Bruton’s Tyrosine Kinase as an Inhibitor of the Fas/CD95 Death-inducing Signaling Complex*

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Bruton’s tyrosine kinase (BTK) is a member of the Src-related Tec family of protein tyrosine kinases. Mutations in the btk gene have been linked to severe developmental blocks in human B-cell ontogeny leading to X-linked agammaglobulinemia. Here, we provide unique biochemical and genetic evidence that BTK is an inhibitor of the Fas/APO-1 death-inducing signaling complex in B-lineage lymphoid cells. The Src homology 2, pleckstrin homology (PH), and kinase domains of BTK are all individually important and apparently indispensable, but not sufficient, for its function as a negative regulator of Fas-mediated apoptosis. BTK associates with Fas via its kinase and PH domains and prevents the FAS-FADD interaction, which is essential for the recruitment and activation of FLICE by Fas during the apoptotic signal. Fas-resistant DT-40 lymphoma B-cells rendered BTK-deficient through targeted disruption of the btk gene by homologous recombination knockout underwent apoptosis after Fas ligation, but wild-type DT-40 cells or BTK-deficient DT-40 cells reconstituted with wild-type human btk gene did not. Introduction of an Src homology 2 domain, a PH domain, or a kinase domain mutant human btk gene into BTK-deficient cells did not restore the resistance to Fas-mediated apoptosis. Introduction of wild-type BTK protein by electroporation rendered BTK-deficient DT-40 cells resistant to the apoptotic effects of Fas ligation. BTK-deficient RAMOS-1 human Burkitt’s leukemia cells underwent apoptosis after Fas ligation, whereas BTK-positive NALM-6-UM1 human B-cell precursor leukemia cells expressing similar levels of Fas did not. Treatment of the anti-Fas-resistant NALM-6-UM1 cells with the leflunomide metabolite analog a-cyano-β-methyl-β-hydroxy-N-(2, 5-dibromophenyl)propenamide, a potent inhibitor of BTK, abrogated the BTK-Fas association without affecting the expression levels of BTK or Fas and rendered them sensitive to Fas-mediated apoptosis. The ability of BTK to inhibit the pro-apoptotic effects of Fas ligation prompts the hypothesis that apoptosis of developing B-cell precursors during normal B-cell ontogeny may be reciprocally regulated by Fas and BTK.

Apoptosis is a common mode of eukaryotic cell death which is triggered by an inducible cascade of biochemical events leading to activation of endonucleases that cleave the nuclear DNA into oligonucleosome-length fragments (1–3). Several of the biochemical events that contribute to apoptotic cell death as well as both positive and negative regulators of apoptosis have recently been identified (1–4). Apoptosis plays a pivotal role in the development and maintenance of a functional immune system by ensuring the timely self-destruction of autoreactive immature and mature lymphocytes as well as any emerging target neoplastic cells by cytotoxic T-cells (1–7). Inappropriate apoptosis may contribute to the development as well as chemotherapeutic resistance of human leukemias and lymphomas (5–7). Therefore, an improved understanding of the molecular basis of apoptosis and the pro-apoptotic versus anti-apoptotic regulatory signals may provide further insights into the pathogenesis of human lymphoid malignancies and have important implications for treatment of leukemias and lymphomas.

The Fas/APO-1 (CD95) cell surface receptor, a member of the tumor necrosis factor (TNF) receptor family, is one of the major regulators of apoptosis in a variety of cell types (8–11). Functional abnormalities of Fas have been associated with pathologic conditions of the immune system homeostasis, including lymphoproliferative disorders, immunodeficiencies, and autoimmunity (10, 11). Identifying the molecules that participate in the apoptotic death signal pathways linked to the Fas receptor and finding ways to modulate the activity of such molecules could provide the basis for innovative treatment programs. Ligation of the cell surface Fas molecule rapidly and dramatically induces apoptosis in many but not all Fas-positive cell types (8). DT-40 is a chicken lymphoma B-cell line that we have used previously to elucidate the molecular mechanism of radiation-induced apoptosis (12). Despite their abundant surface expression of Fas, DT-40 cells, similar to human B-cell precursor leukemia cells, are very resistant to the cytotoxic effects of Fas ligation, indicating the existence of potent negative regulators of Fas-mediated apoptosis.

Bruton’s tyrosine kinase (BTK) is a member of the Src-related Tec family of protein tyrosine kinases (PTK) (13, 14). Mutations in the btk gene have been linked to severe developmental blocks in human B-cell ontogeny leading to human X-linked agammaglobulinemia (15, 16) and less severe deficiencies in murine B-cells leading to murine X-linked immune deficiency (17). Recent studies implicated BTK as a pro-apop-

