell cycle phases: 1, subdiploid; 2,  $G_0/G_1$ ; 3, S; and 4,  $G_2/M$ ). Results represent two independent experiments.

**Role of RAD51, Bcl-xL, and  $G_2/M$  delay in drug resistance of BCR/ABL-positive primary leukemia cells.** Since the role of RAD51, Bcl-xL, and the  $G_2/M$  checkpoint in FTK-dependent drug resistance has been described in cell lines, the relevance of these mechanisms in primary cells needed to be addressed. RAD51 was found to be overexpressed in CML cells (73), which exhibited drug resistance (e.g., an average of  $23.5\% \pm 3\%$  and  $18\% \pm 1.5\%$  of  $CD34^+$  bone marrow cells obtained from three CML-BC patients survived treatment with  $1.5 \mu\text{g}$  of cisplatin and  $0.06125 \mu\text{g}$  of mitomycin C, respectively, in com-

parison to  $7.5\% \pm 2\%$  and  $5\% \pm 2\%$  of bone marrow cells from three normal donors, respectively [data not shown];  $P < 0.05$ ).

Downregulation of RAD51 protein in CML cells by expression of the *RAD51* antisense construct (Fig. 6A, left panel) increased their sensitivity to cisplatin and mitomycin C (Fig. 6A, right panel). Expression of BCR/ABL also induced drug resistance in primary murine bone marrow cells (e.g.,  $50.5\% \pm 5\%$  and  $32\% \pm 5.5\%$  of BCR/ABL-positive cells [Fig. 6C, right panel, bars 3 and 7, respectively] versus  $16.5\% \pm 2.5\%$  and  $2\% \pm 1.5\%$  of normal cells [data not shown]) were able to form



