Bruton’s Tyrosine Kinase Is a Toll/Interleukin-1 Receptor Domain-binding Protein That Participates in Nuclear Factor κB Activation by Toll-like Receptor 4*

In this study we have identified members of the Toll-like receptor (TLR) family (namely, TLRs 4, 6, 8, and 9) as proteins to which the intracellular protein tyrosine kinase, Bruton’s tyrosine kinase (Btk), binds. Detailed analysis of the interaction between Btk and TLR8 demonstrates that the presence of both Box 2 and 3 motifs in the Toll/interleukin-1 receptor domain was required for the interaction. Furthermore, co-immunoprecipitation experiments revealed that Btk can also interact with key proteins involved in TLR4 signal transduction, namely, MyD88, Mal (MyD88 adapter-like protein), and interleukin-1 receptor-associated kinase-1, but not TRAF-6. The ability of Btk to interact with TLR4 and Mal suggests a role for Btk in lipopolysaccharide (LPS) signal transduction. Stimulation of the human monocytic cell line THP-1 with LPS resulted in an increase in the level of tyrosine phosphorylation of Btk (indicative of activation). The autokinase activity of Btk was also stimulated after LPS stimulation. In addition, a dominant negative form of Btk inhibited TLR4-mediated activation of a nuclear factor κB (NFκB)-dependent reporter gene in HEK293 cells as well as LPS-induced activation of NFκB in the astrocytoma cell line U373 and the monocytic cell line RAW264.7. Further investigation revealed that the Btk-specific inhibitor, LFM-A13, inhibited the activation of NFκB by LPS in THP-1 cells. Our findings implicate Btk as a Toll/interleukin-1 receptor domain-binding protein that is important for NFκB activation by TLR4.

The Tec family of protein tyrosine kinases, of which Bruton’s tyrosine kinase (Btk) is a prototypical member, is involved in a vast array of signaling pathways in cells of hematopoietic lineage. Btk is expressed in all hematopoietic cells except T lymphocytes and natural killer cells. It is critically important for B-cell development as well as mature B-cell activation and survival. It has also been shown to be important for IgE-mediated activation of mast cells resulting in allergic reactions. Btk kinase activity and tyrosine phosphorylation have both been shown to increase upon cross-linking or stimulation of the B-cell receptor, the IgE receptor (FeRRII), and a number of cytokine receptors such as those for IL-3, IL-5, IL-6, and IL-10, suggesting a general role for Btk in immune regulation (1–4). Whereas the molecular mechanisms by which the B-cell receptor regulates B-cell proliferation and survival are not well understood, Btk has recently been shown to lie downstream of the B-cell receptor on the pathway regulating activation of the key pro-inflammatory transcription factor NFκB (5–8). The biological importance of the signaling function has been shown by naturally occurring loss of function mutations in Btk in human X-linked agammaglobulinemia and its murine counterpart, X-linked immunodeficiency (Xid). These diseases are characterized by a block in B-cell development and defects in B-cell signaling, and in X-linked agammaglobulinemia patients, mutations in Btk are associated associated with an increased frequency of bacterial infections in all organs (9). Studies in Xid mice have also shown reduced responses to LPS stimulation, with nitric oxide (NO) production decreased, and macrophage effector functions impaired (10–12). This, coupled with the observation that responses to T-independent antigens are impaired in Xid mice, suggests a role for Btk in innate immune responses.

Toll-like receptors (TLRs) have an essential function in both innate and adaptive immunity and have evolved to recognize, with high specificity, diverse microbial pathogens (13). TLR4, as the receptor for the Gram-negative bacterial product LPS, is the prototypical member of the family (numbered TLR1–10 in humans) of type I transmembrane receptors, which are characterized by an extracellular leucine-rich repeat domain and an intracellular Toll/IL-1 receptor (TIR) domain, responsible for signaling. Ligands for other family members (except TLR10) have been identified and include bacterial flagellin and unmethylated bacterial CpG motifs for TLR5 and TLR9, respectively; double-stranded RNA for TLR3; and the antiviral compound R-848 recognizing TLR7 and TLR8. Research into how these receptors signal has identified MyD88 and IL-1-receptor-associated kinases (IRAKs) as key proximal signaling components regulating activation of the pro-inflammatory transcription factor NFκB in response to LPS (reviewed in Ref. 14). Important differences in the proteins recruited to the different TLR members have also been described. Both TLR2 (the receptor for bacterial products such as peptidoglycan) and TLR4 recruit an adapter protein homologous to MyD88, which has been termed Mal (for MyD88 adapter-like protein) or TIRAP (Toll/IL-1 receptor adapter protein), which has been shown to

