Role of the PHTH Module in Protein Substrate Recognition by Bruton’s Agammaglobulinemia Tyrosine Kinase*

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Defects in Bruton’s tyrosine kinase (Btk) are responsible for X chromosome-linked agammaglobulinemia in patients. Mutations in each of the structural domains of Btk have been detected in patients, yet a mechanistic explanation for most of these mutant phenotypes is lacking. To understand the possible role of the unique pleckstrin homology and Tec homology (PHTH) module of Btk, we have compared the enzymatic properties of full-length Btk and a Btk mutant lacking the PHTH module (BtkΔPHTH). Here we show that Btk and BtkΔPHTH have very similar basal catalytic activity but very different abilities to recognize protein substrates. Furthermore, the catalytic domain of Btk is inactive, in contrast to the catalytic domain of the prototypical Src tyrosine kinase that retains full catalytic ability. These data suggest that the PHTH module plays an important role in protein substrate recognition, that Btk and Src likely have different interdomain organizations and regulations, and that alterations in substrate recognition might play a role in X chromosome-linked agammaglobulinemia.

EXPERIMENTAL PROCEDURES

Protein Purification—Full-length Btk, PHTH-deleted Btk, and the catalytic domain of Btk were purified from Sf9 cells. The detailed purification protocols will be published elsewhere. Briefly, the DNA fragments encoding the full-length (amino acids 1–659) or the PHTH-deletion mutant (amino acids 212–659) or the catalytic domain (amino acids 384–659) of Btk were subcloned into pFastBak1 vector (Life Technologies, Inc.). After obtaining the recombinant baculoviruses harboring pFastBak1-Btk, pFastBak1-BtkΔPHTH, or pFastBak1-Btk-cat, Sf9 cells were infected and harvested after 60 h. Btk, BtkΔPHTH, and Btk-cat were purified by chromatography, and the purification was monitored by Coomassie Blue staining and Western blotting with anti-Btk antibody (Santa Cruz Biotechnology).

Kinase Assays—Kinase assay was performed as described previously (9, 10). Purified Btk or BtkΔPHTH kinase (10 nM) in Btk kinase buffer (30 mM Hepes, pH 7.4, 10 mM MgCl2, and 10 μM ATP) was combined with 70 μM Btk substrate peptide. 10 μCi of [γ-32P]ATP (3,000 Ci/mmol) was added, and the mixture (total, 20 μl) was incubated at 30 °C for 8 min. The reaction was stopped by adding Laemmli sample buffer. After heating at 90 °C for 5 min, the substrate peptide was separated on 20% SDS-polyacrylamide gel electrophoresis gel, dried, and autoradiographed. In some experiments, Vav (amino acid residues from 170–375 of mouse Vav) or GST-CDB3 was used as substrate. Purification of these recombinant proteins has been described previously (11, 12).

Enzyme Kinetics Analysis—Enzyme kinetic activity was measured with a coupled spectrophotometric assay (13). In this assay, the production of ADP was coupled to the oxidation of NADH measured as a reduction of absorbance at 340 nm. Reactions contained 20 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM phosphoenolpyruvate, 0.28 mM NADH, 89 units/ml pyruvate kinase, and 124 units/ml lactate dehydrogenase. Reactions were initiated by the addition of 60 nM Btk, and progress curves were monitored by absorbance at 340 nm in a spectrophotometer. When Km( pep) was determined, [ATP] was fixed at 500 μM. When KmA(TP) was determined, the concentration of the peptide substrate was fixed at 100 μM. Initial rates were measured, and the kinetic paramet-

The abbreviations used are: Btk, Bruton’s tyrosine kinase; XLA, X chromosome-linked agammaglobulinemia; PH, pleckstrin homology; TH, Tec homology; SH, Src homology; GST, glutathione S-transferase; PHTH, pleckstrin homology and Tec homology domains.

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RESULTS

**Btk and BtkΔPTH Have Similar Basal Catalytic Activity**—Mutations in the PH and TH domains of Btk have been identified in patients (8) (Fig. 1A); however, it is not clear how these mutations manifest the observed phenotype. Some of the mutations in the PH domain occurred in residues known to be involved in inositol phosphate binding. There were two large deletion mutants in Btk that caused XLA: one involves parts of both the PH and TH domains, and another is within the SH3 domain (8). To understand the possible causal relationship between mutation and disease with the PH and TH deletion, it is essential to understand the role of the PHTH module in Btk function.