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† The abbreviations used are: TNF, tumor necrosis factor; BTK, Bruton’s tyrosine kinase; PTK, protein tyrosine kinase(s); DICS, death-inducing signaling complex; PH domain, pleckstrin homology domain; SH2 and SH3 domains, Src homology 2 and Src homology 3 domains, respectively; GST, glutathione S-transferase; MBP, maltose-binding protein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PI, propidium iodide.
totic enzyme in B-lineage lymphoid cells exposed to ionizing radiation (12) as well as mast cells deprived of growth factors (18). In murine B-cells, BTK has also been shown to act as an anti-apoptotic protein upstream of bcl-xL in the B-cell antigen receptor (but not the CD40 receptor) activation pathway (19). Because of the recently discovered but not well understood ability of BTK to act both as a positive and negative regulator of apoptosis after ionizing radiation, growth factor deprivation, or B-cell antigen receptor signaling and its abundant expression in DT-40 cells (12), we investigated whether BTK plays a role in the pronounced resistance of DT-40 cells as well as human leukemic B-cell precursors against Fas-mediated apoptosis. Our study provides biochemical and genetic evidence that BTK is an inhibitor the Fas/APO-1 death-inducing signaling complex (DISC) in B-lineage lymphoid cells. BTK associates with Fas via its kinase and pleckstrin homology (PH) domains and prevents the FAS-FADD interaction, which is essential for the recruitment and activation of FLICE by Fas during the apoptotic signal. Notably, treatment of human leukemic B-cell precursors with a potent inhibitor of BTK abrogated the BTK-Fas association and sensitized the cells to Fas-mediated apoptosis.

**Fig. 1.** BTK is an inhibitor of Fas-mediated apoptosis in DT-40 lymphoma B-cells. FACS correlated two-parameter displays of wild-type (WT), BTK-deficient (BTK−), LYN-deficient (LYN−) DT-40 cells as well as BTK-deficient DT-40 cells reconstituted with wild-type human btk gene (BTK−, rBTK[WT]) stained with MC540 and PI 24 h after treatment with the control mouse IgG MsIgG (1 μg/ml) or anti-Fas (1 μg/ml). The percentages indicate the fraction of cells at an early stage of apoptosis, as measured by single MC540 fluorescence, and the fraction of cells at an advanced stage of apoptosis, as measured by dual MC540/PI fluorescence (12).
BTK as Inhibitor of Fas/CD95 Death-inducing Signaling Complex

Panel A

The expression levels of BTK and actin in wild-type, BTK-deficient, and human btk gene-reconstituted BTK-deficient DT-40 cells were measured by Western blot analysis using appropriate monoclonal antibodies and the ECL detection system (20, 25) according to the manufacturer's recommendations.

Panel B

Panels C, 1, 2, and 3. Fas protein expression levels of WT and BTK-deficient DT-40 cells were examined by confocal microscopy as detailed under "Experimental Procedures." Green, anti-Fas labeling; blue, Toto-3 stained DNA in nucleus; scale bar, 10 μm.

EXPERIMENTAL PROCEDURES

Cell Lines, Reagents, and Biochemical Assays—The establishment of BTK-deficient DT-40 lymphoma B-cell clones has been described previously (12). To disrupt the btk gene, targeting constructs containing the neomycin resistance gene cassette (i.e. pCBTK-neo) or histidinol resistance gene cassette (i.e. pCBTK-hisD) were sequentially transfected into DT-40 cells. The targeting vectors, pCBTK-neo and pCBTK-hisD, were constructed by replacing the 0.7-kilobase BglII-BamHI genomic fragment containing exons that correspond to human BTK amino acid residues 91–124 with the neo or hisD cassette. pCBTK-neo was linearized and introduced into wild-type DT-40 cells by electroporation. Screening was done by Southern blot analysis using a 3′-flanking probe (0.5-kilobase BglII-BglII fragment). The neo-targeted clone was again transfected with pCBTK-hisD and selected with both G418 (2 mg/ml) and histidinol (1 mg/ml). Southern blot analysis of a BTK-deficient DT-40 clone confirmed the homologous recombination at both btk loci, and hybridization with a neo and hisD probe indicated that the targeted clone had incorporated a single copy of each construct. Lack of BTK expression in BTK-deficient DT-40 cells was confirmed by both immune complex kinase assays and Western blot analysis (12). Mutations in the human btk cDNA were introduced by polymerase chain reaction using Pfu polymerase (Strategene) and confirmed by sequencing. Wild-type and mutant btk cDNAs were subcloned into pApuro expression vector and electroporated into BTK-deficient cells. The PTK activity of BTK immune complexes, as measured in vitro autophosphorylation, was abrogated by the catalytic domain mutation, reduced by the mutation in the PH domain mutation, but not affected by the mutation in the Src homology 2 (SH2) domain. Equal amounts of BTK protein were detected by Western blot analysis in all of the BTK-deficient DT-40 clones transfected with wild-type or mutated human btk genes, but no BTK protein was detectable in the untransfected BTK-deficient DT-40 cells (12). The establishment and characterization of LYN-deficient DT-40 clones were reported previously (12). In addition to these chicken lymphoma B-cells, we also used the following human B-lineage lymphoid cell lines: NALM-6-UM1, a BTK-positive human B-cell precursor (pre-B acute lymphoblastic leukemia) cell line; RAMOS-1, a BTK-deficient human Burkitt's/B-cell leukemia line; and K562, a BTK-positive human Epstein-Barr virus-transformed normal B-lymphoblastoid cell line.

