Transcription Factor STAT5A Is a Substrate of Bruton’s Tyrosine Kinase in B Cells*

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STAT5A is a molecular regulator of proliferation, differentiation, and apoptosis in lymphohematopoietic cells. Here we show that STAT5A can serve as a functional substrate of Bruton’s tyrosine kinase (BTK). Purified recombinant BTK was capable of directly binding purified recombinant STAT5A with high affinity (Kd = 44 nM), as determined by surface plasmon resonance using a BIA-core biosensor system. BTK was also capable of tyrosine-phosphorylating ectopically expressed recombinant STAT5A on Tyr694 both in vitro and in vivo in a Janus kinase 3-independent fashion. BTK phosphorylated the Y665F, Y668F, and Y682F, Y683F mutants but not the Y694F mutant of STAT5A. STAT5A mutations in the Src homology 2 (SH2) and SH3 domains did not alter the BTK-mediated tyrosine phosphorylation. Recombinant BTK proteins with mutant pleckstrin homology, SH2, or SH3 domains were capable of phosphorylating STAT5A, whereas recombinant BTK proteins with SH1/kinase domain mutations were not. In pull-down experiments, only full-length BTK and its SH1/kinase domain (but not the pleckstrin homology, SH2, or SH3 domains) were capable of binding STAT5A. Ectopically expressed BTK kinase domain was capable of tyrosine-phosphorylating STAT5A both in vitro and in vivo. BTK-mediated tyrosine phosphorylation of ectopically expressed wild type (but not Tyr694 mutant) STAT5A enhanced its DNA binding activity. In BTK-competent chicken B cells, anti-IgM-stimulated tyrosine phosphorylation of STAT5 protein was prevented by pretreatment with the BTK inhibitor LFM-A13 but not by pretreatment with the JAK3 inhibitor HI-P131. B cell antigen receptor ligation resulted in enhanced tyrosine phosphorylation of STAT5 in BTK-deficient chicken B cells reconstituted with wild type human BTK but not in BTK-deficient chicken B cells reconstituted with kinase-inactive mutant BTK. Similarly, anti-IgM stimulation resulted in enhanced tyrosine phosphorylation of STAT5A in BTK-competent B cells from wild type mice but not in BTK-deficient B cells from XID mice. In contrast to B cells from XID mice, B cells from JAK3 knockout mice showed a normal STAT5A phosphorylation response to anti-IgM stimulation. These findings provide unprecedented experimental evidence that BTK plays a nonredundant and pivotal role in B cell antigen receptor-mediated STAT5A activation in B cells.

Bruton’s tyrosine kinase (BTK), a member of the Tec family of cytoplasmic protein-tyrosine kinases (PTKs), is intimately involved in signal transduction pathways regulating survival, activation, proliferation, and differentiation of B-lineage lymphoid cells (1–5). BTK participates in signal transduction pathways initiated by the binding of a variety of extracellular ligands to their cell surface receptors (2). Following ligation of B cell antigen receptors, BTK activation by the concerted actions of the PTKs LYN and SYK is required for induction of phospholipase C-γ2-mediated calcium mobilization (2). Mutations in the human btk gene are the cause of X-linked agammaglobulinemia, a male immune deficiency disorder characterized by a lack of mature, immunoglobulin-producing, peripheral B cells (6, 7). In mice, mutations in the btk gene have been identified as the cause of murine X-linked immune deficiency (8).

BTK has an N-terminal region consisting of a pleckstrin homology (PH) domain followed by a proline-rich TEC homology domain. The PH domain is the site of activation by phosphatidylinositol phosphates and G-protein βγ subunits and inhibition by protein kinase C (9). The remaining portion of BTK contains Src homology (SH) domain SH3, followed by SH2 and a C-terminal SH1/kinase domain (KD). The SH2 domain mediates binding to tyrosine-phosphorylated peptide motifs on other molecules, and the SH3 domain mediates binding to proline-rich motifs. Mutations in the SH1 domain, SH2 domain, and PH domain of human BTK have been found to cause maturational blocks at early stages of B cell ontogeny leading to XLA (10). BTK-deficient mice generated by introducing PH domain or SH1 domain mutations in embryonic stem cells exhibit defective B cell development and function (11). Thus, different domains of BTK are important for its physiologic functions.

Proximal events involving Src family PTK and SYK that lead to BTK activation following stimulation of the B cell antigen receptor are well known, but only very few signaling molecules have been identified as downstream substrates of BTK (2, 12). Recent observations suggest the involvement of BTK in signal transduction pathways affecting gene transcription (13). Recent studies have further revealed a nucleocytoplasmic shuttling system for BTK that has implications regarding potential

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† The abbreviations used are: BTK, Bruton’s tyrosine kinase; PTK, protein-tyrosine kinase; PH, pleckstrin homology; SH, Src homology; KD, kinase domain; STAT, signal transducers and activators of transcription; PCR, polymerase chain reaction; CNBr, cyanogen bromide; PFB, pFastBac1; PIFE3, 1,4-piperazinediethanesulfonic acid; JAK, Janus kinase; MBP, maltose-binding protein; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; RU, response unit(s); Inr, initiator; MOPS, 4-morpholinepropanesulfonic acid.
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Notably, Bmx/Etk, another member of the TEC PTK family, was shown to induce activation of the STAT signaling pathway in transactivated COS cells and insect ovary cells (36). More recently, Bmx/Etk was identified as a critical mediator of Src-induced cell transformation and STAT3 activation (37). Expression of Bmx/Etk in a human hepatoma cell line Hep3B resulted in a significant increase in its transforming ability, and this effect was abrogated by dominant negative inhibition of STAT3 (37), indicating that Bmx/Etk links Src to STAT3 activation and that Src-Etk-STAT3 is an important pathway in cellular transformation. While these results set a precedent for activation of STAT proteins by a member of the TEC family, the significance of these findings for the B cell physiology has not been investigated. The purpose of the present study was to examine the role of BTK in STAT5 activation in B cells that follows B cell antigen receptor ligation. Our experimental data presented herein provide unprecedented evidence that STAT5α is a substrate of BTK and that engagement of the B cell antigen receptor activates STAT5α in a BTK-dependent (but not JAK3-dependent) fashion. Thus, our study provides experimental evidence that BTK couples the STAT signaling pathway to the B cell antigen receptor. Our findings prompt the hypothesis that STAT5 links BTK-mediated signaling events that follow the engagement of the B cell antigen receptor to downstream gene activation. A compromised coupling between BTK and STAT5 might lead to aberrant expression of the STAT5α-responsive genes and might contribute to the XLA and XID phenotypes.

