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Src Family Protein Tyrosine Kinases Induce Autoactivation of Bruton's Tyrosine Kinase

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Bruton's tyrosine kinase (Btk) is tyrosine phosphorylated and enzymatically activated following ligation of the B-cell antigen receptor. These events are temporally regulated, and Btk activation follows that of various members of the Src family of protein tyrosine kinases, thus raising the possibility that Src kinases participate in the Btk activation process. We have evaluated the mechanism underlying Btk enzyme activation and have explored the potential regulatory relationship between Btk and Src protein kinases. We demonstrate in COS transient-expression assays that Btk can be activated through intramolecular autophosphorylation at tyrosine 551 and that Btk autophosphorylation is required for Btk catalytic functions. Coexpression of Btk with members of the Src family of protein tyrosine kinases, but not Syk, led to Btk tyrosine phosphorylation and activation. Using a series of point mutations in Blk (a representative Src protein kinase) and Btk, we show that Src kinases activate Btk through an indirect mechanism that requires membrane association of the Src enzymes as well as functional Btk SH3 and SH2 domains. Our results are compatible with the idea that Src protein tyrosine kinases contribute to Btk activation by indirectly stimulating Btk intramolecular autophosphorylation.

Bruton's tyrosine kinase (Btk) is a member of the Tec family of nontransmembrane protein tyrosine kinases (PTKs). This family of cytoplasmic enzymes includes Tec I and Tec II (19, 20), Itk/Tsk/Emt (9, 11, 28, 36), Bmx (29), and Txk/Rlk (10, 13) in addition to Btk (32, 34). Btk contains an amino-terminal pleckstrin homology (PH) domain of approximately 140 amino acids, followed by an 80-residue Tec homology domain (35), a Src homology 3 (SH3) domain of 49 amino acids, a 96-residue SH2 domain, and an SH1 or catalytic domain of approximately 250 amino acids.

Mutations in the Btk gene have been found to lead to human X-linked agammaglobulinemia (XLA) as well as to murine X chromosome-linked immunodeficiency (xid) (23, 30, 32, 34). XLA is a severe immunodeficiency characterized by a lack of immunoglobulin (Ig)-positive B cells in the peripheral blood and lymphoid tissues and very low levels of all classes of Igs in serum (reviewed in references 7 and 31). The number of bone marrow B-cell precursors has been found to be normal in some patients, so that XLA is considered to represent a disease caused by an arrest in B-cell differentiation at the pre-B cell stage (7, 31). xid is less severe than XLA since mature B cells are detected in the periphery, albeit at reduced levels (31). However, B cells from mice with xid do not produce antibodies to polysaccharides or other thymus-independent antigens (31). The mutation that causes xid has been shown to occur at arginine 28 (mutated to the codon for cysteine) within the PH domain of the enzyme (23, 30). The same residue has been shown to be mutated in patients with XLA (7). Of interest are the observations that other mutations in Btk that give rise to XLA can be found in the catalytic domain (22, 32, 34), SH2 domain (7, 26), and SH3 domain (22, 40), as well as the PH domain. These results demonstrate that different regions of Btk are critical for its normal function in B cells and suggest that Btk might interact physically and/or functionally with multiple cellular proteins. Indeed, the PH domain of Btk has been reported to interact with various isoforms of protein kinase C (38) as well as the βγ subunits of heterotrimeric G proteins (31). However, proteins interacting with the Btk SH2 and SH3 domains in B cells have not been reported.

It has been shown that Btk is activated following ligation of the B-cell antigen receptor (BCR) in mature and immature B cells (2, 8, 27) and may be constitutively activated in pre-B cells (2). In addition to Btk activation, surface engagement of the BCR has been shown to activate other classes of nontransmembrane PTKs, including Lyn and Blk (5, 6, 37) (members of the Src family) and Syk (14, 16, 27). Src family kinases are rapidly activated following BCR engagement, and their activation correlates with the initial tyrosine phosphorylation of the immunoreceptor tyrosine activation motifs on the BCR Igα and Igβ subunits (3, 27). Syk activation occurs following the activation of the Src kinases and phosphorylation of the Igα/Igβ immunoreceptor tyrosine activation motifs and correlates with the tyrosine phosphorylation of Syk and the association of Syk with tyrosine-phosphorylated Igα and Igβ (14, 16, 27). Recent evidence indicates that the association of the Syk tandem SH2 domains with the tyrosine-phosphorylated Igα/β immunoreceptor tyrosine activation motifs leads to the stimulation of Syk protein kinase activity through a Syk autophosphorylation-dependent mechanism (25). Btk was also found to be activated following the activation of Src PTKs but preceding the activation of Syk (27). Similar to Syk, activation of Btk protein kinase activity was found to accompany an increase in Btk tyrosine phosphorylation (2, 8, 27), although it is not known whether tyrosine phosphorylation of Btk is a regulatory event or merely a consequence of the activation of Btk or other PTKs. However, unlike Syk, no association of activated Btk with components of the BCR was detected (8, 27). Thus, the mechanisms underlying Btk tyrosine phosphorylation and activation remain unresolved.

