pation is difficult to calculate because electron conduction from simulated hot spots is very fast, dissipating the high temperature on time scales that are short compared to 75 ps. We have assumed that these channels run the full 2-mm length of the target. There is no way to determine if this is actually the case; if the regions are less than 2 mm, then the local gain in those regions will be even higher.

Although laser-plasma instabilities, such as thermal self-focusing, can also give rise to small-scale filaments of high-temperature plasma, this is an unlikely cause of the observed structure because not only does filamentation tend to drive the density down in small filaments and thus, according to Fig. 1, toward regimes of lower gain, but also we do not observe the density fluctuations associated with this mechanism. Hydrodynamic instabilities, such as Rayleigh-Taylor, seem unlikely for the same reason: the density variation is not observed.

It is possible that the ion temperature T_I, not the electron temperature, is the cause of the regions of high local gain. Because the lasing line narrows as T_I decreases, the resulting gain varies as $T_I^{-1/2}$. Small regions of lower T_I, possibly seeded by fluctuations in the driving laser, would be areas of enhanced gain.

The nonuniform structure has a significant effect on XRL coherence. An XRL of this type behaves like an incoherent source over the output aperture of the XRL (*19*, *20*). This persistent lack of coherence has not been understood; one suggested explanation is the development of plasma instabilities, seeded by intensity modulations in the driving laser, which break up the plasma (*21*).

As we have observed from Figs. 5 and 6, the region of amplification in the XRL plasma is indeed highly inhomogeneous, although probably not because of plasma instabilities. Because much of the gain is generated in small isolated regions, the spatial coherence of the XRL cannot be improved without smoothing the temperature field in the plasma. If the isolated gain structure is caused by modulations in the driving laser, smoothing of the driving beam may alleviate the pockets and improve the spatial coherence of the XRL.

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22. This work was performed under the auspices of the Department of Energy by Lawrence Livermore National Laboratory under contract W-7405-ENG-48.

22 April 1996; accepted 24 June 1996

BTK as a Mediator of Radiation-Induced Apoptosis in DT-40 Lymphoma B Cells

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Bruton's tyrosine kinase (BTK) is a member of the SRC-related TEC family of protein tyrosine kinases (PTKs). DT-40 lymphoma B cells, rendered BTK-deficient through targeted disruption of the *btk* gene by homologous recombination knockout, did not undergo radiation-induced apoptosis, but cells with disrupted *lyn* or *syk* genes did. Introduction of the wild-type, or a SRC homology 2 domain or a plecstrin homology domain mutant (but not a kinase domain mutant), human *btk* gene into BTK-deficient cells restored the apoptotic response to radiation. Thus, BTK is the PTK responsible for triggering radiation-induced apoptosis of lymphoma B cells, and its kinase domain is indispensable for the apoptotic response.

Apoptosis, also termed programmed cell death, is a common mode of eukaryotic cell death characterized by distinct ultrastructural features and a ladder-like DNA fragmentation pattern produced by endonuclease-mediated cleavage of DNA into oligonucleosome-length fragments (*1*). Apoptosis plays an important role in the development and maintanance of a functional immune system (*1*). A better understanding of the biochemical events leading to apoptosis may provide further insights into the pathogenesis and treatment of human diseases, such as immunodeficiencies, autoimmune disorders, leukemias, and lymphomas, which are thought to stem from inherited or acquired deficiencies of checkpoints regulating the rate of apoptosis in lymphoid cells.

Reactive oxygen intermediates have been implicated as mediators of apoptosis in B-lineage lymphoid cells (*1*, *2*). The molecular mechanism by which reactive oxygen intermediates can trigger apoptosis has not yet been deciphered, but it may involve activation of as yet unidentified protein tyrosine kinases (PTKs) (*2*). Protein tyrosine kinases play a pivotal role in the initiation of biochemical signaling events that affect proliferation, differentiation, and survival of B-lineage lymphoid cells (*3–7*). In addition to radiation-induced apoptosis (*2*), other apoptotic death signals, including those induced by dexamethasone, monoclonal antibodies to CD3, tumor necrosis factor–α, ceramide, FAS ligand, and Taxol, are triggered by enhanced PTK activity (*8*).