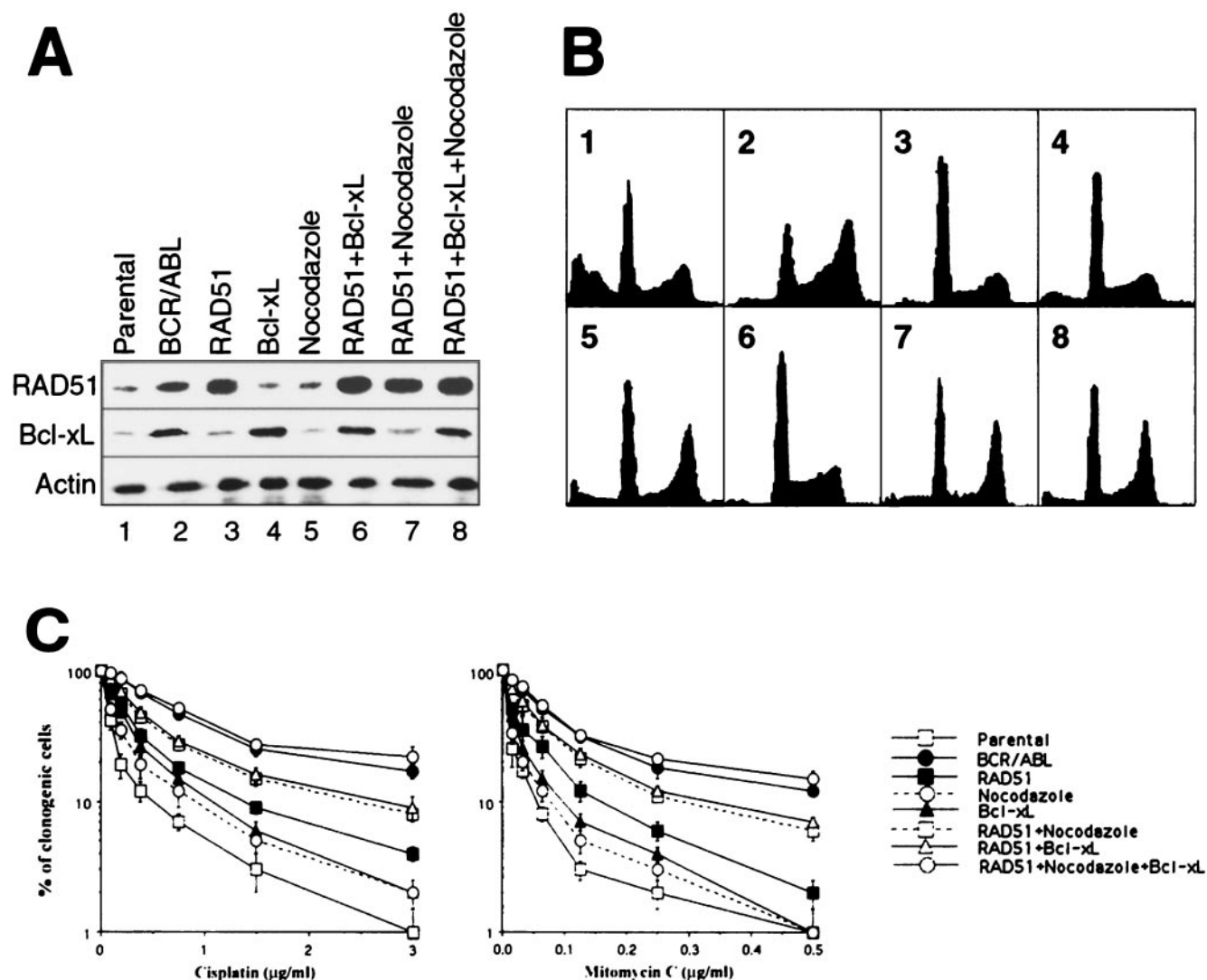


FIG. 5. RAD51, Bcl-xL, and G<sub>2</sub>/M delay worked in concert to induce drug resistance. (A) Expression of RAD51 and Bcl-xL (Western analysis) and (B) cell cycle (propidium iodide staining followed by flow cytometry) were examined in 32Dcl3 parental cells (parental) (lane A1, panel B1) and clones transfected with BCR/ABL (lane A2, panel B2), RAD51 (lane A3, panel B3), Bcl-xL (lane A4, panel B4), or RAD51 and Bcl-xL (lane A6, panel B6). Nocodazole was applied to induce transient G<sub>2</sub>/M arrest in parental cells (lane A5, panel B5) and in cells transfected with RAD51 (lane A7, panel B7) or RAD51 and Bcl-xL (lane A8, panel B8). (C) Cells characterized in panels A and B were plated in methylcellulose in the presence of IL-3 and cisplatin or mitomycin C. Colonies were scored after 7 days. Results represent three independent experiments (mean ± SD).

colonies after treatment with 0.375 μg of cisplatin per ml or 0.1 μg of mitomycin C per ml, respectively;  $P < 0.05$ ).

Overexpression of Bcl-xL is detectable in BCR/ABL-transformed primary murine bone marrow cells (Fig. 6B), and its role in protection of BCR/ABL primary leukemia cells from apoptosis has already been reported (30). In addition, a significant difference in accumulation of BCR/ABL-positive primary murine bone marrow cells and control cells in G<sub>2</sub>/M phase in response to cisplatin treatment was detected (percentages in G<sub>2</sub>/M phase after 24 h and 48 h, 38% ± 7% and 13% ± 3% of control cells versus 61% ± 6% and 35% ± 5% of BCR/ABL-positive cells, respectively;  $P < 0.05$ ) (Fig. 6C, left panel). The kinetics of G<sub>2</sub>/M accumulation of primary cells is delayed in comparison to that of cell lines (cf. Fig. 4B and 6C), probably

reflecting differences in proliferation rates of BaF3 cells in comparison to primary cells.

Prolonged G<sub>2</sub>/M accumulation of BCR/ABL-positive cells in comparison to control cells observed at 48 h after the treatment was accompanied by inhibition of the former cells, exhibiting the signature of apoptosis (subdiploid amount of DNA) (4.5% ± 2.5% in comparison to 30.5% ± 5%, respectively,  $P < 0.05$ ). Disruption of G<sub>2</sub>/M delay by caffeine (for example see Fig. 6C, right panel box) significantly increased drug sensitivity of BCR/ABL cells (Fig. 6C, right panel). In conclusion, all three mechanisms, overexpression of RAD51 and Bcl-xL and G<sub>2</sub>/M delay, seem to work in the primary hematopoietic cells transformed by BCR/ABL and possibly also by other FTKs, such as TEL/ABL, TEL/JAK2, TEL/PDGFR, and NPM/ALK.