MATERIALS AND METHODS

Mice, Cell Lines, Reagents, and Biochemical Assays—Control, XID, and JAK3 knockout mice were purchased from Jackson Laboratories. Mouse splenocytes were isolated, starved in RPMI at a density of 10 × 10^6 cells/ml for 2 h, and then stimulated either with murine anti-IgM or inter-feron-γ-stimulated gene transcription targets (18–20). Different ligands and cell activators employ specific STAT family members (21–23). The basic model for STAT activation suggests that in unstimulated cells, latent forms of STATs are predominantly localized within the cytoplasm. Ligand binding induces STAT proteins to associate with intracellular phosphotyrosine residues of transmembrane receptors (24, 25). Once STATs are bound to receptors, they are phosphorylated by Janus family or Src family PTK (26). STAT proteins then dimerize through specific reciprocal SH2-phosphotyrosine interactions (27) and may form complexes with other DNA-binding proteins (28). STAT complexes translocate to the nucleus and interact with DNA response elements to enhance transcription of target genes (29). STAT5 is a molecular regulator of proliferation, differentiation, and apoptosis in hematopoietic cells (30). B cells abundantly express STAT5, and engagement of the B cell antigen-receptor results in activation of STAT5 (31). B cells from mice bearing subcutaneous mammary adenocarcinoma tumors are severely impaired in their ability to generate antigen-specific responses (32). Interestingly, purified B cells isolated from these immunocompromised tumor-bearing mice exhibited a marked decrease in the expression level of STAT5 without a concomitant change in the expression levels of STAT1, -3, and -6 (32). The observed correlation of the loss of B cell function with the selective decrease in STAT5 expression suggested that regulation of the STAT5 signaling pathway may provide a molecular mechanism for modulating the antigen-specific B cell responses. STAT5α-deficient mice showed decreased proliferation of splenocytes to interleukin (IL)-2 stimulation, which was reported to result from defective induction of IL-2 receptor chain (33). Recently, STAT5α and STAT5β doubly disrupted mice have been generated (34), and Sexl et al. reported that STAT5α/B contribute to interleukin-7-induced B cell precursor expansion (35). The expansion of B cell precursors is disrupted at the level of late pro-B and pre-B cells in STAT5α/B-deficient mice (35). These mice also have reduced numbers of circulating B cells in their blood (35).

Targets inside the nucleus, which may be critical in gene regulation during B cell development and differentiation (14). An unresolved question in B cell immunology is the nature of the molecular coupling mechanisms that link the proximal signals that are triggered by the engagement of the B cell antigen receptor to downstream signal transduction pathways affecting gene transcription. BCR stimulation leads to the activation of transcription factor NF-κB in a BTK-dependent fashion, which in turn regulates genes controlling B cell growth (15). Recently, the BTK substrate phospholipase C-γ2 was shown to couple BTK to the NF-κB signaling pathway (16). BTK has also been shown to regulate the nuclear localization and transactivation of the multifunctional transcription factor BAP-135/TFII-I (17). These results illustrate that the substrates for BTK in B cells is a critical step to a better understanding of the molecular mechanism(s) of lymphocyte activation through the antigen receptor.

Signal transducers and activators of transcription (STAT) proteins are a family of DNA-binding proteins that were initially identified during a search for interferon-α or interferon-γ-stimulated gene transduction targets (18–20). Different ligands and cell activators employ specific STAT family members (21–23). The basic model for STAT activation suggests that in unstimulated cells, latent forms of STATs are predominantly localized within the cytoplasm. Ligand binding induces STAT proteins to associate with intracellular phosphotyrosine residues of transmembrane receptors (24, 25). Once STATs are bound to receptors, they are phosphorylated by Janus family or Src family PTK (26). STAT proteins then dimerize through specific reciprocal SH2-phosphotyrosine interactions (27) and may form complexes with other DNA-binding proteins (28). STAT complexes translocate to the nucleus and interact with DNA response elements to enhance transcription of target genes (29). STAT5 is a molecular regulator of proliferation, differentiation, and apoptosis in hematopoietic cells (30). B cells abundantly express STAT5, and engagement of the B cell antigen-receptor results in activation of STAT5 (31). B cells from mice bearing subcutaneous mammary adenocarcinoma tumors are severely impaired in their ability to generate antigen-specific responses (32). Interestingly, purified B cells isolated from these immunocompromised tumor-bearing mice exhibited a marked decrease in the expression level of STAT5 without a concomitant change in the expression levels of STAT1, -3, and -6 (32). The observed correlation of the loss of B cell function with the selective decrease in STAT5 expression suggested that regulation of the STAT5 signaling pathway may provide a molecular mechanism for modulating the antigen-specific B cell responses. STAT5α-deficient mice showed decreased proliferation of splenocytes to interleukin (IL)-2 stimulation, which was reported to result from defective induction of IL-2 receptor chain (33). Recently, STAT5α and STAT5β doubly disrupted mice have been generated (34), and Sexl et al. reported that STAT5α/B contribute to interleukin-7-induced B cell precursor expansion (35). The expansion of B cell precursors is disrupted at the level of late pro-B and pre-B cells in STAT5α/B-deficient mice (35). These mice also have reduced numbers of circulating B cells in their blood (35).
posed to Kodak XAR-5 film. For electrophoretic mobility shift assays, cell extracts were prepared according to the previously published method (43). Briefly, cells were lysed in WCE lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM EDTA, 0.5 mM Na3VO4, and protease inhibitors). An annealed oligonucleotide corresponding to the DR5 site of STAT5A was end-labeled with T4 polynucleotide kinase using [γ-32P]ATP (Amersham Pharmacia Biotech). A total of 10 μg of cell extract and 0.5 ng of labeled oligonucleotide were used per reaction. The cell extract and 200 ng of poly(dI-dC) (Amersham Pharmacia Biotech) were incubated on ice for 15 min followed by another 15-min incubation on ice after adding the labeled oligonucleotide. The reaction products were run on a 5% polyacrylamide gel in 0.5× Tris borate-EDTA buffer and visualized by autoradiography.

Recombinant Baculovirus Expression System—The murine wild type STAT5A gene in pBluescript SKII + vector was a kind gift from J. N. Ihle (St. Jude Children’s Hospital, Memphis, TN). The STAT5A gene was excised from this plasmid by EcoRI digestion and cloned into pFastBac1 (PFB) donor vector (Life Technologies). The resulting pFast-bac-stat5a recombinant plasmid (PFB-stat5a) was then used to generate the recombinant baculovirus by site-specific transposition in E. coli DH10Bac competent cells (Life Technologies), which harbor a baculovirus shuttle vector (bacmid), bMON14272 with a mini-attTn7 transposon site for site-specific transposition. The bacterial colonies containing recombinant bacmids were identified by disruption of the target site for site-specific transposition. The bacterial colonies containing a baculovirus shuttle vector (bacmid), bMON14272 with a mini-attTn7 transposon site were first incubated on ice for 15 min followed by another 15-min incubation on ice after adding the labeled oligonucleotide. The reaction products were run on a 5% polyacrylamide gel in 0.5× Tris borate-EDTA buffer and visualized by autoradiography.