DT-40 is a radiation-sensitive chicken lymphoma B cell line that we have previously used to explore the mechanisms whereby various PTKs that participate in B cell antigen receptor signaling (*9–11*). DT-40 cells undergo rapid apoptosis after exposure to γ-rays, as measured by a quantitative flow cytometric apoptosis-detection assay (*2*, *12–14*). Radiation-induced apoptosis of DT-40 cells is triggered by activation of radiation-responsive PTKs because it is prevented by the PTK inhibitor genistein (*2*, *12–14*). One of the radiation-responsive PTKs in DT-40 cells is the 77-kD TEC PTK family member Bruton's tyrosine kinase (BTK) (*4*, *15–20*). We now provide experimental evidence that the BTK tyrosine kinase, but not the LYN, SYK, or CSK tyrosine kinases, mediates radiation-induced apoptosis of DT-40 lymphoma B cells. BTK is also responsible for mediating

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the B cell antigen receptor (BCR)–linked apoptotic signals. Whereas the BCR-linked apoptotic signals are triggered by phospholipase C–γ2 (PLCγ2) activation through the concerted actions of BTK and SYK, neither PLC-γ2 nor the SYK kinase is required for radiation-induced apoptosis of lymphoma B cells by BTK.

BTK (*15, 16*), together with TEC I and TEC II (*21*), ITK/TSK/EMT (*22*), BMX (*23*), and TXK/RLK (*24*), is a member of the SRC-related TEC family of PTKs. Mutations in the *btk* gene have been linked to severe developmental blocks in human B cell ontogeny resulting in human X-linked agammaglobulinemia (XLA) (*25*) and less severe deficiencies in murine B cells resulting in murine X-linked immune deficiency (XID) (*26*). To elucidate unequivocally the role of this radiation-responsive PTK in radiation-induced apoptosis of lymphoma B cells, we established a subclone of DT-40 cells lacking the *btk* gene by homologous recombination knockout (*7, 10, 27–29*). Lack of BTK expression in BTK-deficient DT-40 cells was confirmed by both immune complex kinase assays (Fig. 1A) and protein immunoblot analysis (Fig. 1B). By comparison, BTK expression levels of SYK- or LYN-deficient DT-40 cells (*7, 10, 27, 28*) were comparable to those of wild-type DT-40 cells (Fig. 1, A and B). BTK has a unique NH_2-terminal region that contains a plecstrin homology (PH) domain of 137 amino acids, a TEC homology domain of 80 amino acids, a single SRC homology 3 (SH3) domain of 49 amino acids, a single SH2 domain of 96 amino acids, and a catalytic kinase domain of ~250 amino acids (*15, 16, 25, 26*). The PH domain of BTK interacts with various isoforms of PKC as well as the βγ subunits of heterotrimeric GTP-binding proteins (*30*). SH3 domains interact with proline-rich sequences of other proteins, whereas SH2 domains interact with tyrosine phosphorylated proteins (*31*). However, specific proteins interacting with the BTK SH2 or SH3 domains in B-lineage lymphoid cells have not been identified. The catalytic domain of BTK is capable of auto- and transphosphorylation (*19*). Mutations in the catalytic domain, SH2 domain, as well as the PH domain of the BTK result in maturational blocks at early stages of B cell ontogeny in human XLA (*25*). BTK-deficient mice generated by the introduction of PH domain or catalytic domain mutations in embryonic stem cells showed defective B cell development and function (*15*). Thus, different regions of BTK are important for its physiologic functions.

To examine the role of the various domains of BTK in mediating the radiation-induced apoptotic signal, we introduced wild-type human *btk* gene as well as human *btk* genes harboring mutations either in the catalytic domain (Arg^{525}→Gln), SH2 domain (Arg^{307}→Ala), or PH domain (Arg^{28}→Cys) into the BTK-deficient DT-40 cells. The PTK activity of BTK immune complexes, as measured by in vitro autophos-