* This work was supported by Science Foundation Ireland. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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Received for publication, February 11, 2003, and in revised form, April 23, 2003

Published, JBC Papers in Press, April 30, 2003, DOI 10.1074/jbc.M301484200


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Published online for Advance Peer Review, May 27, 2003

Printed in U.S.A.

This paper is available on line at http://www.jbc.org
Btk Is a Transducer of TLR Signaling

EXPERIMENTAL PROCEDURES

**Yeast Two-hybrid Screening**—Full-length Btk was cloned into pPC1 (Clontech) and co-transformed with the Btk bait vector in HFC7 yeast cells.

**Cell Culture, Plasmids, and Reagents**—HEK293 and U373 cells were obtained from the Centre for Applied Microbiology and Research (Porton Down, United Kingdom), and the RAW264.7 cell line was obtained from the American Type Culture Collection (Porton Down, United Kingdom), and the RAW264.7 cell line was obtained from Calbiochem (Nottingham, United Kingdom). All other reagents were obtained from Sigma (Poole, United Kingdom) unless otherwise stated.

**Immunoprecipitation and Western Blot Analysis**—HEK293 or U373 cells were seeded (10^5 cells/ml) onto 96-well plates 24 h before transfection with 80 ng of b-luciferase, 40 ng of Renilla luciferase, and the indicated amount of Btk-expressing plasmid (220 ng total), and RAW264.7 cells were seeded (10^6 cells/ml) onto 24-well plates 24 h before transfection with 250 ng of b-luciferase, 250 ng of Renilla luciferase, and the indicated amount of Btk-expressing plasmid (1000 ng total) using Genejuice (Novagen, Madison, WI) according to the manufacturer's recommendations. The amount of DNA transfected was kept constant by the addition of various amounts of the appropriate empty vector plasmid. For reporter gene assays, cells were lysed for 15 min at room temperature with 50 μl of Passive Lysis Buffer (Promega, Southampton, United Kingdom). After clarification, the supernatant was used to determine luciferase activity, and an equivalent amount was used for Renilla luciferase activity. Firefly luciferase and Renilla luciferase activity was assayed using standard protocols. Levels of firefly luciferase expression were normalized against Renilla activity as a control for transfection efficiency and expressed as fold stimulation over unstimulated empty vector control.

**Reporter Gene Assays**—HEK293 or U373 cells were seeded (10^5 cells/ml) onto 96-well plates 24 h before transfection with 250 ng of b-luciferase, 250 ng of Renilla luciferase, and the indicated amount of Btk-expressing plasmid (1000 ng total) using Genejuice (Novagen, Madison, WI) according to the manufacturer's recommendations. The amount of DNA transfected was kept constant by the addition of various amounts of the appropriate empty vector plasmid. For reporter gene assays, cells were lysed for 15 min at room temperature with 50 μl of Passive Lysis Buffer (Promega, Southampton, United Kingdom). After clarification, the supernatant was used to determine luciferase activity, and an equivalent amount was used for Renilla luciferase activity. Firefly luciferase and Renilla luciferase activity was assayed using standard protocols. Levels of firefly luciferase expression were normalized against Renilla activity as a control for transfection efficiency and expressed as fold stimulation over unstimulated empty vector control.
BTK Is a Transducer of TLR Signaling

Fig. 1. Btk interacts with the TIR domain of TLRs 8, 4, 6, and 9. a, Btk interacts with the TIR domain of TLR8. The top diagram illustrates the domain structure of TLR8. The bottom diagram describes the clone of TLR8 obtained in the yeast two-hybrid assay, comprising its transmembrane and intracellular domains, fused to Gal4-TAD, the transactivation domain of the Gal4 transcription factor. b, mapping of the region within the TIR domain of TLR8 responsible for interaction with Btk. HF7c cells were transformed with deletion mutants of the TLR8 TIR domain, encompassing different TIR domain boxes important for the binding to interaction partners. c, Btk interacts with the TIR domains of TLRs 4, 6, 8, and 9. HF7c cells were transformed with full-length Btk together with the TIR domains of the TLRs indicated. The interaction was determined by both growth on triple drop-out plates and β-galactosidase assays.