Site-directed Mutagenesis—Point mutations were introduced into the stat5a cDNA by PCR (47). The codon for arginine 618 within the SH2 domain was mutated to a codon for lysine. The codons for the conserved tryptophans at positions 571 and 573 within the SH3 domain were mutated to leucine. The codon for tyrosine 701 in murine STAT1, a previously known phosphorylation site by JAK kinases (48), was mutated to a codon for phenylalanine. The codons for the tyrosine residues 665, 668, 682, and 683 that flank tyrosine 694 and appear to be potential phosphorylation sites were also mutated to a codon for phenylalanine. All mutations were confirmed by sequence analysis. Point mutations introduced into the btk gene (39) have been reported earlier.

Pull-down Assays with MBP-BTK and GST-BTK Fusion Proteins—cDNAs encoding full-length BTK and its kinase or PH domains with PCR-generated 5′ and 3′ BamHI sites were cloned into the E. coli expression vector pMAL-C2 with the isopropyl-1-thio-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 maltose-binding protein (MBP). cDNAs encoding the recombinant proteins were excised from pBluescript plasmid, cloned into PFB vector and processed as described above for expression in SF21 cells. The BTK kinase domain (BTK-KD) gene was amplified from wild type BTK by PCR using 5′-primer (AGCCATGGGAGTgactaaagac) with a NcoI site and 3′-primer (ATAAGCTTcaggattcttcatccatcaca) with a HindIII site. The PCR product was cloned into the pFastBac HTb donor plasmid (Life Technologies), followed by digestion with NcoI and HindIII to generate pFastBac HTb: BTKKD. Recombinant viral clones were isolated by gentle centrifugation in a Beckman GS-6 centrifuge at 500 × g for 10 min at room temperature. Cell pellets were harvested by centrifugation at 4500 × g for 1 h at 4°C, and stored at −80°C until purification of the recombinant proteins.

Gel Filtration Analysis—SF21 insect cells expressing STAT5A and BTK were washed twice with assembly buffer (10 mM PIPES, 1 mM EDTA, pH 7.2) and lysed in lysis buffer (0.01 M Tris, pH 8.2, 1 mM EDTA, 0.15 M NaCl, 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 1 mM Na3VO4) for 2 h on ice. Cytosolic fractions were recovered after centrifugation at 4°C for 10 min at 48,000 × g. The cell pellets were washed with 50 μl of the beads for 2 h at 4°C. The beads were washed three times with 1% Nonidet P-40 buffer. Nonidet P-40 lysates of KL2 human Epstein-Barr virus-transformed lymphoblastoid cells were prepared as described (4), and 500 μg of the lysate was incubated with 50 μl of
fusion protein-coupled beads for 2 h on ice. The fusion protein adsorbates were washed with ice-cold 1% Nonidet P-40 buffer and resuspended in reducing SDS sample buffer. Samples were boiled for 5 min and then fractionated on SDS-PAGE. Proteins were transferred to Immobilon-P (Millipore Corp.) membranes, and membranes were immersed in 100 mM Tris-HCl, 1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride, sonicated for 1 min, and centrifuged at 100,000 g for 30 min. Following centrifugation, the supernatant was filtered through a 0.45-μm membrane filter (S100) and applied to a 5 ml membrane filter (S100) and applied to a 5 ml Superdex 200 size exclusion column (Amersham Pharmacia Biotech) preequilibrated with Hepes buffer. Elution of protein was performed with a 20 ml Superdex 200 column (26/60), which was equilibrated with a solution of 20 ml Tris/HCl, pH 8.5, 50 mM NaCl, and 2 mM DTT at a flow rate of 0.3 ml/min.

Monitoring of Binding Interactions Using Surface Plasmon Resonance Technology—A BIAcore 2000 surface plasmon resonance-based biosensor system (Amersham Pharmacia Biotech Biosensor AB) was used to measure the kinetic parameters for the interactions between soluble recombinant STAT5A protein (analyte) and the immobilized recombinant BTK protein (ligand). BTK or bovine serum albumin (control protein) was covalently linked to the dextran on the surface of the CM5 sensor chip at a density of 2.9 ng/cm² using the amine coupling kit from Amersham Pharmacia Biotech according to the manufacturer’s instructions (Amersham Pharmacia Biotech), yielding a resonance signal of 2700 resonance units (RU) (1 RU corresponds to an immobilized protein amount of 1 pg/mm² surface). Unbound moieties on the surface were blocked with ethanolamine. The STAT5A protein samples were diluted in PBS buffer (1 mM KH₂PO₄, 10 mM Na₂HPO₄, 0.137 m NaCl, 2.7 mKCl, 0.005% Tween P-20, pH 7.4) to a final concentration of 50 ng/ml before the injection. Purified STAT5A protein samples in HBS-EP buffer (0.01 Hepes, pH 7.4, 0.15 m sodium chloride, 3 mM EDTA, 0.005% polysorbate 20 (v/v)) was injected in a total volume of 75 μl containing 20–80 μg of purified protein. Samples were injected at 25 °C at a flow rate of 15 μl/min onto the sensor chip surface on which the immobilised protein had been immobilized and onto a control surface on which bovine serum albumin had been immobilized. Binding surface was regenerated by washing with 2 mM NaCl.

The primary data were analyzed using the BIAevaluation software (version 3.0) supplied with the instrument (Biacore, Inc.). To prepare the data for analysis, base lines were adjusted to zero for all curves, and injection start times were aligned. Background sensorgrams were then subtracted from the experimental sensorgrams to yield curves representing specific binding. All of the kinetic data were fit most adequately by assuming a simple bimolecular reaction model for interaction between soluble analyte and immobilized ligand, equivalent to the Langmuir isotherm for adsorption to a surface. For determination of kₐ, only the middle portion of the association curve was used for fitting. For determination of kₐ, only the initial portion of the curve encompassing the first data point was used for fitting. The goodness of fit was assessed by inspecting the statistical value χ² and the residuals (observed – calculated). The χ² values were low (<2), and the residuals randomly distributed about zero.

RESULTS AND DISCUSSION

Physical Interactions between STAT5A and BTK—In co-immunoprecipitation experiments using Triton X-100 whole cell lysates from KL-2 human B cells, we discovered that BTK immune complexes contain STAT5A (Fig. 1A; see A.2, first lane) and STAT5A immune complexes contain BTK (Fig. 1B; see B.1, first lane). These results indicated that BTK is constitutively associated with STAT5A in B cells. Interestingly, this physical association appeared to be markedly reduced after engagement of the B cell antigen receptor. No STAT5A was detected in the BTK immune complexes from whole cell lysates prepared after 20 min of stimulation with the anti-IgM antibody (Fig. 1A.2, lane 2), although there was much more BTK in these immune complexes as it was washed prior to anti-IgM stimulation (Fig. 1A.1), and the Western blot analysis of the whole cell lysates with the same anti-STAT5A antibodies did not indicate any change in the amount of cellular STAT5A (Fig. 1A.3). Similarly, virtually no BTK was detected in the STAT5A immune complexes from whole cell lysates prepared after anti-IgM stimulation.
We next sought to examine the ability of purified recombinant BTK to bind purified recombinant STAT5A by surface plasmon resonance, which permits direct measurements of the association and dissociation kinetics of binding interactions. Fig. 2 shows representative BIAcore sensorgrams of concentration-dependent solution phase >95% pure STAT5A binding to immobilized >95% pure BTK on BIAcore sensor chips. The binding traces were analyzed assuming a single bimolecular