A NMR structure of the proline-rich region (within the TH domain) and the SH3 domain of Itk/Tsk (a member of the Btk family of kinases) showed an intramolecular interaction between these two domains (15). Furthermore, crystal structures of c-Src and Hck revealed that an intramolecular interaction involving its SH3 domain has a major role in keeping Src family tyrosine kinases at a down-regulated state (16–18). This led to an assumption that Btk family tyrosine kinases might use a similar intramolecular interaction to suppress Btk activity.

Therefore, we first compared the enzymatic properties of the full-length Btk and BtkΔPTH (Fig. 1). Both Btk and BtkΔPTH were expressed in Sf9 cells and purified to homogeneity (Fig. 1, A and B). When a small substrate peptide derived from the autophosphorylation site (Tyr-223) of Btk within its SH3 domain (19) was used, we found that the basal catalytic activities of Btk and BtkΔPTH were not substantially different (Fig. 1C). Thus, deletion of the PHTH module had no effect on the kinase activity of Btk.

We next investigated the enzymatic kinetic parameters of Btk and BtkΔPTH. BtkΔPTH were expressed in Sf9 cells and purified to homogeneity (Fig. 1, A and B). When a small substrate peptide derived from the autophosphorylation site (Tyr-223) of Btk within its SH3 domain (19) was used, we found that the basal catalytic activities of Btk and BtkΔPTH were not substantially different (Fig. 1C). Thus, deletion of the PHTH module had no effect on the kinase activity of Btk.

![Table I: Kinetic parameters of Btk and BtkΔPTH](Downloaded from http://www.bjc.org)
than down-regulated c-Src and that Btk and down-regulated c-Src may possibly have different structural configurations between their active sites and activation loops. The above-mentioned enzymatic kinetic data suggest that deletion of the PHTH module did not change the intrinsic catalytic ability of Btk.

Both Tyr-223 and Tyr-551 residues can be autophosphorylation sites. Based on cellular studies, a Btk activation mechanism has been proposed: Src family tyrosine kinases could phosphorylate Tyr-551 at the activation loop of Btk, increase Btk kinase activity, and lead to the autophosphorylation of Tyr-223 within the SH3 domain of Btk (Fig. 2 A) (19, 20). In this model, Src phosphorylation of Tyr-551 is prerequired for Btk activation. However, this proposal has not been tested with purified Btk. Therefore, we examined the possible effect of the deletion of the PHTH module on the ability of Btk to autophosphorylate. Using antibodies specific for Btk phosphorylated at Tyr-223 or at Tyr-551 (21), we investigated the autophosphorylation of purified Btk and BtkΔPHTH. As shown in Fig. 2, both Btk and BtkΔPHTH can autophosphorylate Tyr-223 and Tyr-551 in the presence of ATP (Fig. 2, B–D, lanes 3 and 4), suggesting that both Tyr-223 and Tyr-551 residues can be autophosphorylation sites. We noticed that before incubation with ATP, some purified Btk but not BtkΔPHTH is phosphorylated at Tyr-223 but not Tyr-551, indicating that Tyr-223 might indeed be the primary (or first) autophosphorylation site (Fig. 2, B–D, lanes 1 and 2). Furthermore, Src did not significantly increase the phosphorylation of Tyr-223 or Tyr-551 beyond the autophosphorylation levels (Fig. 2, B–D, lanes 5 and 6). Nevertheless, the ability of Btk to autophosphorylate the Tyr-551 residue at the activation loop is similar to c-Src autophosphorylating Tyr-416 at its own activation loop. This suggests that, similar to Tyr-416 in c-Src, autophosphorylation of Tyr-551 in Btk may correlate with its increased kinase activity by promoting a change in conformation of the activation loop. These autophosphorylation data suggest that, similar to c-Src, factors other than Src tyrosine kinase (such as G proteins) can increase the catalytic activity of Btk and demonstrate that deletion of the PHTH module had no effect on the autophosphorylation of Tyr-223 and Tyr-551 by Btk.

Role of the PHTH Module in Recognizing Downstream Protein Substrates—Remarkably, we observed that Btk and...
Btk has been shown to act upstream of Vav, a guanine nucleotide exchange factor for Rho family small G proteins (22). When a fragment (residues 170–375) of Vav encompassing the catalytic Dbl homology domain was used as substrate (12), it was efficiently phosphorylated by Btk (Fig. 3A, lane 1). BtkΔPHTH was unable to phosphorylate Vav (lane 2). When GST-CDB3 was used as substrate, Btk (lane 1), but not BtkΔPHTH (lane 2), efficiently phosphorylated GST-CDB3. Data are representative of three experiments.