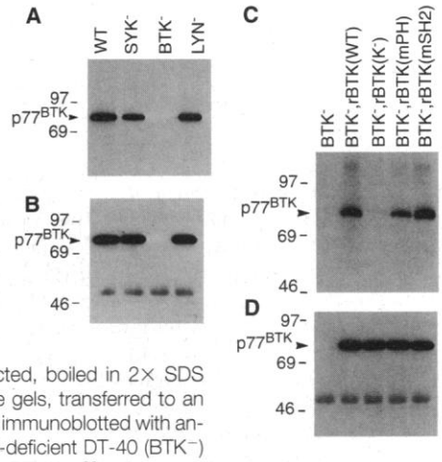

Fig. 1. BTK tyrosine kinase activity and BTK protein expression in wild-type and mutant DT-40 lymphoma B cells. (**A** and **B**) Nonidet P-40 lysates from wild-type and from SYK-, BTK-, and LYN-deficient DT-40 cells were immunoprecipitated with anti-BTK as described (*4, 19, 20*) and divided into two portions. One of the duplicate portions from each sample was used for BTK immune-complex kinase assays (that is, in vitro kinase assays of anti-BTK immunoprecipitates) with [γ-^{32}P]adenosine triphosphate (A) as described (*4, 19, 20*). After the kinase reactions, samples were boiled in 2× SDS reducing sample buffer, proteins fractionated on 15% polyacrylamide gels, and the ^{32}P-labeled BTK bands visualized by autoradiography. The remaining portions were collected, boiled in 2× SDS sample buffer, fractionated on 15% polyacrylamide gels, transferred to an Immobilon polyvinylidene difluoride membrane, and immunoblotted with anti-BTK (B) as described (*4, 19, 20*). (**C** and **D**) BTK-deficient DT-40 (BTK⁻) cells expressing wild-type BTK, BTK(Arg^{525}→Gln), BTK(Arg^{28}→Cys), and BTK(Arg^{307}→Ala) were designated as BTK⁻,rBTK(WT), BTK⁻,rBTK(K⁻), BTK⁻,rBTK(mPH), and BTK⁻,rBTK(mSH2), respectively. Nonidet P-40 lysates from these cells were subjected to BTK immune complex kinase assays (C) and anti-BTK protein immunoblot analysis (D) as described in (A) and (B). The positions of BTK and prestained molecular size markers are shown on the left (in kilodaltons).

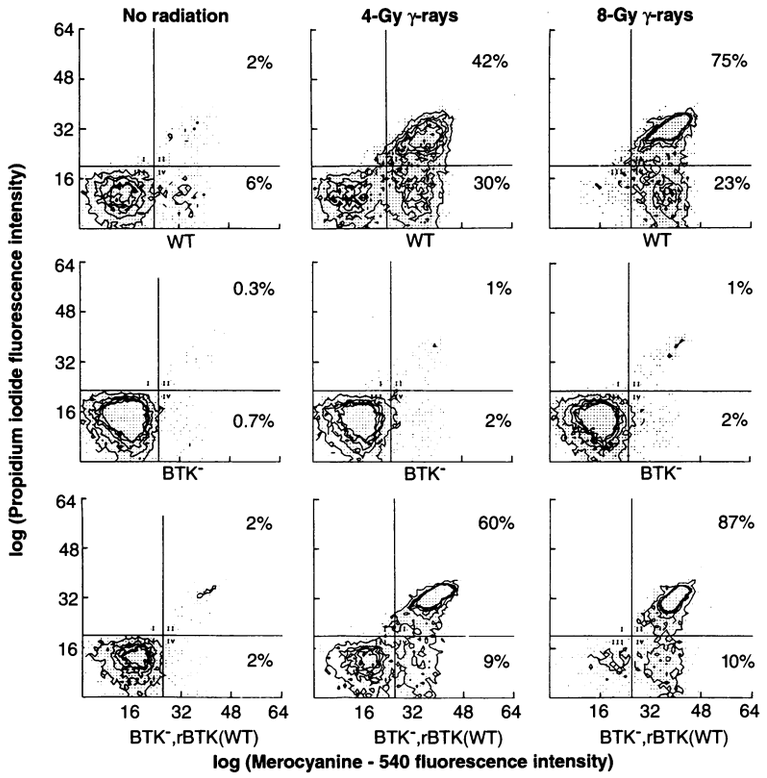

Fig. 2. Ionizing radiation does not induce apoptosis in BTK-deficient DT-40 lymphoma B cells. Flow cytometric analysis correlated two-parameter displays of wild-type (WT), BTK-deficient (BTK⁻) DT-40 cells, and BTK-deficient DT-40 cells reconstituted with the wild-type human *btk* gene (BTK⁻,rBTK(WT) stained with MC540 and PI 8 hours after treatment with phosphate-buffered saline (PBS), pH 7.4 (no radiation), 4-Gy γ-rays, or 8-Gy γ-rays. 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 (*13, 14*).

phorylation, was abrogated by the catalytic domain mutation and reduced by the PH-domain mutation but not affected by the mutation in the SH2 domain (Fig. 1C). Equal amounts of BTK protein were detected by protein immunoblot 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 (Fig. 1D).