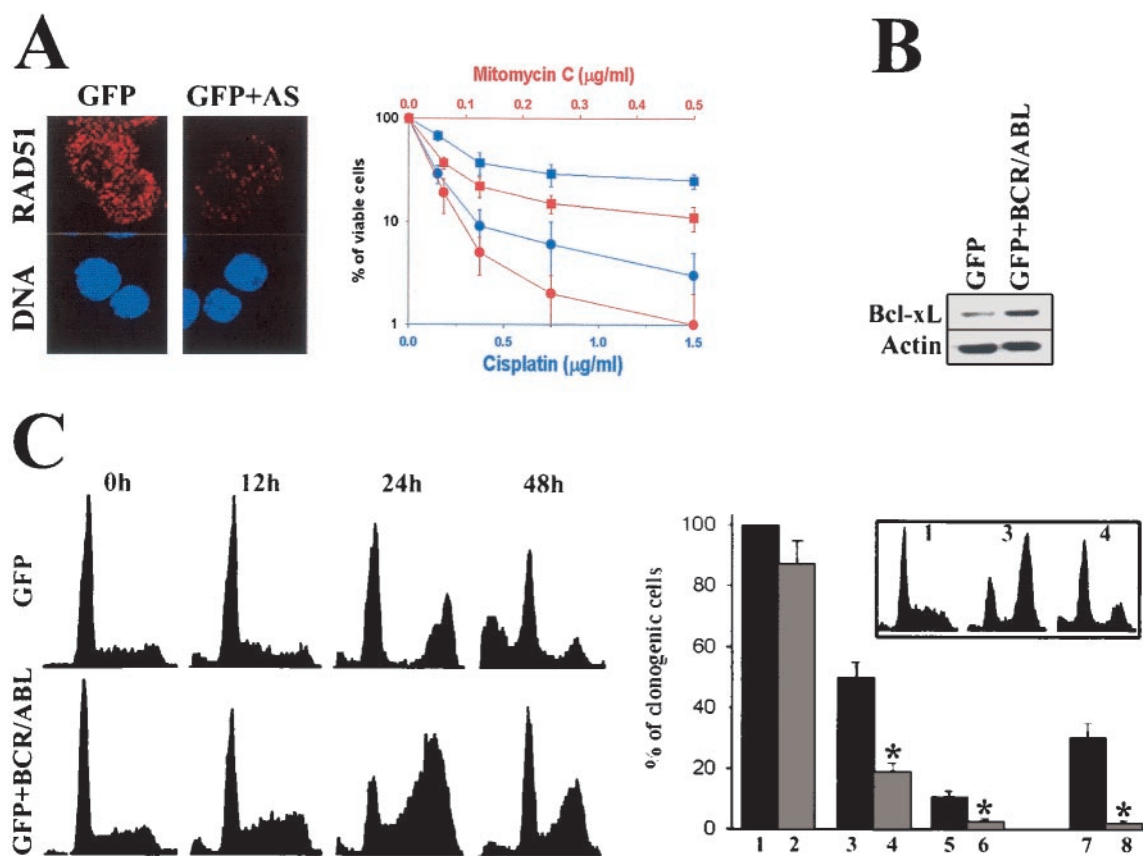


FIG. 6. Overexpression of RAD51 and Bcl-xL and G<sub>2</sub>/M delay are exhibited by BCR/ABL-positive primary leukemia cells: role in drug resistance. (A) Downregulation of RAD51 and Bcl-xL and G<sub>2</sub>/M delay are exhibited by BCR/ABL-positive primary leukemia cells: role in drug resistance. CML-BC patient cells were infected with RAD51(AS)-IRES-GFP virus (GFP+AS, ●) or with IRES-GFP empty virus (GFP, ■). Downregulation of RAD51 in GFP-positive cells was determined by immunofluorescence analysis visualizing the levels of endogenous RAD51 (left panel). Drug sensitivity was assessed by the trypan blue exclusion test after 48 h of exposure to the indicated concentrations of cisplatin or mitomycin C. (B) BCR/ABL enhances expression of Bcl-xL in primary bone marrow cells. Murine bone marrow cells were infected with BCR/ABL-IRES-GFP virus (GFP+BCR/ABL) or IRES-GFP (GFP) empty virus. Bcl-xL expression was examined by Western analysis in GFP-positive cells. (C) BCR/ABL causes G<sub>2</sub>/M delay essential for drug resistance. GFP+BCR/ABL-positive cells and GFP-positive control cells were treated with 0.375 μg of cisplatin per ml in the presence of IL-3, and cell cycle analysis was performed after 0, 12, 24, and 48 h (left panel). GFP+BCR/ABL cells cultured in the presence of IL-3 were left untreated (1) or treated with 2 mM caffeine (2), 0.375 μg of cisplatin per ml (3), 0.375 μg of cisplatin per ml plus 2 mM caffeine (4), 1.5 μg of cisplatin per ml (5), 1.5 μg of cisplatin per ml plus 2 mM caffeine (6), 0.1 μg of mitomycin C per ml (7), or 0.1 μg of mitomycin C per ml plus 2 mM caffeine (8). Twenty-four hours later, cells were plated in methylcellulose, and colonies were scored after 7 days (right panel). Results are presented as the percentage of colonies in experimental samples in comparison to the untreated control sample 1 (602 ± 37 colonies arose from 10<sup>3</sup> untreated GFP+BCR/ABL cells). \*, *P* < 0.05 in comparison to the corresponding group not treated with caffeine. Cell cycle analysis of the selected samples (1, 3, and 4) was performed after 24 h of treatment to confirm that caffeine abolished accumulation of the cells in G<sub>2</sub>/M. Results represent two experiments.

## DISCUSSION

Resistance to DNA damage caused by radiation or cytostatic drugs creates a major problem for effective antitumor therapy. Usually, it does not appear to be a direct consequence of malignant transformation, but rather arises as a result of selection of the tumor cell clones which are able to develop protective mechanisms and to survive genotoxic treatment (22, 27, 28). However, malignancies induced by oncogenic tyrosine kinases such as BCR/ABL, v-SRC and HER2/neu display early drug resistance (4, 43, 53), excluding the selection process. For example, resistance to radiation and cytostatic drugs was observed in the untreated chronic phase of CML at diagnosis (4).