We have also studied the phosphorylation of another protein substrate, GST-CDB3, a GST fusion protein with the cytoplasmic domain of Band 3 protein that has been used as a conventional substrate for Btk kinase assays (10, 11). Again, Btk, but not BtkΔPHTH, efficiently phosphorylated GST-CDB3 (Fig. 3B). Furthermore, Btk, but not BtkΔPHTH, could phosphorylate purified proteins such as Wiskott-Aldrich syndrome protein and type V adenylyl cyclase (data not shown). These data demonstrate that the PHTH module is critical for protein substrate recognition by Btk and suggest a possible role in the development of the disease phenotype.

One possible mechanism to explain why Btk but not BtkΔPHTH could phosphorylate Vav is that the PHTH module contributes to protein substrate binding. To test this, we have examined the interaction of the PHTH module with Vav (Fig. 4). We found that, in addition to the catalytic domain, the PH domain and the PHTH module could also interact with Vav (Fig. 4). Thus, the association of Vav with the catalytic domain alone is not sufficient for Vav phosphorylation. This additional contact with the PH domain might enhance or stabilize the interaction of Vav with Btk and allow Vav to be phosphorylated by Btk. Alternatively, the presence of the PHTH module changes the conformation of the catalytic site of Btk to be suitable to phosphorylate larger protein substrates.

The Btk Catalytic Domain Alone Is Inactive—Many recombinant catalytic domains of protein tyrosine kinases and protein tyrosine phosphatases, such as insulin receptor kinase, Src, Fps, and SHP-1, are essentially fully catalytically active on peptide as well as protein substrates (23–25). One exception is the catalytic domain of tyrosine kinase Csk, which is catalytically inactive even though its crystal structure is very similar to that of c-Src (26, 27). We have investigated the catalytic activity of the catalytic domain of Btk. We found that the catalytic domain of Btk (Btk-cat) could not phosphorylate the peptide or protein substrates, whereas Src-cat did (Fig. 5, A and B).

Substrate

FIG. 3. Role of the PHTH module in recruiting downstream protein substrates. A, when Vav-(170–375) was used as substrate, Btk can efficiently phosphorylate Vav (lane 1). BtkΔPHTH was unable to phosphorylate Vav (lane 2). B, when GST-CDB3 was used as substrate, Btk (lane 1), but not BtkΔPHTH (lane 2), efficiently phosphorylated GST-CDB3. Data are representative of three experiments.

FIG. 4. Binding of Vav-(170–375) to both the catalytic domain and the PHTH domain of Btk. GST fusion proteins were incubated with Vav. The Vav pulled-down by the GST fusion proteins was detected with anti-Vav antibody. Lane 9 represents the input Vav used in the binding reactions. Data are representative of three similar experiments.

FIG. 5. The Btk catalytic domain alone is inactive. A, the catalytic domain of c-Src (Src-cat), but not the catalytic domain of Btk (Btk-cat), phosphorylated the protein substrate Vav-(170–375). B, Btk-cat did not phosphorylate the peptide substrate, whereas Src-cat did. Data are representative of three similar experiments.

FIG. 6. Proposed model of Btk to depict the role of the PHTH module in protein substrate recognition. Y represents the tyrosine residue on the protein substrate to be phosphorylated.
relies on other modules such as the PHTH or the SH3SH2 to recognize substrates or to modulate the accessibility to substrates.

DISCUSSION

Defects in Btk are responsible for XLA in patients. The Btk family of tyrosine kinases is the only tyrosine kinase family to possess a PHTH module. The PH domain of Btk provides a binding site for phosphatidylinositol 3,4,5-trisphosphate, the lipid product of phosphatidylinositol 3-kinase (28). Some PH domain residues mutated in XLA patients (such as R28C) are within the inositol binding region, indicating the importance of the inositol binding and the consequent membrane localization. The TH domain is composed of the N-terminal Btk motif (BM) followed by two proline-rich regions in tandem in Btk. This TH domain has only been found in members of Btk family kinases thus far. The PHTH module has been shown to be important for interaction with Vav, protein kinase C isoforms, G proteins Gβγ and Go12γ, F-actin, tyrosine kinase FAK, phosphorysphatase PTPD1, transcriptional factors Stat 3 and BP-135/TFI-I, and substrate BCR downstream signaling 1 (BRDG1) (1–3). Some of these interactions caused modulation of the Btk kinase activity. It had been proposed that the PHTH module could form intramolecular interactions with other parts of Btk family kinases, leading to suppression of its kinase activity (15, 29). Possible disruption of these intramolecular interactions by other factors might increase the kinase activity. However, we found that the basal catalytic activity of Btk and BtkΔPHTH was not substantially different. It is possible that the PHTH module could relay the binding of other factors to the catalytic domains allosterically. Alternatively, some of these activators might have more than one interaction site on Btk. A third possibility, although there is no evidence for it at the present time, is that there is an unidentified mechanism (or modification) involving the PHTH module that keeps Btk down-regulated in cells that is absent in purified Btk and BtkΔPHTH. Nevertheless, we have demonstrated that the PHTH module is important in protein substrate recognition by Btk (Fig. 6). It is striking that deletion of the PHTH module abolished the phosphorylation of protein substrates but not the phosphorylation of small peptides. Therefore, the PHTH module plays versatile roles in protein-Protein or protein-membrane interactions controlling the function of Btk. It is interesting to note that Dro sophila express both the full-length Btk protein and the PH/BM module-deleted Btk protein from the same gene through alternative RNA splicing (30). Loss of function of both Btk and BtkΔPHTH caused oocyte undergrowth and reduced adult-stage life span, indicating the physiological importance of the PH/TH module.

