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Binding of Bruton's tyrosine kinase to Fyn, Lyn, or Hck through a Src homology 3 domain-mediated interaction

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ABSTRACT **Bruton's tyrosine kinase (Btk) is a recently described B-cell-specific tyrosine kinase. Mutations in this gene lead to human X chromosome-linked agammaglobulinemia and murine X-linked immunodeficiency. Although genetic evidence strongly suggests that Btk plays a crucial role in B-lymphocyte differentiation and activation, its precise mechanism of action remains unknown, primarily because the proteins that it interacts with have not yet been identified. Here, we show that Btk interacts with Src homology 3 domains of Fyn, Lyn, and Hck, protein-tyrosine kinases that get activated upon stimulation of B- and T-cell receptors. These interactions are mediated by two 10-aa motifs in Btk. An analogous site with the same specificity is also present in Itk, the T-cell-specific homologue of Btk. Our data extend the range of interactions mediated by Src homology 3 domains and provide an indication of a link between Btk and established signaling pathways in B lymphocytes.**

Tyrosine phosphorylation is one of the earliest events during B-lymphocyte activation. When the B-cell receptors on the surface of resting splenic B cells or certain B-cell lines are cross-linked by anti-immunoglobin antibody, within 10 min more than a dozen proteins become phosphorylated on tyrosine (1). Among these are certain Src family tyrosine kinases such as Fyn, Lyn, and Hck, which are associated with the B-cell receptor complex in resting B cells (2–4). How clustering of the B-cell receptor complex might lead to activation of protein-tyrosine kinases remains to be shown. Recently, a kinase called Bruton's tyrosine kinase (Btk) has been discovered, which seems to play a central role in B-cell signaling events. Mutations of this gene are responsible for X-linked agammaglobulinemia, a severe human immunodeficiency disease, and murine X-linked immunodeficiency (5–8). It belongs to a subgroup of Src-related kinases including Itk found in T cells and Tec II found in liver and myeloid cells (9, 10). Like all other members of the Src family, Btk contains a tyrosine kinase domain, a Src homology (SH) 2 domain, and an SH3 domain. The long amino-terminal region in Btk is similar to those in Itk and Tec II but is not shared by other Src kinases and contains an apparent pleckstrin homology (PH) domain (11). To understand how Btk is involved in B-cell development and activation, we have searched for proteins with which Btk might interact. We show that Btk has a specific binding site that interacts with the SH3 domains of the Src-related Fyn, Lyn, and Hck kinases. These interactions occur through two proline-containing 10-aa sites in the amino-terminal region of Btk, just carboxyl-terminal to the PH domain. We suggest that Btk is on the pathway of signaling by which fyn, lyn, and hck control cellular events.

MATERIALS AND METHODS

Cloning. To isolate Btk cDNA clones, oligonucleotides corresponding to either the amino terminus or carboxyl terminus of the human Btk protein were made according to the published sequences (5) and used to probe duplicate lifts of λ phage plaques on filters. Ten independent doubly positive clones were isolated from $\approx 1 \times 10^6$ phage plaques. We partially sequenced these 10 cDNA clones and found that they all contained the full-length Btk coding sequence. A DNA fragment containing the amino-terminal portion of a mouse Btk cDNA clone was constructed in the EG202 vector, which contains the LexA DNA binding domain as its amino terminus (12). When this LexA-BtkN bait, along with the reporter plasmid pSH18-34 containing the LexA binding site driving the *lacZ* gene, was transformed into the yeast strain EGY48 (*MATα, trp1, ura3, his, Leu2:plexAop6-leu2*), colonies required leucine to grow and did not turn blue on 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) medium. Thus, the bait did not contain intrinsic transactivation potential and was suitable for library screening. Library plasmids (pJG4-5) containing the B42 acidic activation domain and cDNAs derived from HeLa cells were then transformed into the yeast clone carrying both the LexA-BtkN bait and the reporter plasmid pSH18-34. A total of 4.5×10^6 yeast transformants were selected on Ura⁻, His⁻, Trp⁻, and Leu⁻ X-Gal/galactose plates. Approximately 3000 colonies grew in the absence of leucine, but only 90 turned blue. Because the library vector pJG4-5 used here contained a galactose-inducible promotor, colonies of authentic interacting clones should turn blue when galactose but not glucose is used as a carbon source. Colony replicas of the 90 clones were plated onto both X-Gal/glucose and X-Gal/galactose plates, and 70 showed unambiguous galactose-dependent blue color. Plasmid DNAs were isolated from 12 of these clones, and library plasmids were selected through their tryptophan synthase gene. They were purified and reintroduced back into yeast strain EGY48 along with the reporter plasmid pSH18-34 plus either the pEG202-lexA-BtkN or pEG202 vector. Colonies were replicated onto either glucose/X-Gal or galactose/X-Gal plates. Clones with specific Btk-interacting proteins showed blue color on galactose/X-Gal plates only with pEG202-LexA-BtkN as bait. The insert from one clone was sequenced and found to correspond to the amino-terminal region of the human *FYN* gene.