We next set out to examine the in vivo interactions between ectopically expressed BTK and STAT5A proteins in a heterologous expression system. To confirm that a physical association exists between BTK and STAT5A when they are coexpressed in Sf21 cells, we examined whether BTK and STAT5A are present as native complexes in Sf21 cells cotransfected with MBP-BTK-(1–659) (full-length protein), BTK-(408–659) (kinase domain), and BTK-(2–137) (PH domain) were fused to MBP. BTK-(219–268) (SH3 domain) and BTK-(261–377) (SH2 domain) were fused to GST. MBP-BTK and GST-BTK fusion proteins were used in pull-down binding assays to examine their ability to interact with STAT5A protein in lysates of KL2 human Epstein-Barr virus-transformed B lymphoblastoid cells, as described under “Materials and Methods.” Fusion protein adsorbates and control samples (CON (1), cell lysate + amylase beads with no fusion protein added; CON (2), cell lysate + glutathione-agarose beads, no fusion protein added) as well as a KL2 whole cell lysate sample were resolved by SDS-PAGE, immunoblotted with anti-STAT5A antibody, and developed with ECL. D. gel filtration analysis. Sf21 cells coexpressing BTK and STAT5A were lysed and cytosolic fractions were recovered then analyzed using fast protein liquid chromatography at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and subjected to Western blotting with antibodies specific for BTK and STAT5A. Proteins were visualized using ECL and analyzed by densitometry. The numbers 20–34 correspond to fractions, and arrows below the blot designate the predicted size of the complexes based upon size standards. E. coimmunoprecipitation analysis. STAT5A (STAT5A IP) and BTK immune complexes (BTK IP) as well as whole cell lysates (WCL) were subjected to Western blot (WB) analyses using anti-STAT5A and anti-BTK antibodies. The arrowsheads indicate the positions of the BTK and STAT5A protein bands.

IgM stimulation (Fig. 1B.1, lane 2), although there was as much STAT5A in these immune complexes as in those prepared before anti-IgM stimulation (Fig. 1B.2), and the Western blot analysis of the whole cell lysates with the same anti-BTK antibodies did not indicate any change in the amount of cellular BTK (Fig. 1B.3). These observations are reminiscent of the reported association between BTK and the multifunctional transcription factor BAP-135/TFII-I in unstimulated (but not anti-IgM-stimulated) B cells (17).

We performed “pull-down” experiments with full-length MBP-BTK-(1–659) and truncated MBP-BTK (MBP-BTK-(408–659), BTK kinase domain; MBP-BTK-(2–137), BTK PH domain) or GST-BTK fusion proteins (GST-BTK-(219–268), BTK SH3 domain; GST-BTK-(261–377), BTK SH2 domain) corresponding to different domains of BTK in an attempt to elucidate the structural requirements for BTK binding to STAT5A. Only the full-length BTK and BTK KD were able to bind and pull down STAT5A from lysates of KL2 human Epstein-Barr virus-transformed B lymphoblastoid cells (Fig. 1C). Thus, the kinase domain appears to mediate the binding of BTK to STAT5A.

We next set out to examine the in vivo interactions between ectopically expressed BTK and STAT5A proteins in a heterologous expression system. To confirm that a physical association exists between BTK and STAT5A when they are coexpressed in Sf21 cells, we examined whether BTK and STAT5A are present as native complexes in Sf21 cells cotransfected with PFB-stat5a + PFB-btk. Lysates of Sf21 cells were size-fractionated by fast protein liquid chromatography on a Superdex 200 size exclusion column (Amersham Pharmacia Biotech), and the elution patterns of BTK and STAT5A proteins were examined by immunoblotting with antibodies recognizing the individual proteins. The relative amounts of each protein per fraction were determined by densitometric scanning. More than 90% of the BTK protein eluted in fractions 27–29, corresponding to apparent native molecular masses of 75–150 kDa (Fig. 1, D.1 and D.2). The remainder of the BTK protein eluted in fractions 21–26, which correspond to apparent native molecular masses of >270 kDa. Notably, these fractions also contained STAT5A protein (Fig. 1D). These results suggested the existence of 270–350-kDa native complexes containing BTK and STAT5A. We next used Western blot analyses to evaluate the interaction between BTK and STAT5A in these Sf21 cells coexpressing both BTK and STAT5A. BTK immune complexes from these whole cell lysates contained STAT5A (Fig. 1E.1, lane 2), and STAT5A immune complexes from these whole cell lysates contained BTK (Fig. 1E.2, lane 1). Taken together, these initial findings indicated that, when ectopically coexpressed in Sf21 cells, BTK and STAT5A proteins can physically associate with each other.

We next sought to examine the ability of purified recombinant BTK to bind purified recombinant STAT5A by surface plasmon resonance, which permits direct measurements of the association and dissociation kinetics of binding interactions. Fig. 2 shows representative BIAcore sensorgrams of concentration-dependent solution phase >95% pure STAT5A binding to immobilized >95% pure BTK on BIAcore sensor chips. The binding traces were analyzed assuming a single bimolecular
binding equilibrium between BTK and STAT5A proteins. The maximum resonance signal ($R_{\text{max}}$) increased in a concentration-dependent fashion from 49.6 RU at 20 μg/ml STAT5A to 169 RU at 40 μg/ml STAT5A and 238 RU at 80 μg/ml STAT5A. The kinetics of the binding was independent of the STAT5A concentration. The average on-rate ($k_{\text{on}}$) was $3.0 \pm 0.9 \times 10^4$ M$^{-1}$ s$^{-1}$, the average off-rate ($k_{\text{off}}$) was $11.1 \pm 0.3 \times 10^4$ s$^{-1}$, and the average affinity constant (KD = $k_{\text{off}}/k_{\text{on}}$) was $44.4 \pm 13.0$ nm. These results provide unprecedented experimental evidence that BTK is capable of directly binding STAT5A with a relatively high affinity.