Next we asked whether Btk selectively interacts with TLR8 or whether other TLR-intracellular domains might also show Btk interaction. We therefore tested the TIR domains of TLRs 4, 6, and 9 for their capacity to interact with Btk. The selection was made on the basis of sequence homology and expression pattern. TLR8 shows high sequence homology to TLR4, an important receptor on mast cells and macrophages, and TLR6 and TLR9, which, in addition to macrophages, were also found on B cells (23). As shown in Fig. 1c, Btk can interact with all analyzed TIR domains; nevertheless, the strongest interaction was found with the TIR domain of TLR8. Together, these data indicate that Btk is able to bind to the TIR domains of TLRs. Furthermore, Boxes 2 and 3 of the TIR domains are critical for this interaction.

Btk associates with MyD88, Mal, and IRAK-1—If Btk was to be important for signaling by TLRs, we hypothesized that it might interact with downstream signaling components. We therefore next investigated possible interactions between Btk and MyD88, Mal, and IRAK-1. Fig. 2, a and b, shows HEK293 cells transfected with a plasmid encoding AU1-tagged MyD88 (AU1-MyD88) in combination with a Btk-expressing plasmid. In Fig. 2a, immunoprecipitation of AU1-MyD88 from lysates of cells overexpressing both Btk and AU1-MyD88 resulted in increased detection of Btk in the immunocomplex (lane 4) when compared with lysates from cells expressing either Btk or AU1-MyD88 alone (lanes 1 and 2). Interestingly, Btk was detected after AU1-MyD88 immunoprecipitation from cells expressing AU1-MyD88 alone (Fig. 2a, lane 2), indicating an association between AU1-MyD88 and endogenous Btk. This interaction would appear to be specific due to the fact that Btk was not detected after immunoprecipitation with anti-AU1 in lysates from cells transfected with Btk alone (Fig. 2a, lane 1). To confirm the interaction, the immunoprecipitation was performed in the opposite direction (Fig. 2b). Immunoprecipitation of Btk from lysates of cells overexpressing both Btk and AU1-MyD88 resulted in increased detection of AU1-MyD88 in the immunocomplex (Fig. 2b, lane 3) when compared with lysates from cells expressing AU1-MyD88 alone (lane 1). Again, a band at the correct molecular weight for AU1-MyD88 was detected after immunoprecipitation of complexes using anti-Btk in cells transfected with AU1-MyD88 alone (Fig. 2b, lane 1). Whether this was due to an interaction between endogenous Btk and overexpressed AU1-MyD88 was difficult to determine due to a contaminating nonspecific band present in the IgG control lane (Fig. 2b, lane 4 compared with lane 5).

Fig. 2, c and d, investigated possible interactions between overexpressed Btk and HA-tagged Mal (HA-Mal). Btk and HA-Mal were found to associate when either HA-Mal-containing complexes (Fig. 2c, lane 4) or Btk-containing complexes (Fig. 2d, lane 2) were isolated from cells transfected with both HA-Mal and wild-type Btk. Similar to the interaction with AU1-MyD88, immunocomplexes containing HA-Mal were found to contain endogenous Btk (Fig. 2c, lane 2), and immunocomplexes containing endogenous Btk were found to associate with HA-Mal (Fig. 2d, lane 1), suggesting that endogenous Btk associates with overexpressed HA-Mal. The specificity of this finding was tested in lysates expressing HA-Mal alone, comparing the ability of anti-Btk (Fig. 2d, lane 5) and control IgG (lane 4) to immunoprecipitate HA-Mal. Increased levels of HA-Mal were detected in Btk-containing immunocomplexes when compared with the IgG control complex (Fig. 2d, lanes 5 and 4, respectively), indicating that an interaction between endogenous Btk and overexpressed Mal can be detected.