The Source of the Various SH3 Domains. GST-Abl-SH3 and GST-Src-SH3 have been described (13). The sequences coding for the SH3 domains of mouse Btk (amino acid residues 218–276), bovine Blk (55–113), and chicken Fyn (80–144) were amplified from the corresponding cDNAs, whereas those of mouse Lyn (64–124) and mouse Hck (60–116) were amplified by reverse transcriptase–PCR using polyadenylylated mRNAs isolated from BALB/c mouse spleen. These various DNA fragments were ligated in-frame to the pGex2T

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Abbreviations: Btk, Bruton's tyrosine kinase; SH, Src homology; GST, glutathione *S*-transferase; IPTG, isopropyl β-D-thiogalactoside; PH, pleckstrin homology.

vector and were sequenced using a primer derived from the vector. Glutathione *S*-transferase (GST)-SH3 fusion proteins were purified from isopropyl β-D-thiogalactoside (IPTG)-induced bacterial lysates and were subsequently biotinylated as described (13, 14).

Solution Binding Assays. One liter of IPTG-induced bacteria expressing either GST or GST-Hck-SH3 was lysed in 10 ml of ice-cold lysis buffer (PBS/100 mM EDTA/1% Triton X-100/1% aprotinin/1 mM phenylmethylsulfonyl fluoride) by sonication. The supernatants were incubated with 250 μl of glutathione-agarose for 30 min at 4°C. These GST- or GST-Hck-SH3-saturated agarose beads were then washed four times with the lysis buffer and stored in an equal volume of PBS. To produce and detect Btk, we used a mammalian expression construct, pGD-BTK-FLU, made by ligating the full-length coding region of Btk cDNA in-frame to the 5′ end of the influenza hemagglutinin tag (FLU) in the retrovirus vector pGD-FLU. To create Btk(4A) mutations, PCR mutagenesis was performed (15). Approximately 2×10^7 293 cells either untransfected or transiently transfected with pGD-BTK-FLU or pGD-BTK(4A)-FLU were lysed in 1 ml of the lysis buffer (1% Nonidet P-40/150 mM NaCl/20 mM Hepes, pH 7.5/10 mM NaF/0.4 mM EDTA/50 mM Na_3VO_4/1 mM phenylmethylsulfonyl fluoride/1 mM leupeptin/1% aprotinin) on ice for 30 min. The supernatants were incubated with 5 μl of the glutathione-agarose beads containing either GST or GST-Hck-SH3 for 2 hr in a cold room with rotation. The precipitated complexes were then washed four times with the lysis buffer before Western blot assays with anti-FLU antibody.

RESULTS

To isolate cDNAs encoding proteins that interact with Btk, we used the yeast two-hybrid trapping method (12, 16). Full-length mouse Btk cDNA clones were isolated by screening a λgt10 library made from the mouse pre-B cell line 22D6 with oligonucleotide probes synthesized according to the published sequences (5). To identify proteins that specifically interact with Btk, we used the amino-terminal portion of Btk as bait because this region is conserved among the Itk and Tec subfamily but is distinct from sequences in other Src family members. It contains a PH domain (11). We adopted the yeast two-hybrid system developed in Brent's laboratory (12). The bait was linked to the LexA DNA binding domain and interacted in yeast cells with a galactose-controlled, activation domain-linked HeLa cell cDNA library. The reporters were β-galactosidase and Leu2 directed by LexA binding sites. One of the interacting clones we isolated, when sequenced, was found to represent the amino-terminal region of the human *FYN* gene; it contained the SH3 domain and a portion of the SH2 domain.