Antibodies directed against BTK, SYK, and LYN have been described previously (12, 20, 21). Polyclonal antibodies to BTK were generated by immunization of rabbits with glutathione S-transferase (GST) fusion proteins (Amersham Pharmacia Biotech) containing the first 150 amino acids of BTK. In addition, we used the following anti-BTK antibodies in Western blots of purified fusion proteins: polyclonal goat anti-BTK carboxyl terminus (Santa Cruz Biotechnology), polyclonal goat anti-BTK amino terminus (Santa Cruz Biotechnology), and polyclonal rabbit serum raised against the BTK SH2-SH3 domains (amino acids 219–377). Polyclonal anti-MBP (maltose-binding protein) antibodies were generated by immunizing rabbits. The rabbit polyclonal anti-Fas (sc-715 mixed 1:1 with sc-714), which cross-reacts with both human and chicken Fas proteins, goat polyclonal anti-FADD (sc-1171), goat polyclonal anti-TRADD (sc-1163), and goat polyclonal anti-FLICE (sc-6135) were purchased from Santa Cruz Biotechnology and used according to the manufacturer's recommendations. The monoclonal anti-Fas antibody (F22120) was obtained from the Transduction Laboratories, Inc. (Lexington, KY). Immunoprecipitations, immune complex protein kinase assays, and immunoblotting using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech) were conducted as described previously (12, 20–25). The BTK inhibitor α-cyano-β-methyl-β-hydroxy-N-(2, 5-dibromophenyl)propenamide (LFM-A13; IC_{50} for BTK, 2.5 μM, IC_{50} values for epidermal growth factor receptor, insulin receptor, JAK-1, JAK-2, JAK-3, SYK, HCK, all >300 μM) was a kind gift from Dr. Yaguo Zheng from the Department of Chemistry at the Hughes Institute. The analytical physicochemical data for LMA-13 were: yield: 78% from tetrahydrofuran; mp: 148–150 °C; IR (KBr): 3,353, 2,211, 1,648, and 1,590 cm^{-1}; UV-visible: 220, 245, and 296 nm; 1H NMR (dimethyl sulfoxide-d6): δ 11.41 (s, 1H, NH), 8.57 (m, 1H, ArH), 7.55 (d, J = 8.7 Hz, 1H, ArH), 7.14 (q, J = 6.0 Hz, J_{24} = 2.4 Hz, 1H, ArH), 7.10 (s, 1H, OH), 2.17 (s, 3H, CH3); electron ionization mass spectrometry m/z [M]^+: 358.

Expression and Purification of MBP-BTK and GST-BTK Fusion Proteins—cDNAs encoding full-length BTK and its kinase or PH domains with polymerase chain reaction-generated 5′- and 3′-BamHI sites were cloned into the Escherichia coli expression vector pMAL-C2 with the isopropyl 1-thio-β-D-galactopyranoside-inducible Ptac promoter to create an in-frame fusion between these coding sequences and the 3′-end of the E. coli malE gene, which codes for MBP. cDNAs encoding the SH2, SH3, or SH2+SH3 domains with polymerase chain reaction-generated 5′- and 3′-BamHI sites were cloned into the E. coli expression vector pGEX-2t with the isopropyl 1-thio-β-D-galactopyranoside-inducible Ptac promoter to create an in-frame fusion between these coding sequences and the 3′-end of the E. coli GST gene.

Fig. 2. Fas protein expression levels in wild-type and BTK-deficient DT-40 cells. Panel A, the expression levels of BTK and actin in wild-type, BTK-deficient, and human btk gene-reconstituted BTK-deficient DT-40 cells were measured by Western blot analysis using appropriate monoclonal antibodies and the ECL detection system (20, 25) according to the manufacturer's recommendations. Panel B, the membranes immunoblotted with anti-BTK, and anti-actin antibodies were stripped and rebotted with the monoclonal anti-Fas antibody to compare the Fas protein expression levels in the individual clones. Panel C, 1, 2, and 3. Fas protein expression levels of WT and BTK-deficient DT-40 cells were examined by confocal microscopy as detailed under "Experimental Procedures." Green, anti-Fas labeling; blue, Toto-3 stained DNA in nucleus; scale bar, 10 μm.
recombinant plasmids were transformed into the E. coli strain DH5α. Single transformants were expanded in 5 ml of Luria-Bertani (LB) medium (1% tryptone, 1% NaCl, 0.5% yeast extract) containing ampicillin (100 μg/ml) by overnight culture at 37 °C. Expression of the fusion proteins was induced with 10 mM isopropyl 1-thio-β-D-galactopyranoside. The cells were harvested by centrifugation at 4,500 g for 10 min at 4 °C, lysed in sucrose-lysozyme buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10% sucrose, 1 mM EDTA, 20 mM lysozyme), and disrupted further by sonication. After removal of the cell pellets by centrifugation at 35,000 g for 1 h at 4 °C, GST-BTK fusion proteins were purified by glutathione-Sepharose chromatography (21), whereas MBP-BTK fusion proteins were purified from the culture supernatants by amylose affinity chromatography (26).