We also tested for interactions of Btk with IRAK-1 and TRAF-6. IRAK-1 was detected in a complex with Btk in lysates from HEK293 cells transfected with both wild-type Btk and IRAK-1 (Fig. 3a, lane 3). The specificity of this interaction was tested in lysates expressing IRAK-1 alone, comparing the ability of anti-Btk (lane 5) and control IgG (lane 4) to immunoprecipitate IRAK-1. IRAK-1 was detected in Btk-containing immunocomplexes only and not in the IgG control. An interaction between endogenous Btk and endogenous IRAK-1 was also detected after immunoprecipitation of endogenous Btk from nontransfected HEK293 cells, with a weak but detectable band being detected after Western blotting of separated immunocomplexes with an antibody against endogenous IRAK-1 (Fig. 3b, lane 3). No IRAK-1 was detectable in the IgG control lane (Fig. 3b, lane 1). In contrast, no interaction between Btk and the downstream adapter protein TRAF-6 was detected (Fig. 3c, lane 3), whereas an interaction between TRAF-6 and TAB-1, serving as a positive control, was detected (Fig. 3d, lane 2).
LPS Activates Btk in THP-1 Cells—Our data suggested that Btk might be important for signaling by TLRs. We therefore decided to focus on TLR4 signaling because Btk was found to associate with Mal, an important LPS regulator (15, 17, 18). We first examined the ability of the TLR4 ligand LPS to activate Btk in the human pro-monocytic cell line THP-1. Endogenous Btk was immunoprecipitated from lysates prepared from THP-1 cells stimulated with LPS for various time points. Because activated Btk is phosphorylated on Tyr 551 within the activation loop of the protein (24), immunopurified Btk from each time point was immunoblotted with a phosphotyrosine-specific antibody. Maximal tyrosine phosphorylation of Btk was detected after 5 min of stimulation with LPS (Fig. 4a, top panel). Samples were also blotted for total Btk to ensure equal loading (Fig. 4a, bottom panel).

Phosphorylation of Tyr223 increases the catalytic activity of Btk, and an early substrate for Btk activity is Btk itself, which becomes phosphorylated on Tyr223 (24–27). We therefore tested the ability of immunopurified Btk to become phosphorylated in an in vitro kinase assay, which most likely reflects its autokinase activity. Treatment of THP-1 cells with LPS induced a rapid and transient increase in phosphorylation of Btk, with maximal kinase activity detected 30 min after stimulation (Fig. 4b).

Dominant Negative Btk Inhibits TLR4- and LPS-induced NFkB Activation—The ability of LPS to phosphorylate and activate Btk implies that Btk may play an important role in LPS signaling. To test this, we examined the effect of a kinase inactive form of Btk (Btk(K430R)), which has previously been shown to interfere with Btk signaling (28), on the induction of an NFκB-dependent reporter gene (NFκB-luciferase) by LPS. To do this, we used the transfectable LPS-responsive astrocytoma cell line U373 (Fig. 5a) and the murine monocytic cell line FIG. 2. Btk interacts with MyD88 and Mal. HEK-293 cells (1 × 10^6) were transfected with 4 μg of plasmids expressing wild-type Btk and (a and b) AU1-tagged MyD88 (AU1-MyD88) or (c and d) HA-tagged Mal (HA-Mal) as indicated. Btk was immunoprecipitated using a polyclonal antibody (SC-20, Santa Cruz Biotechnology) recognizing endogenous Btk as indicated, and antibodies recognizing the epitope tags AU1 (Babco) and HA (Santa Cruz Biotechnology) were used to immunoprecipitate AU1-MyD88 and HA-Mal, respectively. Isotype-matched control antibodies were also used in the immunoprecipitates as indicated. The presence of (a and c) Btk, (b) AU1-MyD88, or (d) HA-Mal in the resulting immunocomplex was tested using the relevant antibodies. All results shown are representative of at least three separate experiments.

