Identification of Phosphorylation Sites for Bruton’s Tyrosine Kinase within the Transcriptional Regulator BAP/TFII-I

Ann Marie Egloff and Stephen Desiderio†

From the Department of Molecular Biology and Genetics and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Bruton’s tyrosine kinase (Btk), a member of the Tec family of cytosolic kinases, is essential for B cell development and function. BAP/TFII-I, a protein implicated in transcriptional regulation, is associated with Btk in B cells and is transiently phosphorylated on tyrosine following B cell receptor engagement. BAP/TFII-I is a substrate for Btk in vitro and is hyperphosphorylated on tyrosine upon coexpression with Btk in mammalian cells. In an effort to understand the physiologic consequences of BAP/TFII-I tyrosine phosphorylation following B cell receptor stimulation, site-directed mutagenesis and phosphopeptide mapping were used to locate the predominant sites of BAP/TFII-I phosphorylation by Btk in vitro. These residues, Tyr248, Tyr357, and Tyr462, were also found to be the major sites for Btk-dependent phosphorylation of BAP/TFII-I in vitro. Residues Tyr357 and Tyr462 are contained within the loop regions of adjacent helix-loop-helix-like repeats within BAP/TFII-I. Mutation of either Tyr248, Tyr357, or Tyr462 to phenylalanine reduced transcription from a c-fos promoter relative to wild-type BAP/TFII-I in transfected COS-7 cells, consistent with the interpretation that phosphorylation at these sites contributes to transcriptional activation. Phosphorylation of BAP/TFII-I by Btk may link engagement of receptors such as surface immunoglobulin to modulation of gene expression.

Peripheral B cells are slightly reduced and skewed toward an immature phenotype. Survival of peripheral B cells is diminished, and the peritoneal B-1 B cell population is absent. Notably, xid mice do not exhibit the block in early B cell development observed in patients with X-linked agammaglobulinemia. Btk null mice exhibit a phenotype resembling xid, suggesting that B cell development has a more stringent requirement for Btk in humans than in mice (7–10).

Btk belongs to the Tec family of cytosolic protein-tyrosine kinases, which includes Btk, Itk, Tec, and Bmx. This group of kinases is related to the Src family by the presence of SH3, SH2, and SH1 (catalytic) domains. It is distinguished from the Src family by 1) the presence of pleckstrin homology and Tec homology domains, which serve as binding sites for phospholipids and SH3 domains, respectively; 2) the absence of an N-terminal myristoylation site; and 3) the lack of a regulatory tyrosine residue near the carboxyl terminus (11–14). An atypical member of the Tec family, Rlk/Txk, lacks a pleckstrin homology domain but has 54–62% amino acid identity to Btk in the remainder of its sequence (15, 16).

Btk has been implicated as important in signaling from the B cell receptor for antigen, the interleukin-5 and -6 receptors, and CD38 on B cells (17–19); the FcεRI receptor on mast cells (20); the FcγRI on macrophages; and the thrombin receptor on platelets (21). Upon engagement of the B cell receptor, Btk is phosphorylated by the tyrosine kinase Lyn at residue Tyr551. This permits Btk to undergo autophosphorylation at residue Tyr722, after which kinase activity is increased (22). The activity of Btk can also be modulated by association with Gqα proteins (23), Gβγ proteins (24), or phosphatidylinositol phosphates (25).

A fraction of Btk coimmunoprecipitates with a protein of ~135 kDa termed BAP (for Btk-associated protein); based on this association, BAP was purified, and its cDNA was molecularly cloned (26). BAP is identical to the putative transcription factor TFII-I (27), which was identified by its ability to stimulate transcription from initiator elements (27) and its synergy with Phox I and serum response factor in enhancing transcription from the c-fos promoter (28).

A distinctive feature of BAP/TFII-I is the occurrence of six helix-loop-helix (HLH)-like repeats. In contrast to typical HLH motifs, however, which contain loop regions of between 6 and 20 amino acids (29), the HLH-like repeats of BAP/TFII-I contain extended loop regions of ~70 amino acids (27). Four isoforms of BAP/TFII-I, generated by alternative splicing of primary RNA transcripts, are differentially expressed in various tissues. Amino acid sequence differences among these four isoforms are confined to the interval between the first and second HLH-like domains (30).2 Two sequence motifs resem-

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† To whom correspondence should be addressed: Dept. of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205. Tel.: 410-955-4735; Fax: 410-955-9124.

‡ To whom correspondence should be addressed: Dept. of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, 725 N. Wolfe St., Baltimore, MD 21205.