Confocal Laser Scanning Microscopy—Wild-type and BTK-deficient DT-40 cells treated with anti-Fas antibody (1 μg/ml) by overnight culture at 37 °C were attached to poly-L-lysine-coated coverslips and fixed in ice-cold (−20 °C) methanol for 15 min. After fixation, the coverslips were washed for 15 min in phosphate-buffered saline (PBS) + 0.1% Triton X-100. Cells were stained with a rabbit polyclonal anti-tubulin antibody according to the manufacturer’s recommendations (Sigma) to visualize their cytoplasm. DNA was labeled for 10 min with Toto-3, a DNA specific dye (Molecular Probes, Eugene OR) to visualize the apoptotic changes in the nuclei. MBP-BTK-electroporated BTK-deficient DT-40 cells and non-electroporated BTK-deficient DT-40 cells were labeled with an antibody raised against MBP. The secondary antibody was a goat anti-rabbit fluorescein-conjugated antibody. In some experiments, cells were examined for Fas expression by confocal microscopy. In brief, cells were attached to poly-L-lysine-coated coverslips and fixed for 40 min at 37 °C with 2% paraformaldehyde in PBS. Cells were rinsed in PBS + 115 mM glycine to quench the formaldehyde and then blocked in PBS containing 2% bovine serum albumin (BSA). A monoclonal antibody raised against the extracellular domain of Fas (Transduction Labs, Lexington, KY) was added in PBS + BSA, and the coverslips were incubated for 40 min at 37 °C before rinsing again in PBS. A fluorescein-labeled secondary antibody (Zymed Laboratories Inc., San Francisco) diluted in PBS + BSA was then added to the coverslips, and they were again incubated for 40 min at 37 °C. After another wash, cellular DNA was labeled by incubation in 1 μM Toto-3 for 20 min at room temperature. Coverslips were inverted and mounted onto slides in Vectashield (Vector Labs, Burlington, CA) to prevent photobleaching and were sealed with nail varnish. Slides were examined using a Bio-Rad MRC-1024 laser scanning confocal microscope mounted on a Nikon Eclipse E-800 upright microscope equipped for epifluorescence with high numerical aperture objectives (27). Optical sections were obtained and turned into

**FIG. 3.** BTK inhibits Fas-mediated apoptosis. Panel A, wild-type (WT) cells and BTK-deficient (BTK−) DT-40 cells were treated for 24 h with 1 μg/ml anti-Fas, costained with a rabbit polyclonal anti-tubulin antibody (green fluorescence) and the DNA-specific dye Toto-3 (blue fluorescence), and examined by laser scanning confocal microscopy, as described under “Experimental Procedures.” Unlike WT cells, the majority of BTK-deficient cells show apoptotic changes including nuclear fragmentation (a, b, and d) and shrinkage (c). Bar = 10 μm. Panel B, wild-type and BTK-deficient DT-40 cells were exposed to anti-Fas antibody as detailed under “Experimental Procedures,” harvested, and DNA from Triton X-100 lysates was analyzed for fragmentation, as described (12). Panels C and D, BTK-deficient DT-40 cells reconstituted with wild-type (rWT), kinase domain mutant (rK−), SH2 domain mutant (rmSH2), or PH domain mutant (rmPH) forms of the human btk gene were examined for sensitivity to anti-Fas antibody-induced apoptosis as described under “Experimental Procedures.” Controls were treated with PBS in culture medium for 24 h at 37 °C and 5% CO2 before harvesting. bp, base pairs.
stereomicrographs using Lasersharp software (Bio-Rad). Representative digital images were saved to Jaz disk and processed using Adobe Photoshop software (Adobe Systems, Mountain View CA). Images were printed with a Fuji Pictography thermal transfer printer (Fuji Photo, Elmsford, NY). Digital data were archived and stored on CD-ROM.

Apoptosis Assays—To induce apoptosis, cells were treated with an agonistic anti-Fas/APO-1 antibody (Bender MedSystems) at a 0.1, 0.5, or 1.0 μg/ml final concentration. MC540 binding (as an early marker of apoptosis) and propidium iodide (PI) permeability (as a marker of advanced-stage apoptosis) were measured simultaneously in DT-40 cells 24 h after exposure to anti-Fas antibody, as described previously (12). Whole cells were analyzed with a FACStar Plus flow cytometer (Becton Dickinson, San Jose, CA). All analyses were done using 488 nm excitation from an argon laser. MC540 and PI emissions were split with a 575 nm band pass filter. A 635 nm band pass filter was placed in front of one photomultiplier tube to measure MC540 emission, and a 635 nm band pass filter was used for PI emission.

To detect apoptotic fragmentation of DNA, DT-40, NALM-6-UM1, and RAMOS-1 cells were harvested 24 h after exposure to anti-Fas antibody, and DNA from Triton X-100 lysates was analyzed for fragmentation, as described (12, 24). Anti-Fas treatment induced apoptosis in BTK-deficient cells but not in wild-type or BTK-deficient cells into which MBP-BTK was electroporated. Electroporation of MBP (negative control) had no effect on apoptosis.