**Tyrosine Phosphorylation of STAT5A by BTK**—We have performed a series of experiments to further confirm and extend these initial observations. Sf21 cells transfected with PFB-stat5a expressed the recombinant murine STAT5A protein, as detected by anti-STAT5A immunoblotting of STAT5A immune complexes from whole cell lysates prepared 48 h after transfection (Fig. 3A). By comparison, control SF21 cells transfected with PFB did not show any evidence of STAT5A expression, demonstrating that the anti-STAT5A antibody used for immunoblotting does not recognize an endogenous insect STAT5A protein. The anti-phosphotyrosine Western blot analysis of the STAT5A immune complexes from Sf21 cells transfected with PFB-stat5a did not show any evidence of tyrosine phosphorylation. Thus, insect kinases in SF21 cells do not significantly phosphorylate recombinant STAT5A in vivo. We next sought to determine if coexpression of STAT5A with BTK leads to tyrosine phosphorylation of STAT5A in vivo. Controls included SF21 cells co-transfected with PFB-stat5a and recombinant plasmids for TEC or JAK3. As shown in Fig. 3B.1 (first lane) and Fig. 3C.1 (second lane), anti-phosphotyrosine Western blot analysis of STAT5A immune complexes from Sf21 cells cotransfected with PFB-stat5a + PFB-btk showed tyrosine phosphorylation of STAT5A protein. The level of tyrosine phosphorylation of STAT5A in Sf21 cells coexpressing BTK was similar to that in Sf21 cells coexpressing JAK3. In contrast, no STAT5A tyrosine phosphorylation was observed in Sf21 cells coexpressing TEC kinase (Fig. 3B.1, second lane, and Fig. 3C.1, third lane). STAT5A was expressed at similar levels in each of the transfectants (Fig. 3B.2 and 3C.2), and all three tyrosine kinases were enzymatically active in Sf21 cells, as shown by their ability to phosphorylate an exogenous substrate (GST-IgG for TEC family members and β-casein for JAK3) (Fig. 3B.3). The ability of both BTK and JAK3 to tyrosine-phosphorylate STAT5A was dependent on the enzymatic activity of their respective kinase domains. As shown in Fig. 3D, coexpression of kinase-dead mutants of BTK (Y551F, BTKmKD) or JAK3 (K851E, JAK3mKD) did not result in tyrosine phosphorylation of STAT5A.

We next used in vitro kinase assays to determine if BTK can phosphorylate STAT5A.

BTK immune complexes from whole cell lysates of Sf21 cells transfected with PFB-btk were mixed with purified STAT5A (10 μg) and 10 μCi of ($\gamma$-32P)ATP in the presence of 5 mM each of MnCl$_2$ and MgSO$_4$. TEC from PFB-tec-transfected Sf21 cells and JAK3 from PFB-jak3-transfected Sf21 cells were also included for comparison. The kinase reactions were stopped after 10 min, STAT5A was immunoprecipitated, and its phosphorylation status was assessed by autoradiography. As shown in Fig. 4A, BTK was capable of phosphorylating STAT5A. The phosphorylation level of BTK-treated STAT5A was similar to that of JAK3-treated STAT5A. In contrast to BTK, TEC did not phosphorylate STAT5A. The amount of STAT5A was virtually identical in each kinase reaction (Fig. 4B) and all three tyrosine kinases, including TEC, were enzymatically active, as measured...
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residues at positions 665, 668, 682, 683, and 694 as well as the SH2 domain and a portion of the SH3 domain.

Identification of Tyrosine 694 of STAT5A as the Target Phosphorylation Site for BTK Kinase Domain—We constructed a molecular model of the STAT5A structure (residues 163–703) based on its homology with STAT1 and STAT3β crystal structures (Fig. 5). A flexible or β-strand-like conformation is a desired structural feature for effective phosphorylation of protein substrates by protein kinases. Similar to the structural conformation revealed by the STAT1 and STAT3β crystal structures, our model indicates that the tail segment of STAT5A from residue 693 to residue 703 (694 included) adopts a β-strand-like conformation, which is therefore suitable for binding interactions with the BTK kinase domain. Tyr<sup>694</sup> of STAT5A is in close contact with the BTK catalytic residues including Asp<sup>521</sup>, Arg<sup>525</sup>, and Asn<sup>526</sup>. In addition, Gly<sup>693</sup>, Val<sup>695</sup>, and Lys<sup>695</sup> of STAT5A are near the BTK residues Pro<sup>560</sup> (backbone), Phe<sup>559</sup>, and Lys<sup>558</sup>, respectively. Based on our model, these residues interact with each other and constitute the binding affinity between STAT5A and the BTK kinase domain. Our model also indicates that the SH2 and SH3 domains of BTK may sterically clash with the N-terminal domain of STAT5A (residues 1–150), which is predicted to reside near the coiled-coil domain and may be accessible by the less bulky BTK kinase domain.

According to this model, of the five tyrosine residues in the SH2 domain and tail segment of STAT5A, only Tyr<sup>694</sup> is fully exposed to the solvent environment and therefore is the most accessible tyrosine residue for phosphorylation by BTK. All tyrosine residues except for Tyr<sup>694</sup> are either sterically inaccessible or adopt a helical conformation unsuitable for phosphorylation. For example, Tyr<sup>682</sup> is surrounded by His<sup>588</sup>, Pro<sup>647</sup>, and Lys<sup>681</sup>; Tyr<sup>683</sup> is surrounded by Ser<sup>680</sup> and Lys<sup>675</sup> and possibly sterically occluded by a loop formed by residues 685–700; Tyr<sup>665</sup> is surrounded by Arg<sup>659</sup>, Lys<sup>675</sup>, and Ile<sup>667</sup>; and Tyr<sup>668</sup> is surrounded by Pro<sup>674</sup>, Pro<sup>671</sup>, and Ile<sup>667</sup>. Both Tyr<sup>225</sup> and Tyr<sup>568</sup> adopt a helical conformation that is not suitable for phosphorylation. Tyr<sup>225</sup> is the only tyrosine (other than Tyr<sup>694</sup>) that may be eligible for phosphorylation and more exposed than the aforementioned tyrosine residues. However, Tyr<sup>225</sup> is located on a β-strand as a part of a β-sheet structure on the DNA binding domain. Although sufficiently exposed for phosphorylation, Tyr<sup>225</sup> is situated on a relatively rigid β-sheet structure. More importantly, Tyr<sup>225</sup> is less accessible than Tyr<sup>694</sup> because it is close to the N-terminal domain of STAT5A according to the full model of the STAT1 protein (61). These modeling studies taken together with the CNBr cleavage data prompted the hypothesis that Tyr<sup>694</sup> of STAT5A is the target phosphorylation site for BTK.