Here, we report that members of the family of BCR/ABL-related FTKs [BCR/ABL, TEL/ABL, TEL/JAK2, TEL/

PDGFβR, NPM/ALK, and TEL/TRKC(L)] are able to induce drug resistance. FTK-transformed cells displayed similar or sometimes even more DNA strand breaks than normal cells after incubation with cisplatin or mitomycin C. These drugs do not react with DNA in a manner that would lead directly to strand breaks, and therefore the breaks must arise as indirect, secondary DNA lesions in the attempt to repair primary damage (82). When a replication fork encounters a drug-induced DNA lesion, it causes stalling of the replication process. To resolve this problem, single-strand breaks and/or DSBs occur; the latter are usually lethal if not repaired (34).

DSBs associated with replication forks are predominantly repaired by RAD51-dependent homologous recombination (3). This process is crucial for reinitiation of DNA replication

on collapsed replication forks. We postulate that FTKs can stimulate HRR, resulting in drug resistance. In accordance with this hypothesis, we report that RAD51 was upregulated by various FTKs (BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR, and NPM/ALK) in a STAT5-dependent manner and played an essential role in facilitation of HRR (73; this work). This process might efficiently eliminate DSBs caused by cisplatin and mitomycin C and diminish signaling to apoptosis. In comparison, nontransformed cells express basal levels of RAD51, and thus DSBs are resolved with lower efficiency.

Interestingly, expression of RAD51 and drug resistance of TEL/TRKC(L)-transformed cells were significantly reduced in comparison to those in cells transformed by other FTKs (BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR, and NPM/ALK). Although TEL/TRKC(L) is a potent activator of the phospholipase C $\gamma$ , phosphoinositol 3-kinase, SHC, and mitogen-activated protein kinase, in striking contrast to other FTKs, it does not activate STAT5 (41). The observation implicates the FTK $\rightarrow$ STAT5 $\rightarrow$ downstream effector (e.g., RAD51 and Bcl-xL) pathway in drug resistance (73, 74). This hypothesis is strengthened by the recent evidence that a *STAT5* dominant-negative mutant was able to sensitize BCR/ABL-positive cells to DNA damage-induced apoptosis (29, 65).

In general, there appears to be a fundamental correlation between the expression of RAD51 and experimental resistance to therapeutic drugs (79, 81). Elevated levels of RAD51 have been linked to chlorambucil resistance in B-cell chronic lymphocytic leukemia (10) and with enhanced survival of pancreatic adenocarcinoma after treatment with the DSB-inducing therapeutic drug calicheamicin  $\gamma$ 1 (42). Conversely, downregulation of RAD51 increased the radiosensitivity of prostate cancer cells and malignant glioma cells (13, 49).