We first used a quantitative flow cytometric apoptosis detection assay (*13, 14*) to compare the radiation responses of wild-type and mutant DT-40 cells. MC540 binding and propidium iodide (PI) permeability were simultaneously measured before and after irradiation. Ionizing radiation induced apoptosis in wild-type DT-40 cells in a dose-dependent manner. Eight hours after exposure to 8-Gy γ-rays, 23% of wild-type DT-40 cells showed signs of early apoptosis, as determined by MC540 fluorescence, and 75% were in advanced-stage apoptosis, as determined by MC540/PI double fluorescence (Fig. 2, top panel). Thus, 98% of the wild-type DT-40 cells showed flow cytometric evidence of apoptosis after exposure to 8-Gy γ-rays. By contrast, only 3% of BTK-deficient DT-40 cells underwent radiation-induced apoptosis (Fig. 2, middle panel). Introduction of wild-type human *btk* gene into the BTK-deficient DT-40 cells restored their ability to elicit an apoptotic response after radiation exposure, with 87% of the cells showing evidence of advanced stage apoptosis after exposure to 8-Gy γ-rays (Fig. 2, bottom panel), confirming that BTK plays a pivotal role in radiation-induced apoptosis of DT-40 lymphoma B cells.

On agarose gels, DNA from Triton X-100 lysates of irradiated wild-type DT-40 cells showed a radiation dose–dependent, ladder-like fragmentation pattern consistent with an endonucleolytic cleavage of DNA into oligonucleosome-length fragments at multiples of 200 base pairs (*32–34*), whereas no DNA fragmentation was observed in BTK-deficient DT-40 cells exposed to γ-rays (Fig. 3A). Thus, BTK is required for radiation-induced apoptosis of DT-40 cells. By comparison, targeted disruption of the *lyn* or *syk* genes did not prevent or attenuate radiation-induced apoptosis of DT-40 cells (Fig. 3B). The PTK activity of LYN is negatively regulated by