FIG. 4 Anti-apoptotic properties of BTK confirmed by BTK protein reconstitution of BTK-deficient DT40 cells. MBP or MBP-BTK was electroporated into BTK-deficient DT-40 cells before treatment with anti-Fas antibody, as described under “Experimental Procedures.” Panel A, MBP-BTK-electroporated BTK-deficient DT-40 cells and nonelectroporated BTK-deficient DT-40 cells were labeled with an antibody raised against MBP. The secondary antibody was a fluorescein isothiocyanate-conjugated goat anti-rabbit antibody. Cells were analyzed using a Bio-Rad MRC-1024 laser scanning confocal microscope. Digital images were processed using Adobe Photoshop software and printed using a Fuji Pictography printer. There was no significant staining above background in control nonelectroporated cells (A.1). Arrowheads indicate MBP antibody reactive material in the cytoplasm of cells electroporated with the MBP-BTK fusion protein (A.2). Two populations were observed in the MBP-BTK electroporated cells. Some cells had very bright labeling at the periphery, whereas other cells had large punctate staining inside the cytoplasm. Green, MBP; bar, 10 μm. Panel B, lysates as well as supernatants of BTK-deficient DT-40 cells electroporated with either MBP or MBP-BTK were subjected to Western blot analysis using anti-BTK and anti-MBP antibodies as described under “Experimental Procedures.” The 115-kDa MBP-BTK fusion protein reactive with both antibodies was detected only in lysates (but not supernatants) from MBP-BTK-electroporated cells. Panel C, cells were harvested 24 h after exposure to anti-Fas antibody, and DNA from Triton X-100 lysates was analyzed for fragmentation, as described (12, 24). Anti-Fas treatment induced apoptosis in BTK-deficient cells but not in wild-type or BTK-deficient cells into which MBP-BTK was electroporated. Electroporation of MBP (negative control) had no effect on apoptosis.
examined for the presence of FADD by immunoblotting. Difluoride membrane, and immunoblotted with a monoclonal anti-Fas antibody. Similarly, the FAS immune complexes from the same cells were collected, washed, boiled in 2× SDS sample buffer, fractionated on 12.5% polyacrylamide gels, transferred to an Immobilon polyvinylidene difluoride membrane, and examined for the presence of BTK protein by immunoblotting, as described under “Experimental Procedures.” Panel B, the BTK and FADD immune complexes (as well as the positive control FAS immune complexes) immunoprecipitated from untreated versus Fas-activated BTK-deficient DT-40 lymphoma B-cells, which were reconstituted with wild-type human btk gene, were collected, washed, boiled in 2× SDS sample buffer, fractionated on 12.5% polyacrylamide gels, transferred to an Immobilon polyvinylidene difluoride membrane, and immunoblotted with a monoclonal anti-Fas antibody, as described under “Experimental Procedures.” Similar results were obtained with wild-type DT-40 cells (data not shown). Panel C, FAS, FLICE, FADD, TRADD, and BTK immune complexes from lysates of BTK-positive NALM-6-UM1 human B-cell precursor leukemia cells were subjected to anti-Fas Western blot analysis as in panel B. Panel D, FADD was immunoprecipitated from Nonidet-P-40 lysates of untreated versus Fas-activated (anti-Fas 1 μg/ml × 1 h) BTK-deficient DT-40 cells, as described under “Experimental Procedures.” The immune complexes were collected, washed, boiled in 2× SDS sample buffer, fractionated on 12.5% polyacrylamide gels, transferred to an Immobilon polyvinylidene difluoride membrane, and immunoblotted with a monoclonal anti-Fas antibody. Similarly, the FAS immune complexes from the same cells were examined for the presence of FADD by immunoblotting.

**RESULTS AND DISCUSSION**

In a series of experiments designed to examine the potential negative regulatory role of BTK in Fas-mediated apoptosis, we first compared the effects of Fas ligation on wild-type DT-40 cells with the effects of Fas ligation on a BTK-deficient subclone of DT-40 cells which was established by homologous recombination knockout (12). To this end, we first used a quantitative flow cytometric apoptosis detection assay (12). MC540 binding and PI permeability were measured simultaneously before and after treatment with the agonistic anti-Fas antibody (1 μg/ml × 24 h). Only 5.0% of wild-type DT-40 cells treated with the anti-Fas antibody showed apoptotic changes, whereas 96.3% of BTK-deficient DT-40 cells underwent apoptosis, as determined by MC540 single fluorescence (early apoptosis) or MC540/PI double fluorescence (advanced apoptosis) at 24 h (Fig. 1). Notably, BTK-deficient DT-40 cells reconstituted with a wild-type human btk gene displayed very little flow cytometric evidence of apoptosis, which provided formal evidence that BTK plays a pivotal role in preventing the apoptotic death signal triggered by Fas ligation. In accordance with previously published information regarding the pro-apoptotic function of Src family PTK (29–31) and the reported impairment of Fas-mediated apoptosis in B-cells from LYN-deficient mice (32), very little apoptosis was found in an anti-Fas-treated LYN-deficient subclone of DT-40 cells which was included as a control in these experiments (Fig. 1). As shown in Fig. 2A, no BTK protein was detectable by Western blot analysis in the whole cell lysates of BTK-deficient DT-40 cells, whereas BTK-deficient DT-40 cells reconstituted with a wild-type human btk gene expressed higher levels of BTK than the wild-type DT-40 cells. However, the Fas protein expression levels in these three B-cell clones were virtually identical (Fig. 2B). These findings were confirmed further by confocal microscopy. As shown in panels C1–C3, all three cell lines exhibited similar levels of
punctate Fas staining. Three-dimensional reconstructions of serial optical sections confirmed the expression of Fas both in the cytoplasm and on the surface membrane of all three cell lines without any detectable difference relative to expression levels or pattern. Thus, the resistance of wild-type DT-40 cells or BTK-deficient DT-40 cells reconstituted with wild-type BTK against Fas-mediated apoptosis was not caused by lower expression levels of Fas protein, and the susceptibility of BTK-deficient DT-40 cells to Fas-mediated apoptosis was not caused by augmented Fas protein expression.