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The tyrosine phosphorylation and activation of Btk following the activation of Src PTKs in B cells raises the possibility that the specific activity of Btk is regulated directly or indirectly by Src kinases. To test this hypothesis, we have examined the contribution of tyrosine phosphorylation to the regulation of Btk kinase activity and explored the potential interactions of Btk with members of the Src family of PTKs by using transient coexpression in COS cells. The results of our experiments reveal that Btk autophosphorylation at tyrosine 551 stimulates and is required for Btk protein kinase activity. Btk autophosphorylation in vitro was determined to occur exclusively through an intramolecular mechanism. Coexpression of Btk with Src PTKs but not Syk in COS cells led to the tyrosine phosphorylation and activation of Btk PTK activity. However, Btk was found not to be a direct substrate of Src PTKs, since coexpression of Src PTKs with an ATP-binding mutant of Btk was not tyrosine phosphorylated. To activate Btk enzyme activity, Src PTKs were found to require intact myristylation, ATP binding, and autophosphorylation sites while functional SH2 or SH3 domains were dispensable. Src PTK-dependent activation of Btk did not require an intact PH domain but did require functional Btk SH3 and SH2 domains along with a functional ATP-binding site and tyrosine 551 autophosphorylation site. These results are consistent with the notion that Src family PTKs are capable of indirectly regulating Btk PTK activity by stimulating Btk autophosphorylation.

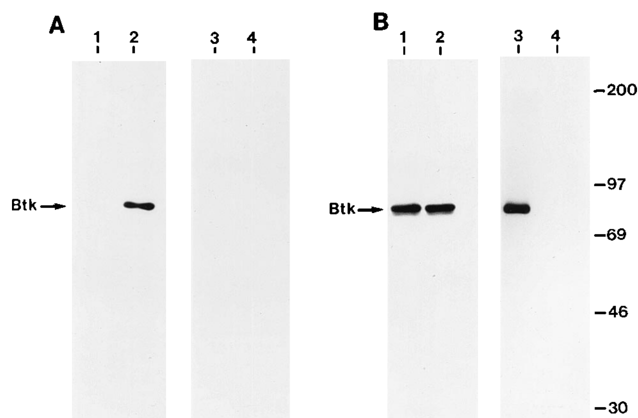

FIG. 1. Expression of Btk in COS cells. (A) pSV7c (lanes 1 and 3) or pSV7cBtk (lanes 2 and 4) was transfected into COS cells, cell lysates were immunoprecipitated with anti-Btk, and the immunoprecipitates were used for immunoblotting with anti-Btk (lanes 1 and 2) or APT (lanes 3 and 4). (B) COS cells transfected with pSV7cBtk were lysed, and Btk was immunoprecipitated with anti-Btk and divided into four aliquots. Immune-complex protein kinase assays (1 mM ATP plus 5 mM $MnCl_2$ for 5 min) were performed on two aliquots (lanes 1 and 3), while the other two samples were incubated with kinase buffer lacking ATP and $MnCl_2$ (lanes 2 and 4). The samples were then used for immunoblotting with anti-Btk (lanes 1 and 2) or APT (lanes 3 and 4). The positions of prestained molecular mass markers (in kilodaltons) (Life Technologies, Inc.) are shown on the right, and the position of Btk is indicated.

MATERIALS AND METHODS

Cloning, mutagenesis, and epitope tagging of murine Btk. The Btk open reading frame was cloned from a mouse A20/2J B-cell cDNA library (Stratagene) by a PCR-based strategy with published Btk sequences (32). Three separate amplification reactions were performed to yield *Bgl*II-flanked Btk fragments representing bp 1 to 728, 729 to 1907, and 1908 to 2100. The individual elements were then ligated at the naturally occurring *Bgl*II sites in the Btk gene into the *Bam*HI site of pBluescript SKII$^+$ vector (Stratagene). Sequencing of the final plasmid demonstrated that the wild-type Btk open reading frame was reconstituted. In vitro transcription/translation demonstrated that the open reading frame could be translated into a protein of the expected size with appropriate PTK activity.

Point mutations were introduced into Btk by PCR with an oligonucleotide encoding the required mutation (39). The following mutations were introduced. In the PH domain, the codons for lysine 26 or arginine 28 were mutated to code for glutamic acid 26 (denoted Btk PH1) or cysteine 28 (Btk PH2), respectively. An additional mutant containing both codon 26 and 28 mutations (Btk 1/2) was also constructed. In the SH3 domain, both conserved tryptophan residues at 251 and 252 were mutated to leucine (Btk SH3). In the SH2 domain, arginine 307 was changed to lysine (Btk SH2). In the catalytic domain, lysine 430 of the ATP-binding site was changed to glutamic acid (Btk ATP$^-$) and the autophosphorylation site at tyrosine 551 was changed to phenylalanine (Btk APS$^-$). All mutations were confirmed by DNA sequence analysis.