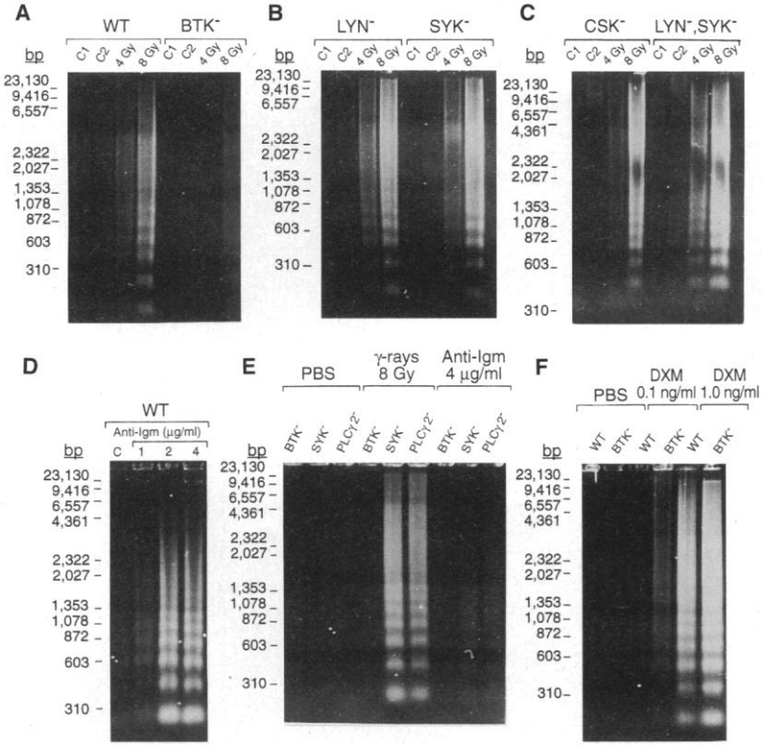

Fig. 3. BTK mediates radiation-induced apoptosis in DT-40 lymphoma B cells. Cells were exposed to various apoptosis-inducing agents, harvested, and DNA from Triton X-100 lysates analyzed for fragmentation as described (*32–34*). (**A** to **C**) Cells were harvested after exposure to 4- or 8-Gy γ-rays (*32–34*). Untreated controls as well as irradiated cells were maintained in culture medium for 8 hours at 37°C and 5% CO_2 before harvesting. C1, unirradiated, untreated cells harvested at time point 0; C2, control (unirradiated, untreated cells harvested at hour 8). (**D**) Cells were harvested after 8 hours of continuous treatment with M4 antibody to surface IgM at concentrations of 1, 2, or 4 μg/ml in culture medium. C, control cells harvested after 8 hours of culture in the same culture medium without added M4 antibody. (**E**) Cells were harvested after 8 hours of sham treatment (PBS), after 8 hours of culture subsequent to 8-Gy γ-irradiation, or after 8 hours of continuous treatment with M4 antibody (4 μg/ml). (**F**) Cells were harvested after 24 hours of continuous treatment with dexamethasone (DXM, 0.1 or 1.0 ng/ml).

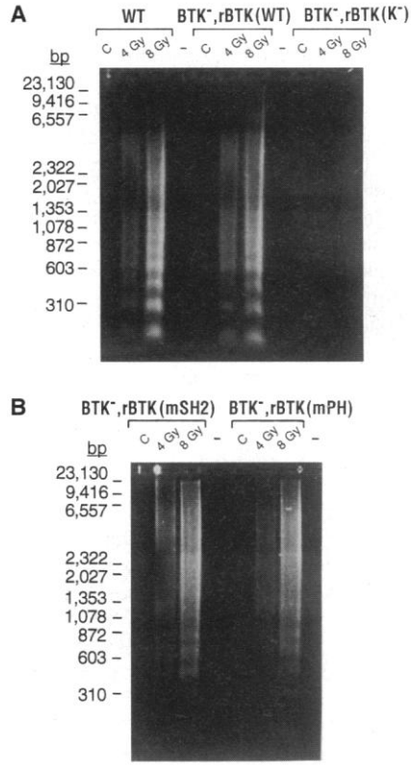

Fig. 4. (**A** and **B**) Radiation-induced apoptosis in BTK-deficient DT-40 lymphoma B cells reconstituted with wild-type or mutant human BTK. Cells were harvested 8 hours after exposure to 4- or 8-Gy γ-rays (*2, 12*), and DNA from Triton X-100 lysates was analyzed for fragmentation as described (*32–34*). Unirradiated controls (C) as well as irradiated cells were maintained in culture medium for 8 hours at 37°C and 5% CO_2 before harvesting. BTK-deficient DT-40 (BTK^-) cells expressing wild-type BTK, BTK(Arg^{525}→Gln), BTK(Arg^{28}→Cys), and BTK(Arg^{307}→Ala) were designated as BTK^-,rBTK(WT), BTK^-,rBTK(K^-), BTK^-,rBTK(mPH), and BTK^-,rBTK(mSH2), respectively. Flow cytometric analysis–correlated two-parameter displays of wild-type (WT), BTK-deficient (BTK^-) DT-40 cells, as well as BTK-deficient DT-40 cells reconstituted with wild-type and mutant human *btk* genes stained with MC540 and PI 8 hours after treatment with 8-Gy γ-rays, showed 96% apoptosis in WT cells, 95% apoptosis in BTK^-,rBTK(WT) cells, 5% apoptosis in BTK^-,rBTK(K^-) cells, 96% apoptosis in BTK^-, rBTK(mSH2)cells, and 93% apoptosis in BTK^-, rBTK(mPH) cells, as determined by double MC540/PI fluorescence (*13, 14*).

phosphorylation at the COOH-terminal tyrosine residue, and CSK kinase phosphorylates this COOH-terminal tyrosine residue (*27, 35*). CSK-deficient DT-40 cells show constitutive activation of LYN and SYK kinases (*27, 35*), and CSK-deficient DT-40 cells were not more sensitive to γ-rays than were the wild-type DT-60 cells (Fig. 3C). Furthermore, a LYN/SYK doubly deficient mutant cell line, which was generated by disruption of the *syk* gene in LYN-deficient DT-40 cells, was as radiation-sensitive as were the wild-type DT-40 cells (Fig. 3C). These results demonstrate that neither LYN nor SYK kinase is required for radiation-induced apoptosis of DT-40 lymphoma B cells. Similarly, H_2O_2 induced apoptosis in wild-type DT-40 cells as well as in LYN/SYK doubly deficient DT-40 cells, but not in BTK-deficient DT-40 cells (*36*).