RAD51 has also been suggested to play an essential role in the repair of chromosome breaks in normal proliferating cells (75). Therefore, a basal level of RAD51 appears to be required for the processing of spontaneous DNA lesions. Oncogenes that alter the expression, phosphorylation, nuclear localization, and/or function of RAD51 may enhance global DNA repair efficiency and lead to drug resistance. In addition to its role in drug resistance, elevated expression or phosphorylation of RAD51 may be responsible for intrachromosomal or interchromosomal deletions and chromosomal translocations, usually mediated by homologous recombination between regions of shared homology (e.g., *Alu* sequences) (for a review, see reference 5). This hypothesis is supported by the recent finding that large submicroscopic deletions in regions with high overall density of *Alu* sequences repeats have been detected in BCR/ABL-positive leukemias (36). RAD51-dependent homologous recombination can also facilitate loss of heterozygosity by gene conversion mechanisms. Moreover, in certain conditions RAD51 can permit exchange between DNA strands containing short nonhomologous sequences (7). Thus, even modest changes in the cellular levels or the activity of RAD51 may not only cause drug resistance but also promote genomic instability.

Elevated expression of RAD51 induced drug resistance, but not of the same magnitude as that observed in FTK-positive cells. This observation suggested that additional mechanisms are stimulated by FTKs which contribute to drug resistance. Previous reports implicated upregulation of Bcl-xL (1) and

G<sub>2</sub>/M delay (4, 46) in BCR/ABL-dependent drug resistance. Bcl-xL can prevent apoptosis by inhibition of the release of cytochrome *c* from mitochondria and subsequent activation of caspase-3 (31). G<sub>2</sub>/M delay seems to provide more time to repair potentially lethal DNA lesions (8). We report here that FTKs [BCR/ABL, TEL/ABL, TEL/JAK2, TEL/PDGFR, and NPM/ALK but not TEL/TRKC(L)] enhanced Bcl-xL expression and that all FTK-positive cells tested displayed G<sub>2</sub>/M delay upon drug treatment. Thus, at least three mechanisms can eventually contribute to drug resistance induced by FTKs: RAD51-mediated HRR, elevation of Bcl-xL expression, and G<sub>2</sub>/M delay. Each mechanism was able to induce modest drug resistance in parental cells. Interestingly, simultaneous overexpression of RAD51 and Bcl-xL and induction of G<sub>2</sub>/M delay caused the strongest drug resistance. These observations indicate that various mechanisms may work in concert to protect leukemic cells from apoptosis induced by DNA damage.

Enhanced expression of RAD51 and Bcl-xL combined with G<sub>2</sub>/M delay induced drug resistance in parental cells of the magnitude similar to that observed in cells transformed by FTKs able to activate STAT5. It is tempting to speculate that combination of these factors is sufficient for the drug resistance, especially because there is genetic evidence suggesting that cells in G<sub>2</sub> phase repair their DSBs by using primarily the HRR mechanism (20). This hypothesis, however, should be tempered by the fact that overexpression of RAD51 and Bcl-xL proteins in parental cells (even if comparable to that observed in FTK-transformed cells) has been driven by viral promoters. Moreover, in some circumstances ectopically overexpressed Bcl-xL could partially downmodulate HRR induced by elevated levels of RAD51 (58). Also, the mechanism of G<sub>2</sub>/M delay caused by nocodazole may be at least partially different from that induced by DNA damage.

G<sub>2</sub>/M phase is generally controlled by two checkpoints, at the G<sub>2</sub> $\rightarrow$ M transition (G<sub>2</sub> checkpoint) and at the metaphase $\rightarrow$ anaphase transition (M checkpoint) (14, 62). G<sub>2</sub> checkpoint depends on the mechanisms regulating the Cdc2-cyclin B1 complex, such as ATM $\rightarrow$ Chk1 $\rightarrow$ serine phosphorylation of Cdc25C phosphatase, driving it outside the nucleus and preventing activation of Cdc2-cyclin B1, and p53 $\rightarrow$ p21 and 14-3-3 $\sigma$ , resulting in inactivation and sequestration of the Cdc2-cyclin B1 complex in the cytoplasm (8). The M checkpoint is controlled by the mitotic spindle assembly mechanisms (62). Genetic and biochemical evidence showed that the G<sub>2</sub> and M checkpoints could be activated by DNA lesions to prolong G<sub>2</sub>/M phase and reduce the cytotoxicity of DNA damage (9, 32, 39, 84).