FIG. 3. Btk interacts with IRAK-1 but not TRAF-6. a, HEK-293 cells (1 × 10^6) were transfected with 4 μg of plasmids expressing wild-type Btk and IRAK-1. Btk was immunoprecipitated from lysates prepared from the cells, and the presence of IRAK-1 in the immunocomplex was tested using an antibody against endogenous IRAK-1. b, Btk was immunoprecipitated from lysates prepared from HEK-293 cells (1 × 10^6), and the resulting immunocomplex was tested for the presence of IRAK-1 by Western blotting. Isotype-matched control antibodies were also used in the immunoprecipitates as indicated. c and d, HEK-293 cells (1 × 10^6) were transfected with 4 μg of plasmids expressing Btk, AU1-TAB-1, or FLAG-tagged TRAF-6 (FLAG-T6) as indicated. After immunoprecipitation of (c) Btk or (d) AU1-TAB-1 from lysates prepared from the cells, the presence of TRAF-6 in the immunocomplex was tested. All results shown are representative of at least three separate experiments.
that the effect of Btk on LPS-induced A13 reduced LPS-induced DNA binding activity of NF/H9260 tested the effect of the Btk-selective kinase inhibitor LFM-A13 on expressed in effect of both Btk(K430R) and an additional mutant form of Btk weaker affinity, suggesting that Btk may play a role in TLR-signaling complex are currently being investigated. Taking the high degree of homology between TIR domain-interacting protein, specifically interacting with TLRs 7, 8, and 9. The precise nature of the downstream target is not clear. We also demonstrate that Btk regulates NFκB activation in response to LPS because inhibitory mutants of Btk and the Btk-specific inhibitor LFM-A13 block NFκB activation by LPS. Interestingly, the inhibitor does not totally inhibit NFκB activation as observed by electrophoretic mobility shift assay, even when used at higher concentrations (data not shown), whereas both mutant forms of Btk completely inhibit the NFκB-dependent reporter gene. This suggests that in addition to its role in regulating DNA binding activity, Btk may also participate in the pathway regulating the transactivation potential of NFκB via phosphorylation. This possibility is strengthened by the fact that Btk can interact with the guanine nucleotide exchange factor, Vav, which regulates the small G protein Rac1, and also the fact that Rac1 lies downstream of Btk on the pathway to c-Jun NH2-terminal kinase activation (36). Rac1 has previously been shown to regulate the transactivation potential of NFκB downstream of TLR domain-containing receptors, namely, the IL-1 receptor complex and TLR2 (37–39).

Taken together, our results therefore show that Btk is a TIR domain-interacting protein, specifically interacting with TLRs 4, 6, 8, and 9. Btk participated in TLR4 signaling to NFκB, and it is possible that it is also involved in signaling by ligands for TLRs 6, 8, and 9. The precise nature of the downstream targets for Btk on the NFκB pathway and its mechanism of recruitment into the TLR-proximal signaling complex are currently under investigation.
**Fig. 5.** Dominant negative Btk inhibits LPS signaling to NFκB via TLR4. 

(a) U373 cells (2.5 x 10⁴) and (b) RAW264.7 cells (5 x 10⁵) were transfected with xB-luciferase (80 ng), Renilla-luciferase (40 ng), and a plasmid expressing Btk(K430R) (50 ng in the case of (a)) as indicated. 18 h after transfection, the cells were stimulated with LPS (1 µg/ml, 6 h) as shown, and luciferase activity was subsequently measured. c and d, HEK293 cells (2.5 x 10⁴) were transfected with xB-luciferase (80 ng) and Renilla-luciferase (40 ng) and plasmids expressing CD4-TLR4 or two mutant forms of Btk as indicated. 18 h after transfection, the cells were stimulated with tumor necrosis factor α (20 ng/ml, 3 h) or left untreated and harvested, and luciferase activity was subsequently measured. Results (mean ± S.D. for triplicate determinations) are represented as fold increase compared with unstimulated controls and are representative of three separate experiments.

e, THP-1 cells (5 x 10⁶) were pretreated with Me₃SO (DMSO) as a control or the Btk-specific inhibitor LFM-A13 as indicated for 1 h. After pretreatment, cells were stimulated with LPS (1 µg/ml) for 2 h as shown, after which time nuclear extracts were prepared, and the DNA binding activity of NFκB was measured by electrophoretic mobility shift assay as outlined in “Materials and Methods.” Results shown are representative of at least three separate experiments.
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