To test whether Fyn can interact directly with Btk, *in vitro* filter binding assays were performed. The cDNA fragment from the isolated clone was transferred into the vector pGex-1ZT, which can express in bacteria GST fusion proteins in the same frame as the LexA fusion proteins of yeast. The GST fusion protein containing the amino-terminal portion of human Fyn (GST-Fyn-N) was purified and biotinylated as described (13, 14). Total IPTG-induced bacterial cell lysates expressing the GST fusion protein containing the full-length Btk (GST-Btk-F) were fractionated by SDS/PAGE, transferred to nitrocellulose, and probed with biotinylated GST-Fyn-N fusion peptides. The GST-Fyn-N probe specifically recognized the GST-Btk-F protein but not the GST alone (Fig. 1), showing that the amino-terminal portion of Fyn interacts directly with Btk.

When GST-Fyn-N was used to probe fragments of Btk, the amino-terminal fragment, Btk-N, was recognized but Btk-H, which lacked only 44 carboxyl-terminal amino acids of Btk-N, did not bind (Fig. 1). A fragment of Itk similar to Btk-N was also tested and was bound by the GST-Fyn-N probe as strongly as was the Btk fragment (9). These results indicate that Btk can interact with Fyn through a relatively small region located immediately amino-terminal to the Btk SH3 domain. The association of Fyn with both Btk and Itk suggests that the two tyrosine kinases may be involved in similar pathways during B- or T-cell development and activation.

The regions of Btk and Itk that contained the binding sites have a common 10-aa sequence, KKPLPPTPEE/D (Fig. 2*A*). (KKPLPPTPEE is designated BB3-1, the first Btk binding site for SH3). In the same region there is another similar sequence KKPLPPEPTA (BB3-2) present in Btk but not in Itk. Both sequences are rich in proline residues and, as shown previously, proline-rich sequences can mediate protein–protein interactions involving certain SH3 domains (14).

Because the clone isolated by the yeast two-hybrid trapping method contained a complete Fyn SH3 domain, it seemed likely that Fyn interacted with Btk through the binding of its SH3 domain to these two proline-rich sequences. To test this possibility, we introduced a DNA fragment containing just the SH3 domain of the chicken *Fyn* gene into the pGex2T vector. The GST fusion protein, GST-Fyn-SH3, was purified from IPTG-induced bacteria and biotinylated. It was used to probe filters containing total bacterial lysates expressing equal amounts of either GST, GST-BB3-1, or GST-BB3-2. The GST-Fyn SH3 probe recognized both GST-BB3-1 and GST-BB3-2 but not GST alone (Fig. 2*B*). In a similar way, we examined the binding spec-

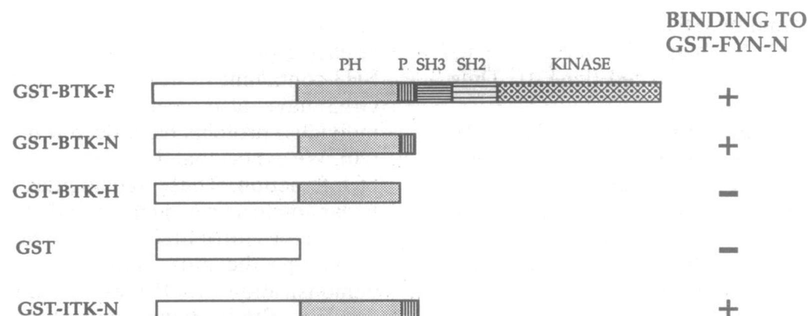

FIG. 1. Mapping of the Fyn binding site on Btk. Various DNA fragments from Btk and Itk were fused in-frame to the GST gene in pGEX. GST-BTK-F contained the full-length Btk protein (residues 1–659), whereas GST-BTK-N and GST-BTK-H contained Btk fragments of 1–218 and 1–174, respectively. GST-ITK-N contained the amino-terminal portion of the mouse Itk protein (1–181). The cDNA fragment from the Fyn clone was transferred into the vector pGex-1ZT, which can express GST fusion proteins in bacteria in the same frame as the LexA fusion proteins of yeast. The GST fusion protein containing the amino-terminal portion of Fyn (GST-Fyn-N) was purified and biotinylated as described (12, 13). Total IPTG-induced bacterial cell lysates expressing GST fusion proteins containing various fragments of Btk or Itk were fractionated by SDS/PAGE, transferred to nitrocellulose, and probed with biotinylated GST-Fyn-N fusion peptides. P, proline-rich region.