The comparative examination of the morphologic features of wild-type versus BTK-deficient DT-40 cells by laser scanning confocal microscopy showed no evidence of apoptosis for wild-type cells after treatment with the agonistic anti-Fas antibody, whereas BTK-deficient cells showed shrinkage and nuclear fragmentation consistent with apoptosis (Fig. 3A). On agarose gels, DNA from Triton X-100 lysates of anti-Fas-treated BTK-deficient DT-40 cells showed a ladder-like fragmentation pattern consistent with apoptosis, whereas no DNA fragmentation was observed in wild-type DT-40 cells (Fig. 3B). These results were reproduced in four independent experiments and provided direct evidence that BTK can inhibit Fas/APO-1-mediated apoptosis.

BTK has a unique amino-terminal region that contains a PH and a Tec homology domain, a single SH3 domain that contains the autophosphorylation site at tyrosine 223, a single SH2 domain, and a catalytic kinase domain that contains the transphosphorylation site at tyrosine 551 (13, 14). The PH
domain of BTK interacts with various isoforms of protein kinase CKβ subunits of heterotrimeric G proteins (13, 14, 33) as well as the BAP-135 protein (34). SH3 domains have been shown to interact with proline-rich sequences of other proteins, whereas SH2 domains interact with tyrosine-phosphorylated proteins (34). However, specific proteins interacting with the BTK SH2 or SH3 domains in B-lineage lymphoid cells have not been reported. Mutations in the catalytic domain, SH2 domain, as well as PH domain of the BTK have been found to lead to maturational blocks at early stages of B-cell ontology in human X-linked agammaglobulinemia (35, 36). BTK-deficient mice generated by introducing PH domain or catalytic domain mutations in embryonic stem cells showed defective B-cell development and function (37). Thus, different regions of BTK are important for its physiologic functions. To examine the participation of the various domains of BTK in negative regulation of Fas-mediated apoptosis, we introduced wild-type human btk gene as well as human btk genes harboring mutations either in the catalytic domain (Arg^{252} → Gln), SH2 domain (Arg^{907} → Ala), or PH domain (Arg^{298} → Cys) into the BTK-deficient DT-40 cells (12). As evidenced in Fig. 3, C and D, BTK-deficient DT-40 cells reconstituted with wild-type human btk gene (rWT) did not undergo apoptosis after treatment with the agonistic anti-Fas antibody, whereas Fas activation of reconstituted BTK-deficient DT-40 cells expressing human BTK with mutations in the kinase (rK), SH2 (rmSH2), or PH (rmPH) domains induced apoptosis as it did in nonreconstituted BTK-deficient DT-40 cells shown in Fig. 3B. Thus, the kinase, SH2, and PH domains of BTK are all important and apparently indispensable for its function as a negative regulator of Fas-mediated apoptosis.

To characterize further the anti-apoptotic function of BTK, we introduced by electroporation an MBP fusion protein containing full-length wild-type BTK into BTK-deficient cells 4 h before treatment with the anti-Fas antibody. Examination of these cells by confocal laser scanning microscopy (Fig. 4A) as well as Western blot analysis using anti-BTK and anti-MBP antibodies (Fig. 4B) confirmed the presence of the electroporated MBP-BTK protein. As shown in Fig. 4C, introduction of wild-type BTK protein by electroporation rendered the BTK-deficient DT-40 cells resistant to the apoptotic effects of Fas ligation, suggesting direct protein-protein interactions between BTK and members of the Fas signal transduction pathway as a possible mechanism for the anti-apoptotic function of BTK.