Engagement of the BCR with an antibody to immunoglobulin M (anti-IgM) triggered apoptosis in wild-type DT-40 cells (Fig. 3D) but not in BTK-deficient DT-40 cells (Fig. 3E). Thus, BTK is required not only for radiation-induced apoptosis, but for BCR-linked apoptosis as well. SYK-deficient (*7, 10, 27, 28*) and PLCγ2-deficient (*7, 10, 37*) cells also failed to undergo apoptosis after anti-IgM stimulation. Because both BTK and SYK are required for the antigen receptor–induced PLCγ2 activation (*7, 9–11, 37*), our results indicate that anti-IgM–induced apoptosis is triggered by PLCγ2 activation, which is regulated by BTK and SYK through their concerted actions. In contrast, radiation-induced apoptotic signals do not depend on SYK kinase or PLCγ2. SYK kinase–deficient as well as PLCγ2-deficient DT-40 cells underwent apoptosis after exposure to ionizing radiation (Fig. 3E). Dexamethasone (DXM) is an apoptosis-inducing chemotherapeutic agent commonly used in the treatment of lymphoid malignancies (*1, 8*). We examined the sensitivity of BTK-deficient DT-40 cells to DXM and found no decrease in sensitivity (Fig. 3F). Thus, BTK is not required for the DXM-induced apoptotic signal.

DT-40 cells expressing the catalytic domain mutant of human BTK [that is, BTK^-, $rBTK(K^-)$] did not undergo apoptosis after γ-ray exposure (Fig. 4A), providing direct evidence that the kinase domain of BTK is indispensable for its function as a mediator of radiation-induced apoptosis in B lineage lymphoid cells. By comparison, γ-ray exposure of DT-40 cells expressing human BTK with mutations in the SH2 (that is, BTK^-,mSH2) or PH domains (that is, BTK^-,mPH) (Fig. 4B) induced apoptosis as readily as in wild-type DT-40 cells or BTK-deficient DT-40 cells reconstituted with wild-type human *btk* gene (Fig. 4A). Thus, neither the SH2 domain nor the PH domain of BTK is essential for its role in radiation-induced apoptosis.

Multiple apoptotic checkpoints in B cell ontogeny are thought to ensure the orderly development and differentiation of B lineage lymphoid cells (*1*). Recent studies in BTK-deficient mice (*15*) and patients with XLA (*25*) provided strong evidence for important BTK roles in B cell development and differentiation. BTK-deficient mice were shown to have increased numbers of immature B cell precursor populations and reduced numbers of mature B cells (*15*). The present study expands our knowledge of BTK functions and reveals the essential role of BTK in triggering a biochemical event required for radiation-induced apoptotic death of lymphoma B cells. This previously unknown function of BTK does not depend on LYN kinase, which is thought to act upstream of SYK and BTK kinases in B cell antigen receptor–linked signaling events (*5, 9–11*); nor does it depend on SYK kinase, which plays a pivotal role in anti–surface IgM–induced apoptosis (*7*), or PLCγ2, which acts downstream of SYK and BTK kinases in BCR-linked signaling events (*5, 9–11*). The mechanism by which exposure of lymphoma B cells to reactive oxygen intermediates triggers activation of BTK remains to be deciphered.