For production of HA-tagged Btk (HA-Btk), the Btk coding sequences were modified at the 5′ end by addition of an oligonucleotide linker including a methionine initiation codon and the codons for the influenza virus HA sequence YPYDVPDYA. The HA sequence can be recognized by monoclonal antibody 12CA5. The modified Btk was subcloned into COS expression vector pSV7c (1). The final construct yielded HA-Btk in which the HA sequences were incorporated in frame in place of the normal Btk initiation codon. For production of c-Src membrane localization sequence-tagged Btk (ST^{G2}-Btk), the Btk coding sequences were modified at the 5′ end by addition of an oligonucleotide linker containing the codons for the sequence MGSSKSKPKDPSQRRRS, which represents the first 17 amino acids of c-Src (24). These sequences are sufficient to direct the myristylation of glycine 2 and membrane localization of members of the Src family of PTKs as well as other proteins (24). As a control, the same linker was added to Btk, except that the second codon was changed from glycine to alanine to code for ST^{A2}-Btk. Mutation of G to A at position 2 abolishes the myristylation and membrane association of c-Src (24). The modified ST-Btks were subcloned into pSV7c. Proteins containing these c-Src sequences can be recognized by monoclonal antibody D710.

Cloning and mutagenesis of B-cell Src PTKs. Mature splenic murine B cells express primarily four members of the Src family of PTKs: Blk, Lyn, Fyn, and Hck (5, 17). The mouse *blk* open reading frame was cloned by reverse transcriptase PCR (21) from mouse spleen RNA with Hot Tub (Amersham) polymerase. Following subcloning into pBluescript SK$^+$ (Stratagene), the gene was further manipulated by overlap extension PCR (12) to generate mutations in the protein. The myristylation mutant was generated by changing glutamic acid to alanine at position 2; the autophosphorylation mutant was generated by replacing tyrosine at position 383 with phenylalanine; the regulatory tyrosine site at 495 was changed to phenylalanine as well; and the ATP-binding site at position 263 was changed from lysine to glutamic acid. Mutations in the SH2 and SH3 domains were made individually and then combined by restriction digestion and ligation to generate mutations in both domains. These mutations consisted of a switch from arginine to lysine at position 145 for the SH2 domain and a switch from either proline to leucine at position 103 or a change of the tryptophan residues at both positions 88 and 89 to leucines for the SH3 mutants. All clones generated by PCR were then verified by restriction digestion and sequencing. The other Src B-cell kinases were cloned by a similar strategy.

COS expression. The various pSV7c-containing plasmids (2 to 20 μg) were transfected into 2×10^6 COS-7 cells in Lipofectamine (GIBCO/BRL), and the cells were harvested 48 h later (25). When coexpression experiments were performed, the amounts of different plasmids were adjusted following preliminary experiments to yield equivalent expression of the necessary proteins.

Antibodies and biochemical analyses. Antibodies directed against various Src family PTKs, Syk, and phosphotyrosine (APT) have been described previously (5, 17). Antibodies to Btk were produced by immunization of rabbits with glutathione *S*-transferase (GST) fusion proteins (Pharmacia Biotech Inc.) containing the first 150 amino acids of Btk. Immunoprecipitations, immune-complex protein kinase assays, and immunoblotting were all conducted as described previously (4, 33). Phosphoamino acid analysis and cyanogen bromide (CNBr) peptide analysis have been described previously (15, 18). Quantitation of radioactive proteins following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed by analysis with a Molecular Dynamics PhosphorImager and ImageQuant software.

RESULTS

Btk activation by intramolecular autophosphorylation at tyrosine 551. The results in Fig. 1A demonstrate that Btk expressed in COS cells is not tyrosine phosphorylated to a detectable level, even though the same Btk molecules are capable of tyrosine autophosphorylation in an immune-complex protein kinase assay following cell lysis (Fig. 1B). These results indicate that enzymatically competent Btk expressed in COS cells does not undergo detectable autophosphorylation or tyrosine phosphorylation by endogenous COS PTKs. As shown in Fig. 2B, phosphoamino acid analysis of the immune-complex Btk autophosphorylation reaction products revealed exclusively phosphotyrosine. CNBr cleavage of the autophosphory-