The downstream pro-apoptotic events initiated by the ligation of Fas or TNF receptor-1 are beginning to be illuminated (3, 38–45). Both Fas and TNF receptor-1 contain a homologous intracellular “death domain,” which plays a pivotal role in ligand-dependent assembly of a pro-apoptotic DISC (38). The death domains of p55 TNF receptor-1 and Fas/Cd95 serve as docking sites that mediate ligand-dependent recruitment of and heteroassociation with other death domain-containing multivalent adaptor proteins: Fas-associated protein with death domain (FADD) and receptor-interacting protein (RIP) in the case of CD95; and TNF receptor-1-associated death domain protein (TRADD) and RIP in the case of TNF receptor-1 (3, 38, 45). FADD is the point of convergence between the Fas/Cd95- and TNF receptor-1-linked apoptotic signal transduction pathways. Whereas Fas/Cd95 directly recruits FADD, TNF receptor-1 binds TRADD, which then acts as an adaptor protein to recruit FADD. The formation of CD95-FADD or TNF receptor-1-TRADD-FADD complexes after ligand binding are important for the induction of apoptosis. The assembly of a pro-apoptotic DISC is completed by the recruitment and concomitant activation of the cytosolic caspase FLICE, a member of the ICE protease family (3, 38–45). Recently, a number of proteins have been identified as inhibitors of Fas- as well as TNF receptor-1-induced apoptosis (3, 39–41). These proteins interact directly with FADD or FLICE, thereby interfering with DISC assembly and function. Notably, the death domain of Fas contains a conserved XXXL motif similar to the immunoreceptor tyrosine-based activation motif sequences as a potential binding site for SH2-containing proteins, and Fas has recently been shown to associate with Fyn and Lek kinases as pro-apoptotic regulators that are required for induction of Fas-mediated apoptosis (30, 31). We therefore postulated that BTK could interact with Fas and prevent the assembly of a pro-apoptotic DISC after Fas ligation.

We first investigated if BTK is capable of a physical association with Fas and other members of DISC by examining the Fas, FLICE, FADD, and TRADD immune complexes from the Nonidet P-40 lysates of untreated DT-40 cells for the presence of BTK. BTK was detected by Western blot analysis in Fas (but not the other) immune complexes by anti-BTK immunoblotting (Fig. 5A). Similarly, Fas was detected by anti-Fas immunoblotting in BTK immune complexes from wild-type DT-40 cells as well as BTK-deficient DT-40 cells reconstituted with wild-type human btk gene (Fig. 5B). The constitutive association of BTK with Fas protein was also found in the human B-cell precursor leukemia cell line NALM-6-UM1 (Fig. 5C). Taken together, these results demonstrated that BTK is capable of associating with Fas protein, and this physical association does not require prior engagement of the Fas receptor. As shown in Fig. 5D, Fas is associated with FADD in BTK-deficient DT-40 cells, as evidenced by detection of Fas in FADD immune complexes, and this physical interaction was enhanced markedly after Fas ligation. In Fas-activated BTK-deficient DT-40 cells, Fas-asso-
associated FADD molecules could be detected by anti-FADD immuno blotting (Fig. 5D). In contrast to BTK-deficient DT-40 cells, very little Fas-FADD association was found in untreated or anti-Fas-treated BTK-deficient DT-40 cells reconstituted with wild-type human BTK (Fig. 5D). Similarly, Fas ligation failed to enhance the Fas-FADD association in human NALM-6-UM1 leukemia cells (Fig. 5C). Thus, BTK associates with Fas and impairs its interaction with FADD, a protein that is essential for the recruitment and activation of FLICE by Fas during the apoptotic signal. Although these results do not exclude the possibility that BTK may alter the fate of the apoptotic signal triggered by Fas ligation by multiple mechanisms including modulation of the function of positive or negative regulators of apoptotic signal transduction, they do provide at least one plausible explanation for the observed anti-apoptotic function of BTK.
To elucidate further the physiologic significance of the observed BTK-Fas association in human leukemic B-cell precursors, we compared the sensitivities of BTK-positive NALM-6-UM1 human pre-B leukemia cell line and BTK-deficient RAMOS-1 human B-cell leukemia cell line to Fas-mediated apoptosis. As shown in Fig. 6A, these two cell lines express similar levels of Fas protein. BTK-deficient RAMOS-1 cells underwent apoptosis after Fas ligation with the agonistic anti-Fas antibody, but BTK-positive NALM-6-UM1 cells did not (Fig. 6B). We next examined the effects of the leflunomide metabolite analog LFM-A13, a potent inhibitor of BTK, on BTK-Fas association and resistance to Fas-mediated apoptosis in NALM-6-UM1 cells. Anti-BTK and anti-Fas Western blot analyses of whole cell lysates from LFM-A13-treated NALM-6-UM1 cells showed no reduction in BTK (Fig. 6C1) or Fas protein (Fig. 6C2) expression levels. There was substantially less Fas protein in the BTK immune complexes, providing direct evidence that inhibition of BTK by LFM-A13 abrogates the BTK-Fas association (Fig. 6C3). Notably, a 4-h treatment with LFM-A13 did not induce apoptosis in NALM-6-UM1 cells but rendered these highly resistant human leukemia cells sensitive to Fas-mediated apoptosis (Fig. 6D). These results provided further support for our hypothesis that BTK is a physiologically important negative regulator of Fas-mediated apoptosis.