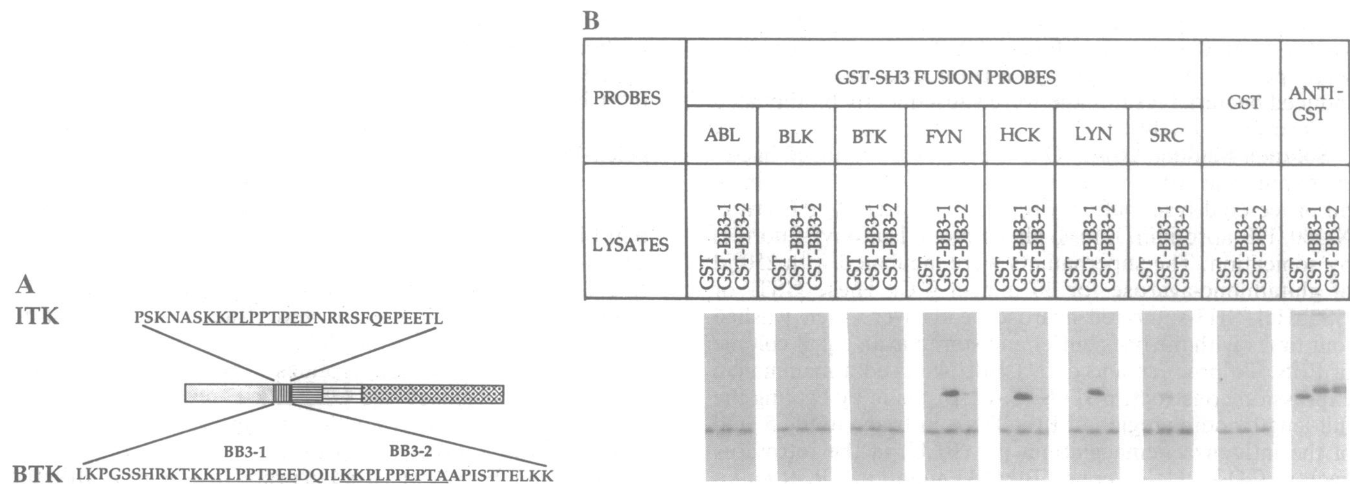

FIG. 2. Determination of the specificities of the SH3 binding sites in Btk by *in vitro* filter binding assays. (*A*) Amino acid sequences of Btk and Itk corresponding to the putative Fyn binding site. (*B*) Proteins from bacterial lysates expressing equal amounts of GST, GST-BB3-1, or GST-BB3-2 were probed with biotinylated GST-Abl-SH3, GST-Blk-SH3, GST-Btk-SH3, GST-Fyn-SH3, GST-Hck-SH3, GST-Lyn-SH3, GST-Src-SH3, GST, or anti-GST antibody (1 μg/ml) as described (12, 13).

ificities of BB3-1 and BB3-2 to a variety of SH3 domains. The Btk sites were not bound by Abl-SH3, Blk-SH3, Btk's own SH3, or Crk1 SH3 (data not shown). However, they bound to the SH3 domains of both Lyn and Hck at least as strongly as they bound to Fyn SH3 (Fig. 2*B*). A weak interaction between Src SH3 and BB3-1 or BB3-2 was revealed after longer exposures than were required to see the other interactions (we estimate it to be <10% of the strong interactions). Src SH3 is closer in sequence to Fyn SH3 than to others, highlighting the ability of similar SH3s to distinguish among potential binding sites.