We have shown that purified Btk and BtkΔPHTH are capable of autophosphorylating the Tyr-223 and Tyr-551 residues. The ability of Btk to autophosphorylate the Tyr-551 residue at the activation loop is similar to c-Src autophosphorylating Tyr-416 at its own activation loop. This suggests that autophosphorylation of Tyr-551 in Btk may correlate with its regulated kinase activity. Indeed, phosphorylation of Tyr-551 by Src family kinases was proposed to increase Btk kinase activity (20). Although mutation of Tyr-223 had no direct effect on the kinase activity of Btk, phosphorylation of Tyr-223 could affect the interaction of Btk with other proteins (1). These autophosphorylation data suggest that, similar to c-Src, factors other than tyrosine kinases can increase the catalytic activity of Btk because Btk is able to autophosphorylate the Tyr-551 residue at the activation loop.

In comparison with the down-regulated c-Src tyrosine kinase, Btk seems to have a different structural domain organization. Although both Btk and c-Src have similar linear organization of the SH3-SH2 catalytic domains, they respond very differently to some peptide activators. A phosphorysphorysphatase-containing peptide, which is an SH2-binding peptide, increased the kinase activity of c-Src (31) but had no significant effect on the activity of Btk or BtkΔPHTH. A proline-rich peptide, which is an SH3-binding peptide, also enhanced the kinase activity of c-Src (31) but had no significant effect on the activity of Btk or BtkΔPHTH. These may imply that the interdomain organization is different in c-Src and Btk.

Recently, an x-ray crystal structure of the catalytic domain of Btk in its unphosphorylated state was determined (32). Our enzymological data are consistent with the crystal structural data. Due to the inactive conformation of the critical helix c2 in the N-terminal lobe, the catalytic domain alone is not active (32). The structures of the activation loops of Btk and down-regulated Src kinases are markedly different. The activation loop in Btk adopts a unique conformation. Unlike Src, the peptide substrate binding site is not occluded by other portions of the catalytic domain of Btk; this might explain why Btk has a lower Km for small peptide substrates than c-Src (Table I). The apparent higher Km for ATP-Mg2+ by Btk than down-regulated Src is consistent with the failure of binding of Btk to the ATP affinity column(2). Given the difference in catalytic activity of BtkΔPHTH and Btk-cat, the SH3 and/or SH2 domains presumably have a major effect on the configuration of the active site of Btk. It was further proposed that Btk has a unique activation mechanism (32). Phosphorylation of Tyr-551 could lead to Btk activation by triggering an exchange of hydrogen-bonded pairs from Glu-445/Arg-544 to Glu-445/Lys-430 and subsequent rotation of helix c2 within the N-terminal lobe of the catalytic domain (32).

Recognition of upstream modulators and downstream substrates and subcellular localization of protein tyrosine kinases are essential components of their biological function. Unlike enzymes involved in metabolism, tyrosine kinases appear to have diverse upstream regulators as well as multiple downstream substrates. Metabolic networks are generally very stable and resilient because most molecules in the metabolic pathways are thought to be sparsely connected. On the other hand, signaling networks are generally more dynamic and plastic and prone to modulation. Although the active site of the catalytic domain of these protein tyrosine kinases may have some degree of substrate specificity, these tyrosine kinases use their multi-modular domains, such as the PHTH module, the SH3 domain, or the SH2 domain, to interact with regulators and/or substrates in the context of specific signaling processes and the cellular environment.

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