The mechanisms of G<sub>2</sub>/M delay in FTK-positive cells have not been characterized, but regulation of Cdc2-cyclin B1 could be involved (46). Although there is no direct evidence that FTKs can affect the M checkpoint, CML cells contain elevated levels of MAD2 and BUB1 (T. Skorski, unpublished observation), which inhibit the anaphase-promoting complex and cause mitotic spindle arrest (62). Thus, it seems reasonable to speculate that both the G<sub>2</sub> and M checkpoints may contribute to G<sub>2</sub>/M delay in FTK-positive cells treated with DNA-damaging agents. Nocodazole, a mitotic spindle inhibitor (83), does not affect the Cdc2-cyclin B1 complex, but requires BUB1 and MAD2 to exert cell cycle delay (46, 80). Therefore, the G<sub>2</sub>/M delay induced in parental BaF3 cells by nocodazole probably

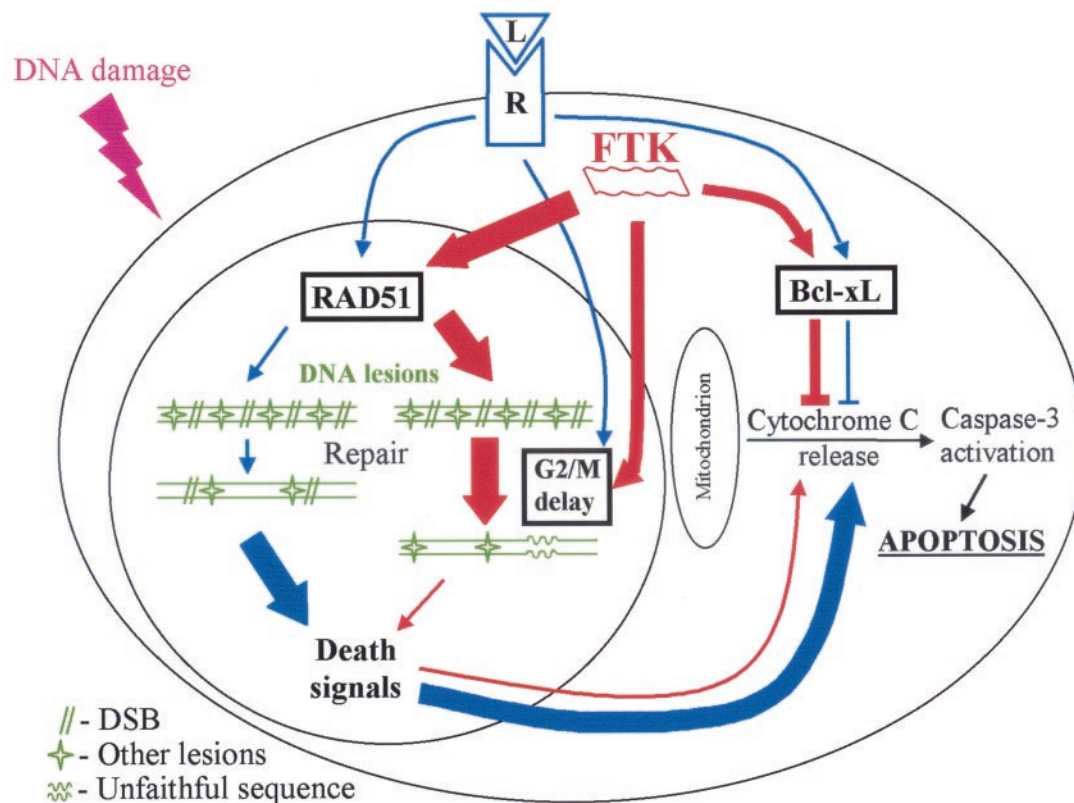


FIG. 7. Model of drug resistance induced by FTKs. The basal level of RAD51 in normal hematopoietic cells is regulated by the physiological signaling from the ligand (L)-receptor (R) complex. It can be further modified by DNA damage (tyrosine phosphorylation). RAD51-mediated HRR does not efficiently repair DSBs during a short  $G_2/M$  phase. Remaining DSBs and other lesions trigger death pathways. This pronounced signal could not be inhibited by the basal levels of Bcl-xL resulting in the release of cytochrome *c* from mitochondria and activation of caspase-3. In contrast, FTK-transformed cells contain high levels of RAD51 modified by tyrosine phosphorylation. HRR is pronounced in these cells, and transient arrest in the  $G_2/M$  phase provides additional time for repair. Thus, DSBs are repaired with high efficiency, and apoptotic signaling is significantly diminished. Modest apoptotic signaling in FTK-positive cells can be inhibited by elevated levels of Bcl-xL, which prevent the release of cytochrome *c* from mitochondria and activation of caspase-3. Because of this “antiapoptotic umbrella” provided by Bcl-xL, FTK-transformed cells can tolerate other (possibly mutagenic) DNA lesions. In addition, dysregulated HRR may result in unfaithful repair of DSBs. Taken together, FTK-positive cells can accumulate DNA lesions leading to genomic instability and malignant progression of the disease. Blue, ligand-receptor-induced events; red, FTK-induced events.

does not exactly mimic that observed in FTK-positive cells after DNA damage, but may demonstrate some similarities. Although nocodazole arrests cells after chromosome condensation, cells can decondense their chromosomes and return to  $G_2$  phase before reentering M phase after removal of nocodazole (56). A similar decondensation of chromosomes occurs when  $G_2/M$  checkpoints are triggered by DNA damage (57), perhaps allowing better access of DNA repair machinery to the damaged DNA. In agreement with these