In order to determine the site of BTK-mediated phosphorylation in STAT5A, tyrosine residues present in the 18-kDa CNBr fragment at positions 665, 668, 682, 683, and 694 were mutated to phenylalanine, as described under “Materials and Methods.” In addition, mutations were also made in the SH2 (R307K) and SH3 (Y251L,Y252L) domains of STAT5A to determine the role of these domains for in vivo recognition and phosphorylation of STAT5A by BTK. The various STAT5A mutants were coexpressed in Sf21 cells with wild type BTK. Fig. 6A shows that BTK tyrosine phosphorylates the Y665F, Y668F, and Y682F,Y683F mutants of STAT5A but not the Y694F mutant of STAT5A. Notably, STAT5A mutations in the SH2 (R307K) and SH3 (Y251L,Y252L) domains did not alter the BTK phosphorylation of STAT5A. STAT5A mutants were expressed at similar levels (Fig. 6B), and the enzymatic activity levels of BTK in the presence of the various STAT5A proteins were comparable (Fig. 6C). Similarly, BTK immunoprecipitated from whole cell lysates of PFB-btk-transfected with PFB-btk, PFB-tec, or PFB-jak3, Δ, cell lysates from various transfectants were immunoprecipitated with specific antibodies, and the immune-complexes were mixed with purified STAT5A and [γ<sup>32</sup>P]ATP. The kinase reactions were stopped by adding radioimmune precipitation buffer (20 mM MOPS, pH 7, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS), centrifuged at 12,000 rpm, and the supernatants were immunoprecipitated with an anti-STAT5A antibody. The phosphorylation status of the immunoprecipitated STAT5A was determined by autoradiography. B, the amount of STAT5A in each kinase reaction was determined by STAT5A immunoprecipitation and anti-STAT5A immunoblotting. C, the enzymatic activity of the immunoprecipitated tyrosine kinases was measured by autophosphorylation in immune complex kinase assays. D–F, phosphoamino acid analysis (PAA) and cyanogen bromide (CNBr) cleavage analysis of BTK-phosphorylated STAT5A. BTK was immunoprecipitated from lysates of PFB-btk-transfected Sf21 cells and an immune complex kinase assay was performed with purified STAT5A and [γ<sup>32</sup>P]ATP. The reaction was stopped with radioimmune precipitation buffer, and STAT5A was immunoprecipitated and run on a polyacrylamide gel. The <sup>32</sup>P-labeled STAT5A band was isolated and subjected to PAA (shown in D) or CNBr cleavage analysis (shown in E). The positions of ninhydrin-stained phosphoamino acid standards (phosphoserine (PS), phosphotyrosine (PY), and phosphothreonine (PT)) are indicated with circles. The CNBr cleavage map of STAT5A is depicted in F, with arrowheads pointing to the positions of methionines.
Tyrosine phosphorylation of STAT5A by JAK3 results in enhanced DNA binding activity. In order to determine if the BTK-mediated tyrosine phosphorylation also augments the DNA binding activity of ectopically expressed STAT5A in a heterologous expression system, we performed electrophoretic mobility shift assays using a $^{32}$P-labeled oligonucleotide representing a GAS-like element from the β-casein gene promoter region. As shown in Fig. 7A, extracts from untransfected SF21 cells (lane 1) or SF21 cells transfected with PFB-stat5a alone (lane 2) did not contain any proteins capable of binding the probe causing a mobility shift. By comparison, a significant mobility shift of the $^{32}$P-labeled oligonucleotide probe was observed in extracts from SF21 cells co-expressing wild type BTK and STAT5A (lane 5). These findings indicate that BTK-mediated tyrosine phosphorylation of ectopically expressed STAT5A enhances its DNA binding activity. The level of STAT5A activation in these extracts was virtually identical to that observed in extracts of SF21 cells co-expressing wild type JAK3 and STAT5A (compare lanes 5 and 10). The observed mobility shifts of the oligonucleotide probe were not caused by endogenous insect STAT proteins, because no mobility shifts were observed when we used extracts from SF21 cells expressing wild type BTK alone (lane 3) or wild type JAK3 alone (lane 9). BTK-mediated activation of STAT5A was dependent on the enzymatic activity of BTK, since no mobility shifts were observed in extracts of SF21 cells co-expressing STAT5A and a kinase-inactive mutant of BTK (lane 6). In accordance with the above detailed data identifying Tyr$^{694}$ as the BTK phosphorylation site of STAT5A, co-expression of BTK was unable to activate the Y694F mutant of STAT5A (lane 8). These results demonstrate that STAT5A is a functional target for BTK.

We next used the baculovirus expression system to compare the ability of mutant BTK proteins with point mutations of conserved residues in the PH (R28C), SH1/kinase domain (Y551F, autophosphorylation site mutant; K430E, ATP binding site mutant), SH2 (R307K), and SH3 (Y251L,Y252L) domains to phosphorylate STAT5A in vivo. As shown in Fig. 7B, recombinant BTK proteins with mutant PH, SH2, or SH3 domains were capable of tyrosine phosphorylating the co-expressed wild type STAT5A (lane 1). However, none of the mutants were capable of tyrosine phosphorylating the Y694F mutant of STAT5A (lane 2). These results further support the role of Tyr$^{694}$ in BTK-mediated tyrosine phosphorylation of STAT5A.}

Fig. 5. Molecular model of STAT5A. Molecular model of the STAT5A structure is shown as a surface representation (A) and ribbon representation (B). The structure of STAT5A (residues 163–703) was constructed based on its homology with STAT1 and STAT3β crystal structures. Different domains are shown in different colors: the coiled-coil domain in red, the DNA binding domain in gold, the linker domain in green, and the SH2 domain in blue. The DNA double strand is used to mark the DNA binding location and is shown as a stick model (A, multicolor) and a space-filling model (B, yellow for phosphate and blue and white for two different DNA strands, respectively). All five tyrosine residues on the SH2 domain and tail segment that were subjected to phenylalanine mutations are shown in different colors and labeled accordingly. Among them, only Tyr$^{694}$ (red) is fully exposed to the solvent environment. The other four tyrosine residues are at least half-buried, consistent with our experimental data suggesting that Tyr$^{694}$ is most accessible to BTK phosphorylation. Other tyrosine residues including Tyr$^{225}$ and Tyr$^{568}$ (not shown) are situated on a helix of the coiled-coil domain.
type STAT5A protein, whereas recombinant BTK proteins with SH1/kinase domain mutations were not. The expression levels of STAT5A (Fig. 7C) and mutant BTK proteins (Fig. 7D) were similar for all Sf21 cells co-expressing STAT5A and a mutant BTK protein. No BTK kinase activity was detected in BTK immune complexes from Sf21 cells expressing the kinase domain mutants of BTK (Fig. 7E). Taken together with the results of the pull-down experiments, these findings indicate that the PH, SH2, and SH3 domains of BTK do not significantly contribute to its physical and functional interactions with STAT5A. The ability of BTK to phosphorylate STAT5A requires both an intact ATP-binding site and an intact autophosphorylation site within the kinase domain.

We next examined the ability of purified recombinant BTK-KD to phosphorylate purified recombinant STAT5A using in vitro kinase assays. As shown in Fig. 7F.1, STAT5A did not show any significant level of phosphorylation in the absence of BTK-KD, and BTK-KD was capable of tyrosine-phosphorylating STAT5A and STAT5A (Y694F-Y) in the presence of [γ-32P]ATP. The in vitro kinase reactions were stopped by adding radioimmune precipitation buffer, and the phosphorylated STAT5A protein was visualized by autoradiography. E, after stopping the kinase reactions, the kinase reaction mixtures were centrifuged at 12,000 rpm, and the supernatants were immunoprecipitated with an anti-STAT5A antibody. The immune complexes were subjected to Western blot analysis with the anti-STAT5A antibody. F, the enzymatic activity of BTK in the kinase reactions was measured by examining its autophosphorylation as well as its ability to phosphorylate GST-Ig in immune complex kinase assays. G–J, tyrosine phosphorylation of STAT5A by ectopically expressed BTK is independent of JAK3. Wild type BTK, JAK3 and their kinase-inactive mutants were co-expressed with STAT5A in Sf21 cells. Lysates were immunoprecipitated with anti-STAT5A (G and H), anti-JAK3 (I), or anti-BTK (J) antibodies and subjected to immunoblotting with anti-phosphotyrosine antibodies (G), anti-STAT5A antibody (H), anti-JAK3 antibody (I), or anti-BTK antibody (J).
STAT5A and BTK-KD. STAT5A immunoprecipitated from whole cell lysates of Sf21 cells expressing STAT5A alone showed no tyrosine phosphorylation (Fig. 7G, lane 1). When compared with this STAT5A protein, STAT5A immunoprecipitated from whole cell lysates of Sf21 cells coexpressing STAT5A and BTK-KD showed markedly enhanced tyrosine phosphorylation (Fig. 7G, lane 2). Taken together, these findings provided direct evidence that the KD of BTK is capable of tyrosine-phosphorylating STAT5A both in vitro and in vivo. Thus, the KD of BTK is sufficient for the functional interactions between BTK and STAT5A.