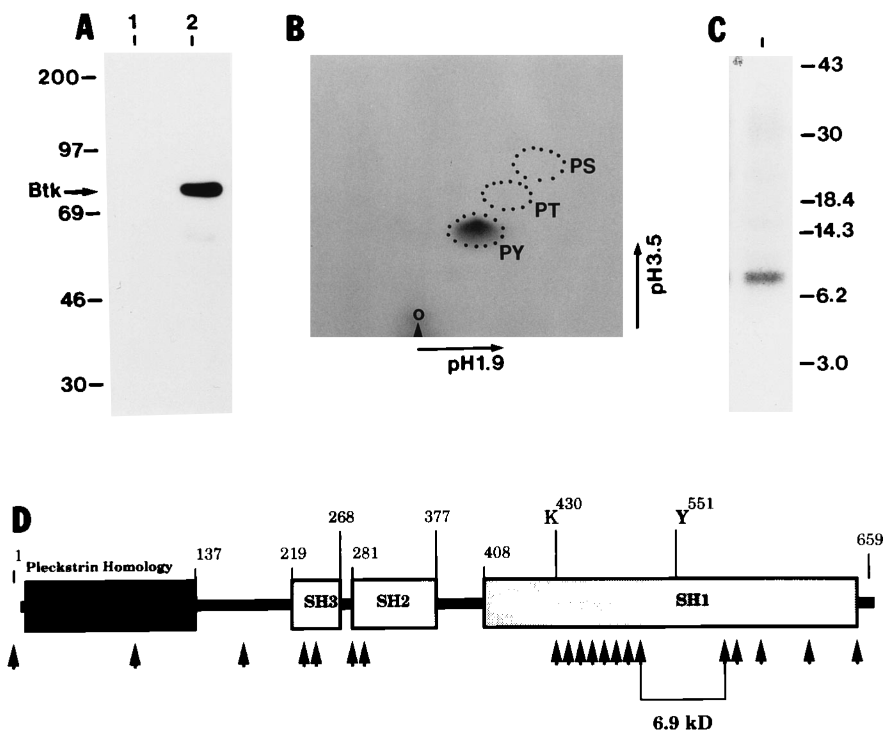

FIG. 2. Characterization of Btk immune-complex kinase reaction products. (A) Btk was immunoprecipitated from lysates of pSV7c- (lane 1) or pSV7cBtk (lane 2)-transfected COS cells, and immune-complex kinase assays were performed with [γ-^{32}P]ATP. (B and C) The ^{32}P-labeled Btk bands from parallel immune complexes were isolated, and phosphoamino acid analysis (B) or cyanogen bromide cleavage analysis (C) was conducted. (D) Diagram of methionines present in the Btk sequence. The positions of prestained molecular mass markers (in kilodaltons) and Btk are indicated in panels A and C. The buffer pH, direction of electrophoresis, pattern of ninhydrin-stained phosphoamino acid standards (PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine), and origin (o) for two-dimensional phosphoamino acid analysis are shown in panel B.

lated Btk was found to yield a single phosphopeptide of approximately 7 kDa (Fig. 2C), which was most compatible with a potential site of phosphorylation at tyrosine residue 551. This speculation was confirmed by site-specific mutation of tyrosine 551 to phenylalanine, which abolished all detectable Btk autophosphorylation (Fig. 3A and B). Mutation of the same residue back to tyrosine restored Btk autophosphorylation capability (Fig. 3A and B). Interestingly, the tyrosine-to-phenylalanine mutation of Btk was also found to destroy the capability of the enzyme to phosphorylate exogenous substrates such as GST-Igα (Fig. 3C) or rabbit muscle enolase (data not shown).

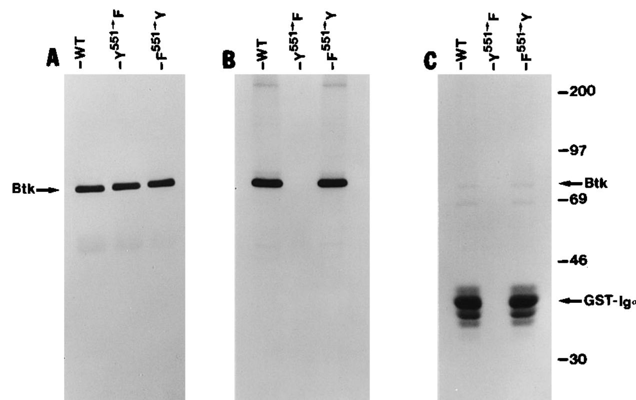

FIG. 3. Analysis of Btk site-specific mutations at tyrosine 551. Wild-type Btk (WT), Btk tyrosine 551 to phenylalanine (Y^{551}→F), and Btk phenylalanine 551 to tyrosine (F^{551}→Y) were expressed in COS cells, immunoprecipitated with anti-Btk, and divided into three aliquots. The samples were then immunoblotted with anti-Btk (A) or used in immune-complex kinase assays with [γ-^{32}P]ATP without (B) or with (C) GST-Igα as an exogenous substrate. The positions of Btk, GST-Igα, and prestained molecular mass markers (in kilodaltons) are indicated.

To determine whether Btk autophosphorylation affected Btk enzyme activity, Btk was immunoprecipitated from COS cell lysates and incubated with 1 mM ATP and 5 mM $MnCl_2$ for different lengths of time from 30 s to 1 h. The results of these experiments show that tyrosine phosphorylation of Btk increased as a function of reaction time and correlated at later times with diminished electrophoretic mobility in SDS-gels (Fig. 4A and B). Representative samples from the control (no ATP-$MnCl_2$ preincubation) and from Btk incubated for 30 min with ATP-$MnCl_2$ were then used for immune-complex protein kinase assays with GST-Igα as an exogenous substrate. A time course of GST-Igα tyrosine phosphorylation by control (Fig. 4C) or autophosphorylated (Fig. 4D) Btk revealed that autophosphorylation of Btk resulted in a significant increase in the specific activity of the enzyme. Quantitation of these experiments demonstrated that the difference in Btk activity was over 20-fold at 5 min and approximately 5-fold at 10 min (Fig. 4E). Reduction in relative activity at the later time point is most probably due to changes in specific activity of the "control" Btk as it becomes increasingly autophosphorylated over the course of the experiment. Point mutations within the Btk PH, SH3, or SH2 domains yielded Btk proteins that were found to be as capable as wild-type Btk of undergoing autophosphorylation-dependent activation (data not shown).