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11. The predominant PTKs that are abundantly expressed in B lineage lymphoid cells include the TEC PTK family member BTK (*3, 4, 15, 16*), the cytoplasmic PTK SYK (*3, 4, 6, 7*), and the SRC PTK family member LYN (*3–5*). DT-40 cells express high levels of BTK, SYK, and LYN kinases, whereas SRC, LCK, FYN, BLK, YES, and HCK kinases are not expressed at detectable levels. Stimulation of wild-type as well as BTK-deficient DT-40 cells with M4 anti-IgM (1 to 4 μg/ml) induces tyrosine phosphorylation of multiple substrates (M. Takata and T. Kurosaki, unpublished observations). In wild-type DT-40 cells, anti-IgM stimulation activates LYN, SYK, and BTK kinases (*9, 10*) [M. Takata *et al.*, *EMBO J.* **13**, 1341 (1994); K. Nagai, M. Takata, H. Yamamura, T. Kurosaki, *J. Biol. Chem.* **270**, 6824 (1995); D. J. Rawlings *et al.*, *Science* **271**, 822 (1996)].
12. DT-40 cells (5×10^5 per milliliter in plastic tissue culture flasks) were irradiated (^{137}Cs irradiator; J. L. Shephard, Glendale, CA, Model Mark I) with 4 or 8 Gy γ-irradiation at a dose rate of 1 Gy/min under aerobic conditions as described [F. M. Uckun *et al.*, *Cancer Res.* **53**, 1431 (1993); F. M. Uckun *et al.*, *J. Clin. Invest.* **91**, 1044 (1993); F. M. Uckun *et al.*, *J. Biol. Chem.* **271**, 6389 (1996)]. Before irradiation, cells were sometimes incubated with the tyrosine kinase inhibitor genistein (30 μg/ml for 1 hour) at 37°C to examine the role of PTK in radiation-induced apoptosis as described (*2*) [F. M. Uckun *et al.*, *ibid.* **90**, 252 (1993)].
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14. Phosphatidylserine, which is normally confined to the intracellular compartment, is exposed on the surface of apoptotic cells because of a loss of plasma membrane phospholipid asymmetry [V. A. Fadok *et al.*, *J. Immunol.* **148**, 2207 (1992)]. The randomized packing of membrane phospholipids allows such cells to exhibit increased binding of the lipophilic dye merocyanine (MC) 540 (Sigma) and to be detected by flow cytometry [B. J. Del Bruno *et al.*, *J. Cell Physiol.* **138**, 61 (1989)]. Loose packing of membrane phospholipid head groups and cell shrinkage precede DNA fragmentation in apoptotic cells, thereby providing MC540 binding as an early marker for apoptosis (*13*). Plasma membrane permeability to PI (Sigma) develops at a later stage of apoptosis (*13*). MC540 binding and PI permeability were simultaneously measured in DT-40 cells 8 hours after exposure to 4- or 8-Gy γ-rays as described (Y. Messinger *et al.*, *Clin. Cancer. Res.*, in press). Stock solutions of MC540 and PI, each at 1 mg/ml, were passed through a 0.22-μm filter and stored at 4°C in the dark. Shortly before analysis, suspensions containing 1×10^6 cells were suspended in MC540 (5 μg/ml) and PI (10 μg/ml) and kept in the dark at 4°C. Whole cells were analyzed with a FACStar Plus flow cytometer (Becton Dickinson). All analyses were done at 488-nm excitation from an argon laser. MC540 and PI emissions were split with a 600-nm short-pass dichroic mirror; a 575-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. When DT-40 cells were exposed to 4-Gy γ-rays, the percentage of apoptotic cells, as determined by dual MC540/PI fluorescence, increased within 8 hours after radiation from 7 to 65%. By comparison, only 17% of DT-40 cells preincubated with genistein (30 μg/ml) for 1 hour at 37°C before radiation exposure were apoptotic at 8 hours after radiation.
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18. Exposure of DT-40 cells to 4-Gy γ-rays stimulates the enzymatic activities of LYN, SYK, and BTK kinases within 1 min and within the next 5 min enhances tyrosine phosphorylation of multiple electrophoretically distinct substrates (*4, 17, 19, 20*). Exposure of LYN-, SYK-, and BTK-deficient DT-40 cells to 4-Gy γ-rays or 10 mM H_2O_2 results in enhanced tyrosine phosphorylation, but the magnitude of this response is smaller than that of the enhanced tyrosine phosphorylation in wild-type DT-40 cells.