To examine whether the SH3 domains of Fyn, Lyn, and Hck interact with full-length Btk in solution, we bound the SH3 region of Hck to beads and collected proteins that bound to the beads. To produce and detect Btk, we used a mammalian expression construct, pGD-BTK-FLU, made by ligating the full-length coding region of Btk cDNA in-frame to the 5′ end of the influenza hemagglutinin tag (FLU) in the retrovirus vector pGD-FLU. The construct was transiently transfected into 293 cells and, after 2 days, immunoprecipitation and Western blots of total cell lysates with anti-FLU antibody showed that Btk was present in transfected but not in nontransfected cells (Fig. 3*B*, lanes 7 and 8). The lysates were then incubated with glutathione-agarose beads coupled either to GST-Hck-SH3 or to GST alone. Bound proteins were solubilized and analyzed in a Western blot developed with anti-FLU monoclonal antibody (12CA5). A single band the size of Btk-FLU was bound by GST-Hck-SH3 but not by GST (Fig. 3*B*, lanes 2 and 5). No band was detected when untransfected 293 cell protein was analyzed (lane 4). Thus, Hck SH3 binds to Btk in solution.

To show that the interaction of Btk with the Hck SH3 domain is through the BB3-1 and BB3-2 sites, we made a construct, BTK(4A), containing point mutations in both sites by converting the codons for four proline residues into those for alanine (Fig. 3*A*) (15). These mutations did not interfere with Btk's kinase activity as shown by *in vitro* kinase assays (data not shown). The wild-type and mutant proteins were made as GST fusions to the same level in bacteria (Fig. 3*C*, lanes 1 and 3), but the mutations completely abolished the binding by Hck SH3 to the filter-bound proteins (Fig. 3*C*, lanes 4 and 6). In 293 cells, the mutated Btk was made (Fig. 3*B*, lane 9) but not bound by GST-Hck-SH3 (Fig. 3*B*, lane 6). Thus, the association of the SH3 domain of Hck with full-length Btk requires the proline residues in the BB3-1 and BB3-2 sites of Btk.

DISCUSSION

Our studies have uncovered an affinity between three Src-related proteins and Btk or Itk. This affinity both shows how SH3 binding specificity can determine protein–protein associations and suggests that the critical Btk kinase is integrated into the signaling pathway that involves kinases closely related to Src. From the point of view of SH3 specificity, the present work as well as that of others shows that SH3 can mediate highly selective interactions. Thus BB3-1 and BB3-2 are clear examples of binding sites specific for particular SH3 domains in that they are recognized by SH3s from Fyn, Lyn, and Hck but not those from other Src family members such as Btk and Blk. It has been shown that polyproline is not a good binding site for SH3 domains so that amino acids other than proline must be required for SH3 binding. These studies, as well as earlier ones on the Abl SH3 binding specificity (13, 14) and recent ones on sites in Abl that preferentially interact with Crk and Nck SH3 domains (R. Ren, Z.-S.Y., and D.B., unpublished results), show that amino acid residues around the proline residues confer binding selectivity. In fact, BB3-1 (KKPLPPTPEE) and BB3-2 (KKPLPPEPTA) differ only in their carboxyl-terminal three amino acids and yet display at least a 10-fold difference in affinity for the Fyn, Lyn, and Hck SH3s. A recently published study shows that a Pro-Xaa-Xaa-Pro motif as well as flanking amino acids interacts with SH3 domains (17). SH3s are known to mediate protein–protein interactions in various circumstances, including the binding of an adapter molecule, Grb2, to SOS, a guanine-nucleotide releasing protein (18). Interactions of dynamin with several SH3-containing proteins such as p85α, phospholipase C-γ, and Grb2 have also been shown (19). So far, more than 30 individual proteins have been found to contain SH3 domains (20). We expect that they represent many different specificities of interaction. These specificities are all, thus far, for short linear amino acid sequences and resemble in their diversity the specificities attributed to antibodies for their epitopes.

Perhaps the most important aspect of this work is the demonstration that Btk and Itk can interact with Fyn, Lyn, and Hck. Btk is unique among the protein-tyrosine kinases in B cells in that it has been proved by mutagenesis to be critical to human B-cell development and to signaling in the mature mouse B cells. Most B cells in X-linked agammaglobulinemia patients are blocked at the V_H-to-DJ_H rearrangement stage (21). Interestingly, dominant-negative mutations of the Lck protein-tyrosine kinase gene in transgenic mice block T-cell development at the equivalent stage (V_β-to-DJ_β joining) (22).