Given the apparent importance of tyrosine 551 autophosphorylation in regulating Btk protein kinase activity, we sought to determine whether the in vitro reaction mechanism was intramolecular, intermolecular, or both. For these exper-

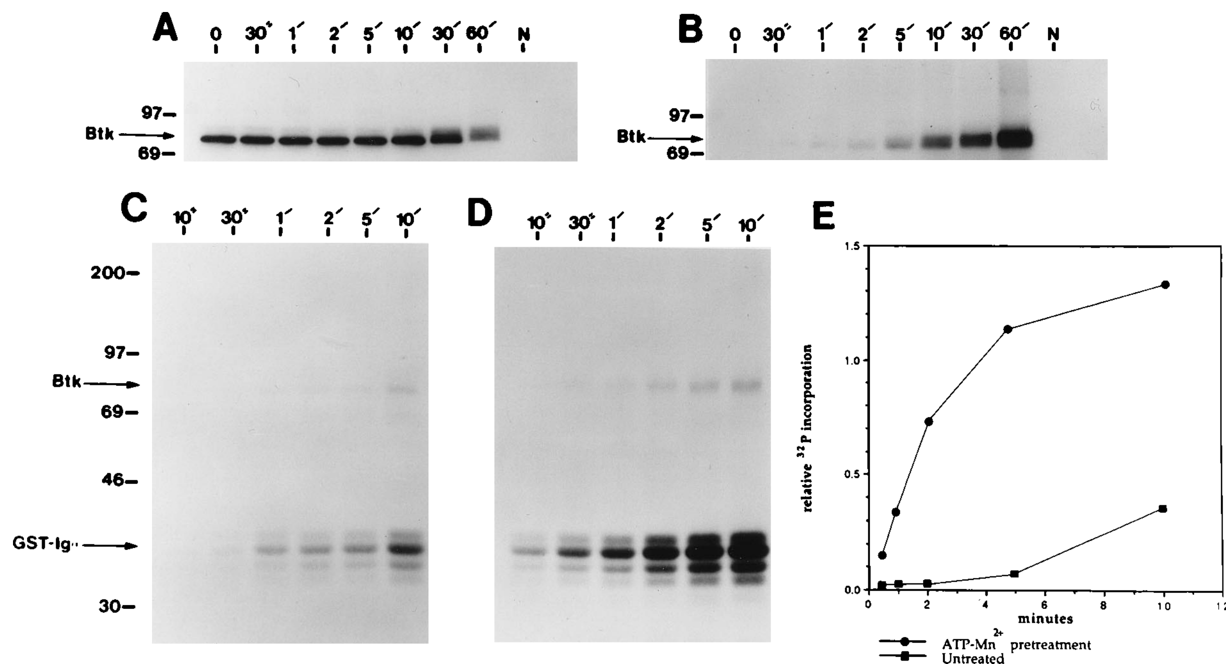

FIG. 4. Btk enzyme activation by autophosphorylation. (A and B) Btk was expressed in COS cells and immunoprecipitated with anti-Btk or preimmune rabbit serum (N), and immune-complex kinase assays were conducted with 1 mM ATP and 5 mM $MnCl_2$ for the indicated times. At each time point, the Btk immunoprecipitates were divided into two aliquots and immunoblotted with anti-Btk (A) or APT (B). (C and D) Btk immunoprecipitates from untreated (C) or ATP-Mn^{2+}-treated (30-min) (D) samples were used in immune-complex kinase time course (10 s to 10 min) assays with GST-Igα as an exogenous substrate. Quantitation of the relative ^{32}P incorporation into GST-Igα as a function of time is shown in panel E. The positions of Btk, GST-Igα, and prestained molecular mass markers (in kilodaltons) are indicated.

iments, two versions of HA-Btk which contained the 9-amino-acid HA epitope in the amino terminus of the Btk enzyme were generated. In one set, the Btk enzyme was wild type, while in the other, the Btk ATP-binding site at lysine 430 was mutated to glutamic acid. As shown in Fig. 5B (lanes 2 and 3), following expression in COS cells and immunoprecipitation with anti-Btk, the lysine-to-glutamic acid mutation abolished all detectable Btk kinase activity. Neither of the HA-Btk chimeras were found to be tyrosine phosphorylated when expressed either alone or together in COS cells (data not shown). Wild-type Btk was then coexpressed in COS cells with the ATP-binding-site mutant of HA-Btk, and both coimmunoprecipitated with anti-Btk. Btk immunoblots demonstrate that both forms were precipitated (Fig. 5A, lane 4). Immune-complex protein kinase assays were conducted in parallel aliquots. As shown in Fig. 5B, lane 4, all of the ^{32}P incorporation was found in the wild-type Btk. Dissociation of the initial immune complex following the in vitro kinase reaction and reimmunoprecipitation of the products with anti-HA (Fig. 5A and B, lanes 5) revealed that only the kinase active enzyme was phosphorylated. These results suggest that Btk autophosphorylation in vitro takes place primarily through an intramolecular mechanism.