Thus, all three PTKs contribute to the enhanced tyrosine phosphorylation in irradiated DT-40 lymphoma B cells. Ionizing radiation or H_2O_2 do not have a direct activation effect on any of these three PTKs immunoprecipitated from DT-40 cells. Thus, radiation or H_2O_2 activation of BTK is mediated by as yet unknown mechanisms (*17*).
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28. Chicken *btk* cDNA clones were isolated from a chicken spleen cDNA library (Clontech, Palo Alto, CA) with human *btk* cDNA (HGMP Resource Centre, Harrow, Middlesex, UK) as a probe under low-stringency conditions. Using chicken *btk* cDNA, we obtained chicken *btk* genomic clones by screening a genomic library constructed by ligation of Eco RI–digested DT-40 genomic DNA (range, 6 to 9 kb) with vector arms of the λ ZAP II (Strategene). For disruption of the *btk* gene, targeting constructs containing the neomycin-resistance gene cassette (pcBTK-neo) or histidinol-resistance gene cassette (pcBTK-hisD) were sequentially transfected into DT-40 cells (M. Takata and T. Kurosaki, unpublished data). The targeting vectors, pcBTK-neo and pcBTK-hisD, were constructed by replacement of the 0.7-kb Bgl II–Bam HI genomic fragment containing exons, which correspond to human BTK amino acid residues 91 to 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 (DNA) blot analysis by means of a 3′ flanking probe (0.5-kb Bgl II–Bgl II 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 the BTK-deficient DT-40 clone confirmed that homologous recombination occurred 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. The establishment of LYN-deficient (*9*), SYK-deficient (*7*), and CSK-deficient (*27*) DT-40 clones have been described. We also established a mutant DT-40 clone that is deficient in both LYN and SYK by disrupting *syk* gene in LYN-deficient DT-40 cells (M. Takata and T. Kurosaki, *J. Exp. Med.*, in press). For generation of these LYN/SYK doubly deficient cells, the targeting vector pSYK-bsr was constructed by replacement of the *neo* gene of pSYK-Neo with blasticidin S resistance gene (*bsr*) (Funakoshi, Tokyo, Japan) and transfected into LYN-deficient DT-40 cells.
29. Mutations in the human *btk* cDNA were introduced by polymerase chain reaction through use of Pfu polymerase (Strategene) and confirmed by sequencing. Wild-type and mutant *btk* cDNAs were subcloned into pApuro expression vector [M. Takata *et al.*, *EMBO J.* **13**, 1341 (1994)] and electroporated into BTK-deficient cells.
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38. Supported by National Institutes of Health grants R01-CA-42111 and R01-CA-42633. F.M.U. is a Stohlman Scholar of the Leukemia Society of America.

19 March 1996; accepted 25 June 1996

Corticofugal Modulation of Time-Domain Processing of Biosonar Information in Bats

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The Jamaican mustached bat has delay-tuned neurons in the inferior colliculus, medial geniculate body, and auditory cortex. The responses of these neurons to an echo are facilitated by a biosonar pulse emitted by the bat when the echo returns with a particular delay from a target located at a particular distance. Electrical stimulation of cortical delay-tuned neurons increases the delay-tuned responses of collicular neurons tuned to the same echo delay as the cortical neurons and decreases those of collicular neurons tuned to different echo delays. Cortical neurons improve information processing in the inferior colliculus by way of the corticocollicular projection.

The processing of auditory information has been considered to be based on neural interactions occurring within the ascending auditory system (*1*). The contribution of the massive corticofugal system to auditory information processing has been given little consideration. Neurons in the deep layers of the auditory cortex (AC) project to the inferior colliculus (IC), the medial geniculate body (MGB) (*2–4*), or the subcollicular auditory nuclei (*5*). The corticofugal projections originating from the low- and high-frequency–tuned regions of the AC terminate at the low- and high-frequency–tuned regions of the MGB and IC, respectively (*3*). Electrical stimulation of the AC can inhibit or facilitate the auditory responses of neurons in the IC (*6–8*) and MGB (*6*, *9*). However, the response properties of neurons at the stimulation and recording sites were not examined in these studies. Therefore, it is not known how the inhibition and facilitation evoked by electrical stimulation contribute to auditory information processing.

In the visual system, however, an important functional role of the corticofugal projection in processing visual images has recently been identified. Through the corticothalamic projection, cortical visual neurons tuned to a particular orientation of a moving contour synchronize activities of thalamic visual neurons having receptive fields that are aligned appropriately to signal that particular orientation of a moving contour. This synchronized activity is hypothesized to facilitate the detection of the stimulus feature by the cortical visual neurons (*10*). Because corticofugal projections are part of the neural net shared by the auditory, visual, and somatosensory systems, the corticofugal projections in the auditory system presumably have a function similar to that of those found in the visual system.

The central auditory system of the Jamaican mustached bat (*Pteronotus parnellii parnellii*) is highly developed for processing different types of biosonar information in a parallel and hierarchical way. Its auditory cortex consists of many functional areas (*1*). Among these areas, the FM-FM area (FM, frequency-modulated) is particularly interesting because it has a map for a systematic representation of echo delays corresponding to target distances. Therefore, we studied the functional role of the corticofugal projections in the processing

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