The ability of the BTK inhibitor LFM-A13 to abrogate the BTK-Fas association provided circumstantial evidence that the kinase activity of BTK plays an important role for the formation of the BTK-Fas complexes. To establish further whether or not the association of BTK with Fas is dependent on its kinase activity, we next examined BTK-Fas interactions in BTK-deficient DT-40 cells reconstituted with either wild-type human BTK (BTK-, rBTK[WT]) or kinase inactive mutant (Arg625→Gln) human BTK (BTK-, rBTK[K]). As shown in Fig. 7A, Western blot analysis of whole cell lysates from these two cell lines with anti-BTK or anti-Fas antibodies did not reveal any substantial differences (i.e. in both of two independent experiments, we observed slightly higher BTK and Fas expression levels in BTK-, rBTK[K] cells). Fas immune complexes from lysates of BTK-, rBTK[WT] cells contained BTK protein, and this BTK-Fas association was enhanced further by treatment of cells with the anti-Fas antibody (Fig. 7B, first two lanes). In contrast, no BTK protein was detectable in Fas immune complexes from BTK-, rBTK[K] cells regardless of treatment with the anti-Fas antibody (Fig. 7B, third and fourth lanes). These results provide corroborating evidence that the association of BTK with Fas is dependent on the kinase activity of BTK.

We next performed binding experiments with full-length MBP-BTK and truncated MBP-BTK and GST-BTK fusion proteins corresponding to various domains of BTK (Fig. 8, A–C) to elucidate the structural requirements for BTK association with Fas. MBP-BTK 1–659 (full-length BTK) as well as MBP-BTK 408–659 (BTK kinase domain) and MBP-BTK 2–137 (BTK PH domain) were able to bind and pull down Fas from lysates of BTK-deficient DT-40 cells (Fig. 8D). These results provide further support for our hypothesis that BTK is a physiologically important negative regulator of Fas-mediated apoptosis.

Although the crystal structure of full-length BTK has not been reported, the recently published structures of the PH domain and BTK motif (46) provide useful information applicable to the binding capability of BTK and its PH domain. FADD has been reported to interact with the cytoplasmic domain of Fas, which is largely composed of a death domain consisting of six antiparallel α-helices assembled from residues 230–314 (47). The XXXL sequence of the Fas death domain has been speculated to resemble ITAMs and be recognized by an SH2 domain of a PTK upon tyrosine phosphorylation or by other mechanisms (30, 31). An analysis of the conformation of this XXXL sequence shows that it is located in the middle of an α-helix, and unless a substantial conformational change of that α-helix would occur to make the tyrosine residue more accessible, it may be too rigid for interaction with a PTK. Thus, the structural geometry of the XXXL sequence would likely prevent Fas and BTK from adopting a binding mode such as that of CD3-ε ITAM/ZAP-70 as was suggested (31). The inability of the BTK SH2 domain to pull down Fas from whole cell lysates further supports this notion. How then does BTK associate with Fas? BTK and Fas may associate via complementary electrostatic attractions and hydrogen bond interactions, which could involve the previously reported charged residues on the surfaces of the α-helices of the Fas death domain. This association could be mediated by a third protein that forms an interface between Fas and BTK. The importance of the SH2 and kinase domains of BTK for its anti-apoptotic function prompts the hypothesis that a tyrosine-phosphorylated substrate of BTK may provide such an interface. Further studies will be required to elucidate the exact structural basis for the BTK-Fas interactions.

The ability of BTK to inhibit the pro-apoptotic effects of Fas ligation prompts the hypothesis that apoptosis of developing B-cell precursors during normal human B-cell ontogeny may be regulated reciprocally by Fas and BTK. The absence of BTK or mutations in its kinase, PH, and SH2 domains could lead to inappropriate apoptotic cell death of pre-B-cells, leading to the phenotype of X-linked agammaglobulinemia (13, 15, 16). Inappropriate apoptosis may underlie the pathogenesis as well as drug resistance of human leukemias and lymphomas, which makes control of apoptosis an important potential target for therapeutic intervention. The fate of leukemia/lymphoma cells exposed to chemotherapeutic agents such as vincristine and daunorubicin may reside in the balance between the opposing pro-apoptotic effects of caspases activated by DISC and an upstream negative regulatory mechanism involving BTK and/or its substrates. Therefore, inhibitors of BTK are likely to enhance the drug sensitivity of B-lineage leukemia/lymphoma cells.

Our findings provide unique biochemical evidence to link a death receptor physically to an anti-apoptotic tyrosine kinase and corroborate the growing evidence that there are multiple counterregulatory mechanisms in B-cell precursors which operate to preserve cell survival and growth, thereby ensuring their orderly development and differentiation (4, 6, 7). BTK is the first tyrosine kinase to be identified as a dual function regulator of apoptosis which promotes radiation-induced apoptosis but inhibits Fas-activated apoptosis. We have reported previously that BTK but not LYN is required for radiation-induced apoptosis (12). Here, we showed that targeted disruption of lyn gene does not abrogate the anti-apoptotic activity of BTK. Thus, the pro- as well as anti-apoptotic functions of BTK do not depend on LYN kinase, which is thought to act upstream of BTK in B-cell antigen receptor-linked mitogenic signaling (14, 22). Indeed, our findings taken together with previously published role of LYN as a promoter of Fas-activated apoptosis (32) suggest that these two tyrosine kinases may play opposite...
roles in regulation of Fas-linked death signaling. We believe that the identification of BTK as a dual function regulator of apoptosis will significantly increase our understanding of both the biological processes involved in programmed cell death and diseases associated with dysregulation of apoptosis.

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