Btk activation by coexpression with Src PTKs. The peak of Btk enzyme activation in B cells occurs following the activation of Src family members and prior to that of Syk (27). To determine if Src PTKs or Syk might influence Btk kinase activity, we coexpressed in COS cells wild-type Btk with either Blk, Lyn, Fyn, Hck (four members of the Src family that are normally expressed in B cells), or Syk and analyzed Btk tyrosine phosphorylation and enzyme activity. As shown in Fig. 6, expression of Btk with any of the Src enzymes resulted in Btk tyrosine phosphorylation (Fig. 6B) and significant (approximately 10-

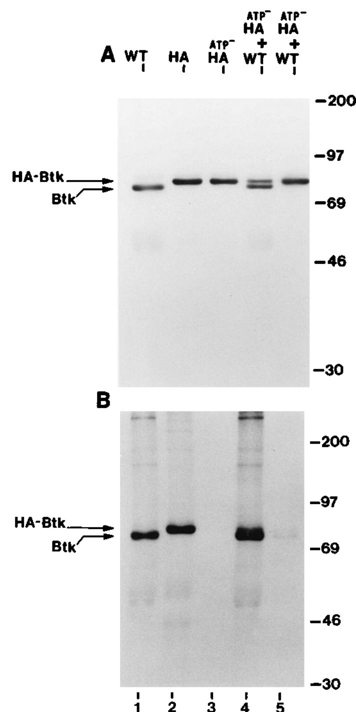

FIG. 5. Btk autophosphorylation is intramolecular. Wild-type Btk (WT), HA-tagged Btk (HA), and the HA-tagged lysine 430-to-glutamic-acid Btk mutant (ATP^-HA) were expressed in COS cells individually or together as indicated. Cell lysates were immunoprecipitated with anti-Btk and divided into two aliquots. Immune-complex kinase assays with [γ-^{32}P]ATP were performed with one set (B), and Btk immunoblots were performed with the other (A). The samples in lanes 5 were immunoprecipitated with anti-Btk and then reimmunoprecipitated with anti-HA. The positions of Btk, HA-tagged Btk (HA-Btk), and prestained molecular mass markers (in kilodaltons) are indicated.

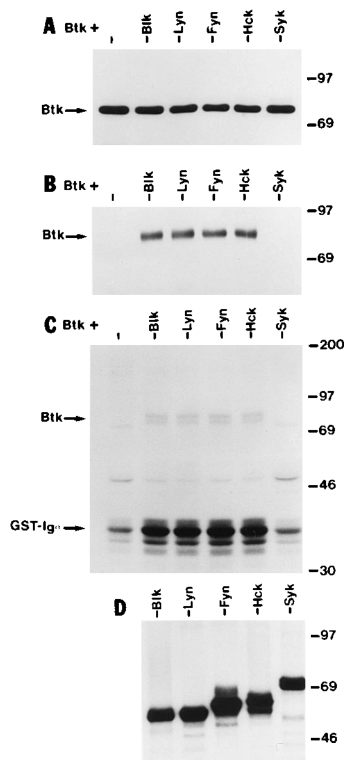

FIG. 6. Coexpression of Btk with Src PTKs and Syk. Btk was expressed in COS cells alone, with the indicated members of the Src family, or with Syk. (A to C) Btk was immunoprecipitated, and Btk immunoblots (A), APT immunoblots (B), or Btk immune complex kinase assays ([γ-^{32}P]ATP) with GST-Igα (C) were performed. (D) The various members of the Src family of Syk coexpressed with Btk in COS cells were immunoprecipitated with antibodies specific for each, and immune complex kinase assays with [γ-^{32}P]ATP were conducted. The positions of Btk, GST-Igα, and prestained molecular mass markers (in kilodaltons) are indicated.

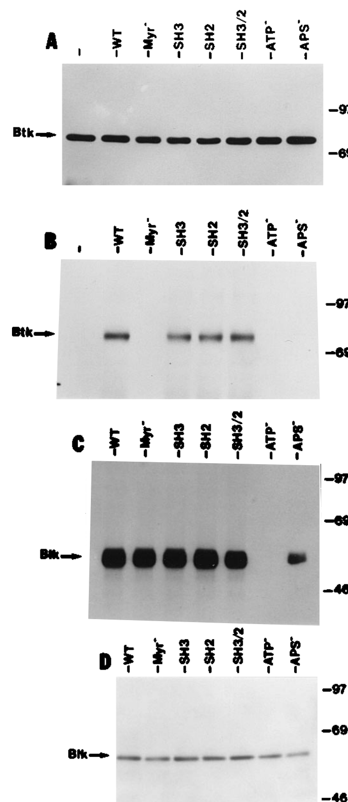

FIG. 7. Coexpression of Btk with Blk mutants. Btk was expressed in COS cells alone or with the indicated site-specific mutants of Blk. (A and B) Btk was immunoprecipitated with anti-Btk, and Btk immunoblots (A) or ATP immunoblots (B) were performed. (C and D) Blk was immunoprecipitated with anti-Blk, and immune-complex protein kinase assays with [γ-^{32}P]ATP immunoblots (C) or Blk immunoblots (D) were performed. The positions of Btk, Blk, and prestained molecular mass markers (in kilodaltons) are indicated.

fold) Btk enzyme activation (Fig. 6C). However, coexpression with Syk did not affect Btk tyrosine phosphorylation or enzyme specific activity.