Activation of a BTK-dependent and JAK3-independent Mechanism of STAT5A Tyrosine Phosphorylation in B Cells after Engagement of the B Cell Antigen Receptor—B cells abundantly express STAT5 and engagement of the B cell antigen-receptor results in activation of STAT5 (31). We first sought to confirm these results by examining the phosphorylation status of anti-STAT5A immunoreactive protein (presumed chicken STAT5) in DT40 chicken B cells after stimulation with the anti-chicken IgM antibody M4. As shown in Fig. 8A, M4 stimulation resulted in enhanced tyrosine phosphorylation of STAT5 (lane 2). Notably, pretreatment of DT40 cells with the BTK inhibitor FIG. 7.

A, activation of STAT5A by ectopically expressed BTK. Wild type or Y694F mutant STAT5A was coexpressed with wild type or kinaseinactive mutant BTK, or wild type JAK3. Control Sf21 cells were either not transfected or transfected with expression plasmids for STAT5A, mutant STAT5A, BTK, mutant BTK, or JAK3 alone. Whole cell extracts were prepared and tested for the presence of active STAT5A by electrophoretic mobility shift assays using an annealed oligonucleotide corresponding to the GAS-like STAT5 binding site in the β-casein gene promoter (5′-AGATTTCTAGGAATTCAATC-3′). The position of shifted complex of activated STAT5A is indicated with an arrowhead.

B–E, requirement for an intact BTK kinase domain for the in vivo tyrosine phosphorylation of STAT5A by ectopically expressed BTK. STAT5A was coexpressed in Sf21 cells with the indicated site-specific mutants of BTK. STAT5A was immunoprecipitated and STAT5A immune complexes were subjected to either anti-phosphotyrosine (B) or anti-STAT5A (C) immunoblotting. BTK was immunoprecipitated, and BTK immune complexes were subjected to in vitro kinase assays with [γ-32P]ATP (E) or anti-BTK immunoblotting (D). F and G, recombinant BTK kinase domain phosphorylates STAT5A. F, purified BTK-KD was examined for its ability to phosphorylate purified STAT5A in in vitro kinase assays (F.1). Control reactions contained no STAT5A (lane 1) or no BTK (lane 2). Samples were examined for BTK-KD content (F.2) and STAT5A content (F.3) by Western blot analysis. G, BTK-KD and STAT5A were coexpressed in Sf21 cells, and STAT5A immunoprecipitated from Sf21 whole cell lysates was examined for tyrosine phosphorylation by anti-phosphotyrosine (APT) Western blot analysis. Lane 1, STAT5A immunoprecipitates (IP) from Sf21 cells transfected with PFB-stat5a; lane 2, STAT5A immunoprecipitate from Sf21 cells transfected with PFB-stat5a plus PFB-btk-kd; lane 3, BTK immunoprecipitate from Sf21 cells transfected with PFB-btk-kd; lane 4, whole cell lysate Sf21 cells transfected with PFB-stat5a plus PFB-btk-kd.
STAT5A as a Substrate of BTK

Fig. 8. A and B, pretreatment of chicken B cells with the BTK inhibitor LFM-A13 prevents anti-IgM-induced tyrosine phosphorylation of STAT5A. Cells were stimulated with anti-IgM (M4) antibody (10 μg/10^6 cells/ml) in the presence of 100 μM LFM-A13 (a BTK inhibitor that does not inhibit JAK3) or 100 μM HI-P131 (a JAK3 inhibitor that does not inhibit BTK) for 20 min. Cells were lysed and immunoprecipitated with STAT5A, and STAT5A immune complexes were subjected to Western blot analyses with anti-phosphotyrosine (B) or anti-STAT5A (A) antibodies.

We next studied the role of BTK in B cell antigen receptor-mediated STAT5 phosphorylation by comparing the STAT5 tyrosine phosphorylation levels after M4 antibody stimulation in BTK-deficient DT40 cells reconstituted with wild type human BTK (C–F) and BTK-deficient DT40 chicken B cells reconstituted with inactive kinase domain-mutant human BTK (C–F) were stimulated with M-4 anti-chicken IgM antibody (20 μg of M-4/ml/10^6 cells) and lysed at the indicated times. Cell lysates were immunoprecipitated with anti-BTK (C, D, E, and F) or anti-STAT antibodies (E, F, E', and F') and immunoblotted with anti-phosphotyrosine (C, E, C', and E'), anti-BTK (D and D'), or anti-STAT5A antibodies (F and F') as indicated. G and H, B cell antigen receptor-mediated versus IL-2 receptor-mediated tyrosine phosphorylation of STAT5A in B cells from wild type, XID, and JAK3 knockout mice. Splenocytes from normal and XID mice (G) and normal and JAK3 knockout mice (H) were stimulated with an anti-mouse IgM antibody (10 μg/10^6 cells/ml) or recombinant IL-2 (20 ng/10^6 cells/ml) for 10 min. Cells were lysed and immunoprecipitated with an anti-STAT5A antibody, and the STAT5A immune complexes were subjected to immunoblotting with anti-phosphotyrosine (G and H, upper panels) or anti-STAT5A (G and H, lower panels) antibodies.

LFM-A13, which does not inhibit JAK3 (5) abrogated the M4-induced STAT5 phosphorylation (lane 3). By comparison, the JAK3 inhibitor HI-P131, which does not inhibit BTK (40), did not prevent the M4-induced STAT5 phosphorylation in DT40 cells (lane 4). Anti-STAT5A immunoblotting confirmed that the amounts of immunoprecipitated STAT5A in these samples were comparable (Fig. 8B). These findings provided circumstantial evidence that BTK (but not JAK3) plays an important role in STAT5 tyrosine phosphorylation after B cell antigen receptor ligation.

We next examined the role of BTK in B cell antigen receptor-mediated STAT5 phosphorylation by comparing the STAT5 tyrosine phosphorylation levels after M4 antibody stimulation in BTK-deficient DT40 cells reconstituted with wild type human BTK versus BTK-deficient DT40 cells reconstituted with kinase-inactive mutant BTK. Notably, tyrosine phosphorylation of BTK (Fig. 8C) preceded the tyrosine phosphorylation of STAT5A (Fig. 8E) in BTK-deficient DT40 cells reconstituted with wild type human BTK. By comparison, B cell antigen receptor ligation failed to trigger tyrosine phosphorylation of BTK (Fig. 8C') or STAT5 (Fig. 8E') in BTK-deficient DT40 cells reconstituted with kinase-inactive mutant BTK. Immunoblotting with anti-BTK and anti-STAT5 antibodies confirmed that the samples corresponding to the different time points after M4 stimulation contained comparable levels of BTK (Fig. 8, D and D') and STAT5 (Fig. 8F and F') proteins.