observations, we (this work) and others (32) showed that nocodazole-induced  $G_2/M$  delay reduced the genotoxic effect of drugs such as ethyl methanesulfonate, cisplatin, and mitomycin C when examined after 48 h (apoptosis) and 7 days (clonogenicity). In contrast, colcemid did not provide any protective effect shortly (6 h) after DNA damage induced by irradiation (12). This discrepancy may depend on differences in the experimental protocols and/or in the DNA lesions caused by cytostatic drugs and irradiation.

Therefore, results from our experimental model may lead to overestimation of the role of RAD51, Bcl-xL, and  $G_2/M$  delay in

the induction of resistance of FTK-positive leukemias and lymphomas to the cross-linking and radiomimetic drugs. We cannot rule out other mechanisms, such as  $G_1$  cell cycle arrest (14), elevated expression of Bcl-2 (55), and repair of DSBs by nonhomologous end joining (47). However, we (this work) and others (4, 46) did not detect a pronounced  $G_1$  arrest in FTK-transformed cells in response to DNA damage; conversely, the role of  $G_2/M$  was emphasized. Bcl-xL but not Bcl-2 was reported to have a significant inhibitory effect on the activation of caspase-3 in BCR/ABL-transformed cells treated with cytostatics (1), but we cannot exclude that other antiapoptotic pathways may also contribute to drug resistance (59). The recent finding that BCR/ABL downregulates DNA-protein kinase catalytic subunits (15) diminishes the potential role (if any) of nonhomologous end joining in the drug resistance of FTK-transformed cells. Although RAD51-mediated HRR seems to represent the major pathway of repair of lethal DNA lesions in these cells (3), mechanisms such as single-strand annealing, nucleotide excision repair, and base excision repair may be also regulated by the FTKs and contribute to DNA repair.



The collaboration between RAD51, Bcl-xL, and G<sub>2</sub>/M delay to induce drug resistance was described for murine hematopoietic cell lines. Since these three components are also functional in primary leukemia cells expressing BCR/ABL, it seems reasonable to speculate that they may collaborate to induce drug resistance in these cells and possibly in other FTK-positive hematological malignancies.

In conclusion, we hypothesize that FTKs, depending on their ability to activate STAT5, may induce common mechanisms of drug resistance, consisting of the stimulation of RAD51 (facilitated DSB repair), Bcl-xL (inhibition of caspase-3 activation), and/or prolongation of G<sub>2</sub>/M cell cycle phase (more time for DNA repair). These mechanisms can work in concert to participate in therapeutic drug resistance in leukemia/lymphoma cells and to promote genomic instability leading to a more malignant phenotype of the disease (Fig. 7).

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