To determine which elements of the Src enzymes were required to activate Btk, site-specific mutations were generated in Blk. For these experiments, point mutations were created at the Blk myristylation site, in the Blk SH3 domain, in the Blk SH2 domain, in the SH3-plus-SH2 domain, at the Blk ATP-binding site, at the SH1 autophosphorylation site, and at the carboxy-terminal regulatory tyrosine phosphorylation site (see Materials and Methods for details). Wild-type Blk and the various Blk mutants were then coexpressed with wild-type Btk in COS cells. As shown in Fig. 7, the Blk proteins were expressed at similar levels (Fig. 7D) and all had similar levels of protein kinase activity (Fig. 7C, lanes 1 to 5) with the exception of the ATP-binding-site mutant, which had no detectable activity (Fig. 7C, lane 6), and the SH1 autophosphorylation site mutant (Fig. 7C, lane 7), which had reduced levels of autophosphorylating activity. As shown in Fig. 7B, Blk-induced tyrosine phosphorylation of Btk required an intact myristylation site along with an intact ATP-binding site and site of autophosphorylation. As observed previously, Btk tyrosine phosphorylation correlated with Btk enzyme activity (data not shown). Blk mutations in the SH3 or SH2 domains, alone or in combination, did not alter Blk activation of Btk. Site-specific mutation of Blk at the carboxy-terminal regulatory tyrosine phosphorylation site also did not affect Blk activation of Btk (data not shown). These results suggest that to activate Btk in COS cells, members of the Src family must be membrane associated, possess catalytic activity, and require an intact autophosphorylation site.

To determine which elements of Btk were required to be activated by Src PTKs, various point mutations were introduced into Btk as described in Materials and Methods. These included two different PH domain mutations (including the xid mutation), an SH3 mutation, an SH2 mutation, a mutation of the ATP-binding site, and the above-described autophosphorylation site mutation at tyrosine 551. The various Btk mutants were then coexpressed in COS cells with wild-type Blk. As shown in Fig. 8, Blk-induced tyrosine phosphorylation and activation of Btk was PH domain independent but required intact Btk SH3 and SH2 domains. Preincubation of the SH3 and SH2 Btk mutants with unlabeled ATP and $MnCl_2$ was found to be capable of inducing Btk activation in immune complexes and was indistinguishable from that of wild-type Btk (data not shown). Mutation of the Btk ATP-binding site or mutation of the autophosphorylation site (mutations which abolished detectable Btk enzyme activity) resulted in Btk proteins that were not tyrosine phosphorylated when coexpressed with Blk. These results imply that Btk is not a direct substrate for Src enzymes. Furthermore, these observations indicate that Btk tyrosine phosphorylation and enzymatic activation in COS cells results from Btk autophosphorylation, which can be stimulated by Src coexpression through a Btk SH3- and SH2-

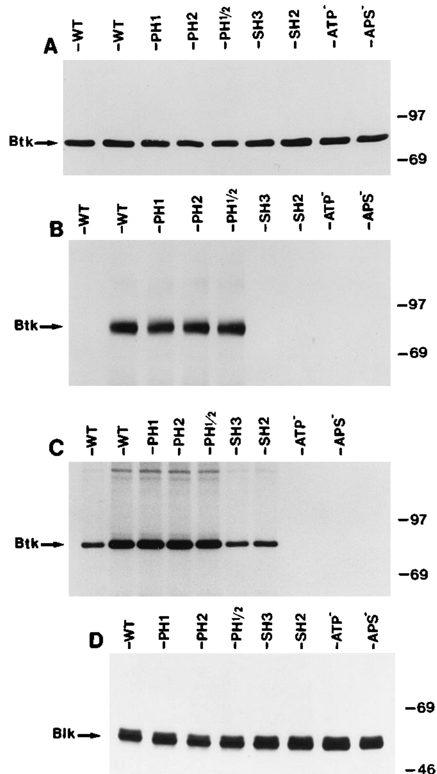

FIG. 8. Coexpression of Blk with Btk mutants. Wild-type Btk (WT) or the indicated site-specific mutants of Btk were coexpressed with wild-type Blk in COS cells. (A to C) Btk was immunoprecipitated with anti-Btk, and Btk immunoblots (A), APT immunoblots (B), or Btk immune-complex autophosphorylation assays with [γ-^{32}P]ATP (C) were conducted. (D) Blk was immunoprecipitated with anti-Blk followed by immune complex autophosphorylation assays with [γ-^{32}P]ATP. The positions of Btk, Blk, and prestained molecular mass markers (in kilodaltons) are indicated.

dependent mechanism. In other experiments, we found that expression of Blk with wild-type Btk and the HA-Btk ATP-binding-site mutation resulted in tyrosine phosphorylation of only the wild-type Btk, suggesting that Btk autophosphorylation in COS cells also appears to take place through an intramolecular mechanism (data not shown).

DISCUSSION

The results presented above demonstrate that Btk enzyme activity is positively regulated through an autophosphorylation-dependent process which appears to involve an intramolecular mechanism. The primary site of Btk autophosphorylation in our immune-complex protein kinase assays was determined to be at tyrosine residue 551, which is present in the SH1 or catalytic domain of the enzyme. However, we cannot rule out that Btk might be capable of phosphorylating additional sites under different experimental conditions. Since site-specific mutation of this tyrosine residue to phenylalanine abolished all detectable Btk protein kinase activity, phosphorylation of tyrosine 551 appears to play an integral role in the Btk catalytic process. Stimulation of Btk enzyme activity by autophosphorylation or autoactivation was independent of a functional Btk SH2 domain, indicating that this type of Btk activation does not depend upon intramolecular or intermolecular complexes of phosphorylated tyrosine 551 with Btk SH2s. Btk autoactivation was also found to be independent of functional SH3 domains as well as intact PH domains. As the Btk PH domain appears to have several potential subdomains that may interact with different proteins and/or lipids, we cannot rule out that other mutations in the PH domain might influence Btk autoactivation. Additionally, since we have not created mutations within the TH domain, it is possible that this region of the enzyme also plays a role in the Btk autoactivation process.