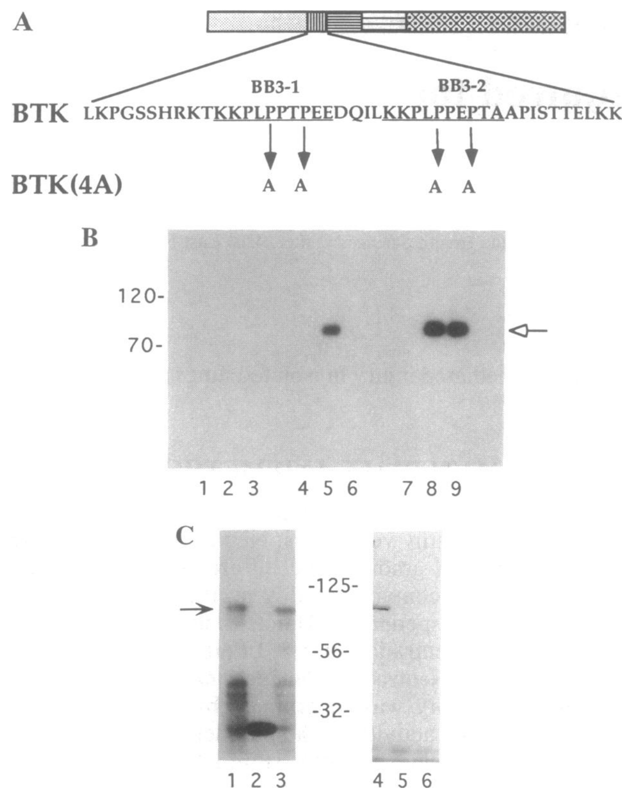

FIG. 3. Binding of full-length Btk protein and the Hck SH3 domain in solution. (*A*) Schematic representation of Btk with the amino acid sequences around the SH3 binding sites, BB3-1 and BB3-2 (underlined). BTK(4A) is the mutant form of Btk where four proline residues in the SH3 binding sites have been mutated to alanine residues. (*B*) Solution binding assays. Cell lysates from untransfected 293 cells (lanes 1, 4, and 7), 293 cells transfected with pGD-Btk-Flu (lanes 2, 5, and 8), and 293 cells transfected with pGD-Btk(4A)-Flu (lanes 3, 6, and 9) were precipitated with GST (lanes 1–3), GST-Hck-SH3 (lanes 4–6), or anti-FLU antibody (lanes 7–9). The precipitated complexes were Western blotted and probed with anti-FLU antibody. The arrow indicates the full-length BTK-FLU or BTK(4A)-FLU. (*C*) Filter binding assays. Proteins from bacterial lysates expressed GST-Btk (lanes 1 and 4), GST (lanes 2 and 5), and GST-Btk(4A) (lanes 3 and 6) were probed with either anti-GST antibody (lanes 1–3) or the biotinylated GST-Hck-SH3 (lanes 4–6). The arrow indicates the intact GST-BTK or GST-BTK(4A). Molecular size markers (in kDa) are indicated.

It is possible that Lck acts through Btk's homologue, Itk, during T-cell development. The role of Btk in B-cell activation is unclear. It is largely located in the cytoplasm and is not tyrosine phosphorylated in the pre-B cell line, 70Z, as well as in resting splenic B cells, suggesting that it may require other proteins for its activation (6–8). On the other hand, Fyn, Lyn, and Hck have been shown to be associated with the B-cell receptor complex in unactivated B cells and are rapidly phosphorylated on tyrosine after cross-linking with anti-IgM antibody (2–4). Our studies suggest that they are good candidates for carrying out Btk activation. We have, however, been unable to convincingly demonstrate that Btk becomes phosphorylated when WEHI 231 cells are activated by anti-IgM antibody (G.C. and D.B., unpublished results).

Interestingly, Btk protein did not interact detectably with full-length Fyn protein in 293 cells (data not shown), although it interacted with Fyn SH3 in yeast cells and in solution. Possibly, in the full-length Fyn protein the SH3 domain is cryptic and unavailable for binding to Btk in the native state and becomes exposed only after activation.

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