We next examined the effects of anti-IgM stimulation on the tyrosine phosphorylation status of STAT5A in murine B cells from BTK-competent wild type mice versus functionally BTK-deficient XID mice. Whereas the anti-IgM stimulation resulted in enhanced tyrosine phosphorylation of STAT5A in BTK-competent B cells from wild type mice (Fig. 8G, lanes 1 and 2; Fig. 8H, lanes 1 and 2), no STAT5A phosphorylation was induced in BTK-deficient B cells from XID mice (Fig. 8G, lanes 3 and 4). These findings provide experimental evidence that BTK plays a pivotal role in B cell antigen receptor-mediated STAT5A phosphorylation in B cells. In contrast to B cells from XID mice, B cells from JAK3 knockout mice showed a normal STAT5A phosphorylation response to anti-IgM stimulation (Fig. 8H, lanes 3 and 4). Thus, JAK3 is not required for the B cell antigen receptor-mediated STAT5A phosphorylation in B cells.

BTK is essential for the physiologic B cell responses to stimulation of the B cell antigen receptor (1–6) and is required for normal B cell development, since defects in BTK lead to XID in mice and XLA in humans (7, 8). Recent observations suggest the involvement of BTK in signal transduction pathways affecting gene transcription (13). Recent studies have further revealed a nucleocytoplasmic shuttling system for BTK that has implications regarding potential targets inside the nucleus, which may be critical in gene regulation during B cell development and differentiation (14). An unresolved question in B cell immunology is the nature of the molecular coupling mechanisms that link the proximal signals that are triggered by the engagement of the B cell antigen receptor to downstream signal transduction pathways affecting gene transcription. The recognition of an antigen by the B cell antigen receptor triggers a...
signal transduction cascade that culminates in activation of multiple genes controlling activation, proliferation, differentiation, and survival of B cells. BCR stimulation leads to the activation of transcription factor NF-κB, which in turn regulates genes controlling B cell growth. BTK is essential for activation of the NF-κB/Rel family of transcription factors via the B cell antigen receptor (15, 49). BTK also has been shown to regulate the nuclear localization and transcriptional activity of the multifunctional transcription factor BAP-135/TFI-I (17). BAP-135/TFI-I is a ubiquitously expressed multifunctional transcription initiation factor capable of binding to several promoter elements, including initiator (Inr) elements (50–52) that is tyrosine phosphorylated in B cells after engagement of the B cell antigen receptor (12). TFI-I can function both as a basal factor through the Inr element (50–53) and as an activator in the absence of a functional Inr element (53–55). Thus, TFI-I is likely to participate in the regulation of the transcription of several genes in B cells (50–55). TFI-I might establish an additional link between Btk-mediated signaling and transcription because TFI-I possesses Inr-dependent transcription properties and because many of the genes that are important for normal B cell development are Inr-containing genes (e.g. VpreB, TdT, and possibly RAG, CD5, Bcl-2, and Bcl-xL); a subset of these might be potentially TFI-I-responsive. Interference with this coupling mechanism may contribute to the B cell deficiencies observed in XLA patients or XID mice. Here we now show that BTK couples the STAT signaling pathway to the B cell antigen receptor. It is possible that STAT5A links BTK-mediated signaling events to downstream gene activation. A compromised coupling between BTK and STAT5A might lead to aberrant expression of the STAT5A-responsive genes and might contribute to the XLA and XID phenotypes.

BTK belongs to the Tec family of nonreceptor tyrosine kinases that includes TecI, TecII, Itk, Bmx/Etk, and DSrc28C (found in Drosophila) (56). Members of this family contain SH1, SH2, and SH3 domains but lack the typical myristoylation site and negative regulatory tyrosine of Src family members. A distinctive feature of these kinases is the presence of a PH domain in the N-terminal region, followed by a unique Tec homology domain. PH domains are involved in membrane localization, signal transduction, and cytoskeletal structure of several proteins (57–59). The BTK PH domain has also been implicated as a protein interaction domain (9, 60). Yang and Desiderio (12) provided evidence that the PH domain participates in the association of Btk and BAP-135/TFI-I. The PH domain of Btk is primarily responsible for its physical interaction with TFI-I, but an intact kinase domain of BTK is also required to enhance transcriptional activity of TFI-I in the nucleus. By comparison, our data presented herein indicate that only the kinase domain of BTK appears to be essential for its physical and functional interactions with STAT5A.

Co-immunoprecipitation experiments presented herein indicated that BTK is associated with STAT5A in unstimulated B cells but that association is reduced after anti-IgM stimulation. These results are reminiscent of the reported interactions between BTK and TFI-I (17). The physical association of BTK with TFI-I in B cells is reduced 4-fold after engagement of the B cell antigen receptor (17). It has been proposed that after anti-IgM stimulation, activated BTK induces tyrosine phosphorylation of TFI-I, which in turn reduces the constitutive association of BTK and TFI-I and allows dissociated TFI-I to translocate to the nucleus in active tyrosine-phosphorylated form (17). It is conceivable that a similar molecular mechanism governs the BTK-STAT5A interactions.

In summary, our results demonstrate a functional interaction between the transcription factor STAT5A and BTK. BTK is the first cytoplasmic non-Janus kinase to be identified as a positive regulator of STAT5A in B cells. Our findings point to a novel pathway through which B cell-specific signals mediated by BTK might control target genes via STAT5A. Thus, mutations in the BTK kinase domain impairing the physical and/or functional association between STAT5A and BTK may result in diminished STAT5A-dependent transcription and contribute to defective B cell development and/or function. Our findings prompt the hypothesis that STAT5A links BTK-mediated signaling events that follow the engagement of the B cell antigen receptor to downstream gene activation. A compromised coupling between BTK and STAT5A might lead to aberrant expression of the STAT5A-responsive genes and might contribute to the XLA and XID phenotypes. Similarly, the B cell precursor defects observed in STAT5A/5B-deficient mice may in part be due to impaired coupling of BTK-mediated proximal signals to downstream transcription programs controlling the orderly expansion of B cell precursors. However, the absence of significant defects in antigen receptor-mediated mature B cell responses of STAT5A/5B-deficient mice indicates that alternative pathways can develop and almost completely compensate for STAT5A deficiency when B cells are forced to develop and function without STAT5A expression. Our results corroborate the growing evidence that multiple counterregulatory mechanisms exist in B cells and operate to preserve cell survival and growth, thereby ensuring their orderly development and differentiation. To our knowledge, STAT5A deficiency has not been linked to a human immunodeficiency. Therefore, the physiologic significance of our findings should be interpreted with due caution.

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