The abbreviations used are: Btk, Bruton’s tyrosine kinase; SH1, SH2, and SH3, Src homology 1, 2, and 3, respectively; HLH, helix-loop-helix; MBP, maltose-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; PVDF, polyvinylidene difluoride; TR, thymidine kinase.
blinding the Src kinase autophosphorylation consensus sequence (EDKDY) are present within this interval and are preserved in all four BAP/TFII-I isoforms.

BAP/TFII-I is transiently phosphorylated on tyrosine following B cell receptor engagement, with kinetics that closely follow tyrosine phosphorylation of Btk (26). Increased tyrosine phosphorylation of BAP/TFII-I is also observed upon cotransfection with Btk into fibroblastoid cells. This response is dependent upon Btk kinase activity, since cotransfection with the kinase-inactive mutant Btk (K430E) fails to enhance tyrosine phosphorylation of BAP/TFII-I (26). These observations and the physical association of Btk with BAP/TFII-I in B lymphoid cells (26) suggested that BAP/TFII-I is a physiologic substrate for phosphorylation by Btk following B cell receptor stimulation.

Several lines of evidence suggest that phosphorylation modulates the ability of BAP/TFII-I to stimulate transcription. First, dephosphorylation impairs the ability of BAP/TFII-I to stimulate transcription from a Vβ promoter in vitro, while sparing its ability to bind the Vβ initiator element (31). Second, stimulation of Vβ transcription by BAP/TFII-I in transfected cells is enhanced by wild-type Btk but not by a kinase-inactive mutant (32). Third, the transcripional activity of BAP/TFII-I in transfected cells is impaired by mutation of a site conforming to the Src autophosphorylation consensus (31). Fourth, epidermal growth factor, which enhances c-fos promoter activity, induces tyrosine phosphorylation of BAP/TFII-I (26). These observations and the identity of c-Myc epitope. Tyrosine to phenylalanine point mutations were introduced at Tyr248, Tyr357, or Tyr462 to phenylalanine, consistent with the "c-fos" promoter was cloned into the HindIII site upstream of the firefly lucerase gene within the Promega PGL3 basic vector, and orientation was confirmed by sequence analysis. The herpes simplex thymidine kinase Renilla vector (pRL-TK) was purchased from Promega.

The human embryonic kidney fibroblast cell line 293 and the simian fibroblast cell line COS-7 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 μg/ml streptomycin, and 50 units/ml penicillin. Purification of Recombinant Btk and Btk(K430E)—E. coli BL21, transformed with either pMal-C2Btk or pMal-C2Bke, was cultured overnight at 37 °C in 50 ml of rich medium containing 2 g/liter glucose and 50 μg/ml carbenicillin. The overnight cultures were diluted 1:20 in the same medium and grown at 37 °C until A_{600} was 0.5. Expression was then induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 0.3 mM, and incubation was continued for 3 h at 37 °C. Cells were collected by centrifugation and resuspended in 25 ml of buffer F (50 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin, and 2 μg/ml antipain) per 500-ml culture. Lysozyme and phenylmethylsulfonyl fluoride were added to 1 mg/ml and 1 mM, respectively. After incubation for 30 min on ice, bacterial suspensions were frozen in liquid nitrogen and stored at −80 °C.

MBP-Btk and MBP-Btk(K430E) fusion proteins were purified by amylose affinity chromatography (New England Bioslabs). Frozen bacterial suspensions were thawed in a 30 °C water bath with gentle agitation and then placed on ice. Ice-cold EDTA was added to a final concentration of 2 mM, and EGTA was added for a final concentration of 10 mM. After mixing by inversion, the lysate was sonicated for three cycles of 10 pulses each with a Branson microtip sonifier (power output 5, duty cycle 50%). An equal volume (25 ml) of lysate dilution buffer (300 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.2 mM dithiothreitol, 2% Nonidet P-40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin, and 2 μg/ml antipain) was added to 25 ml of bacterial suspension. Suspensions were mixed by inversion, incubated on ice for 20 min, and then clarified by centrifugation at 30,000 × g for 20 min at 4 °C. The supernatant was loaded twice over a 2-ml amylose resin, pre-equilibrated with wash buffer 1 (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM EDTA, 0.1 mM dithiothreitol, 1% Nonidet P-40, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 2 μg/ml chymostatin, 2 μg/ml pepstatin, and 2 μg/ml antipain). The column was washed sequentially with 10 ml of wash buffer 1, 10 ml of wash buffer 2 supplemented to 1 mM NaCl, and 10 ml of wash buffer 2 (100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.1 mM dithiothreitol, 10% glycerol). Bound protein was eluted with 3 ml of elution buffer (100 mM NaCl, 50 mM Tris-HCl (pH 7.4), 10 mM malate, 0.1 mM dithiothreitol, and 10% glycerol). Eluates were dialyzed against elution buffer lacking malate, frozen in liquid nitrogen, and stored at −80 °C.