In our experiments, the wild-type Btk expressed in COS cells was not detectably tyrosine phosphorylated and possessed low catalytic activity in immune-complex protein kinase assays. Since Btk was found to be activatable in immune complexes following addition of ATP and Mn^{2+}, our results suggest that Btk activity may be suppressed in vivo. Since dramatic overexpression (relative to the level found in B cells) of Btk did not promote Btk tyrosine phosphorylation in the COS cells, the suppression of Btk autophosphorylation in cells may be the result of either abundant or highly active Btk suppressors (e.g., phosphotyrosyl phosphatases). Clearly, Btk could act as its own suppressor through some intra- or intermolecular mechanism. It is possible that the binding of antibodies to the Btk PH domain causes the Btk conformation to be altered in immune complexes, thereby promoting autophosphorylation and enzyme activation. We did find that HA-tagged Btk immunoprecipitated with anti-HA antibodies performed the same as wild-type Btk in similar experiments (data not shown). However, even in this case, we cannot rule out that binding of antibodies to the HA tag immediately amino-terminal to the PH domain did not promote the same level of putative conformational change as binding of antibodies directly to the PH domain.

Regardless of the cause underlying Btk enzymatic quiescence, coexpression of Btk with Src family PTKs in COS cells resulted in Btk tyrosine phosphorylation and Btk enzyme activation. All Src family members normally expressed in B cells demonstrated an ability to stimulate Btk phosphorylation and activation. We have tested all of the other mammalian Src PTKs (Yes, Lck, Fgr, and Src) in this assay and found that they all possessed roughly equal capacity to stimulate Btk. As a model for Src PTK-Btk interactions, we evaluated the contribution of various Blk elements by creating site-specific mutants. Our results demonstrated that Btk activation required that Blk possess an intact myristylation site, an autophosphorylation site, and catalytic activity. Functional Blk SH3 and SH2 domains were not required for Btk activation in these assays. The carboxy-terminal regulatory tyrosine phosphorylation site was also found to be dispensable. Taken together, these results indicate that Src PTK membrane association and catalytic activity are the most important attributes needed to stimulate Btk tyrosine phosphorylation and activation. It is clear from the Btk mutational analysis that Btk does not appear to be a direct substrate of Src PTKs or a substrate for other cellular PTKs potentially stimulated by Src PTKs. Since the SH2 domain of Btk was found to be important for Src PTK-induced Btk activation, it is possible that Btk interacts directly with the activated, autophosphorylated Src PTKs through SH2-phosphotyrosine-dependent complexes. However, we have been unable to detect Btk-Src PTK complexes in these cells even under conditions in which the presence of the potentially co-immunoprecipitating Src PTKs and Btk was evaluated by assaying for respective enzyme activities.

The results of these experiments with COS cells are compatible with the previously observed order of activation of Src PTKs and Btk in B cells following BCR ligation (27). Our data suggest that activation of Src PTKs results in the phosphorylation of a protein(s) in or around the plasma membrane that function(s) to promote Btk autoactivation through an auto-

phosphorylation-dependent process. The nature of this Src PTK substrate(s) is not known, but it apparently interacts with the Btk SH3 domain and/or SH2 domain to promote Btk autophosphorylation and is present not only in B cells but also in COS cells. Interestingly, tyrosine-phosphorylated proteins can be coimmunoprecipitated with tyrosine-phosphorylated Btk from B cells and COS cells (unpublished results), although we have no evidence to support the notion that these represent the putative Btk activators.

Since we have not observed significant translocation of Btk to the plasma membrane by immunofluorescence analysis following coexpression with Src PTKs (unpublished results), it is possible that the putative Src substrate(s) is itself translocated from the membrane to the cytoplasm following phosphorylation or is a primarily cytoplasmic substrate(s) that is readily phosphorylated by Src PTKs. Clearly, other variations on this theme cannot be ruled out at present. We have conducted experiments in which the Src membrane association domain (the SH4 domain) was placed in the amino terminus of wild-type Btk as well as in the Btk SH3 and SH2 mutants. When coexpressed with Src PTKs, the Src SH4 domain was unable to rescue Src-dependent activation of the SH3 or SH2 Btk mutants (unpublished results). These results suggest that Src-induced activation of Btk may be more complex than simply the promotion of Btk membrane association or may imply that if membrane association (even transient) is the key step in Btk activation, perhaps Btk needs to be associated with specialized membrane regions not available to the SH4 tagged molecules.

In summary, our results indicate that Src PTKs can modulate the activity of Btk through an indirect mechanism which appears to involve intramolecular autophosphorylation of Btk. As communication between Src PTKs and Btk was maintained in the xid PH mutant of Btk but not in the SH3 and SH2 mutants of Btk, our observations suggest that mutations in the Btk SH3 and/or SH2 domains leading to XLA may be blocked along this Src-dependent pathway.

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