Expression and Purification of Polyhistidine-tagged BAP/TFII-I Fragments—Wild-type and mutant BAP/TFII-I fragments, tagged at the amino terminus with polyhistidine, were expressed from the BAP pBAD HIS constructs in E. coli TOP10 (Invitrogen). Bacterial cultures were grown overnight at 37 °C in 2× YT containing 50 μg/ml carbenicillin, diluted 1:10 in the same medium, and incubated for 1 h at 37 °C. Expression was induced by the addition of arabinose to 0.002% (v/v) and further incubation for 4 h at 37 °C. Cells were collected by centrifugation and resuspended in buffer F (25 ml per 500-ml culture). After the addition of lysozyme to 1 mg/ml and phenylmethylsulfonyl fluoride to 1 mM, bacterial suspensions were incubated on ice for 30 min, frozen in liquid nitrogen, and stored at −80 °C.

Bacterial suspensions were thawed with gentle agitation in a 30 °C water bath and placed on ice immediately upon thawing. Samples were sonicated for three cycles of 10 pulses each with a Branson microtip sonifier (power output 5, duty cycle 50%). One volume of 2× imidazole wash buffer (500 mM NaCl, 100 mM Tris-HCl (pH 7.4) and 40 mM
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**RESULTS**

Expression and Purification of BAP/TFII-I and BAP/TFII-I Fragments—The overall strategy used in these phosphorylation site mapping studies was 1) to determine which portions of BAP/TFII-I are phosphorylated by Btk in vitro; 2) to define the specific tyrosine residues phosphorylated by Btk in vitro by a combination of phosphopeptide mapping and site-directed mutagenesis; and 3) to verify that the same tyrosine residues are hyperphosphorylated in vivo upon coexpression of BAP/TFII-I with active Btk.

The shortest BAP/TFII-I isoform, BAP/TFII-I A, contains all 24 tyrosine residues present in the larger isoforms (Fig. 1A). This isoform and its derivative polypeptide fragments were therefore used for in vitro phosphorylation site mapping. The following BAP/TFII-I protein fragments, fused at the amino terminus to polyhistidine and the carboxyl terminus to a c-Myc epitope, were expressed in bacterial cells and purified by Ni²⁺-nitritolactiic acid affinity chromatography (Fig. 1B): BAP...
counting, is plotted as a function of [Me$^2$]Btk and MBP-Btk(K430E) preparations were incubated with cation substrate BAP N-2R. Increasing amounts of the MBP-Btk, a kinase-inactive mutant, was prepared in parallel. Fusion proteins were assayed for autokinase activity in vitro. 2 µl (lanes 1 and 4), 5 µl (lanes 2 and 5), or 10 µl (lanes 3 and 6) of MBP-Btk (lanes 1–3) or MBP-Btk(K430E) (lanes 4–6) were incubated with 10 µCi of [$\gamma$-$^{32}$P]ATP for 30 min at 37 °C in a buffer containing 20 mM Tris-HCl (pH 7.2), 2 mM MgCl$_2$, and 10 mM MnCl$_2$. Products were fractionated by SDS-PAGE and detected by PhosphorImager (left panel) or silver stain (right panel). The arrows indicate the position of MBP-Btk and MBP-Btk(K430E). The product of a contaminating bacterial protein kinase is marked by an asterisk; B, phosphorylation of the BAP/TFII-I N-2R fragment by Btk in vitro. MBP-Btk (10 µl, lanes 1–3), MBP-Bke (10 µl, lanes 5–7), or kinase dialysis buffer (10 µl, lane 4) was combined with 10 µl (lanes 1, 4, and 5) or 2 µl (lanes 2 and 6) BAP/TFII-I N-2R. BAP/TFII-I N-2R was omitted from reactions analyzed in lanes 3 and 7. Reaction conditions were identical to those in A. Following fractionation by SDS-PAGE, protein was transferred to PVDF and treated with 1 N KOH for 2 h at 55 °C. Radiolabeled protein was detected by PhosphorImager. The positions of MBP-Btk and BAP/TFII-I N-2R are indicated by arrows. A contaminating phosphoprotein is indicated by an asterisk. C, metal dependence of MBP-Btk activity. MBP-Btk (10 µl, lanes 1–5 and 11–15) or MBP-Bke (10 µl, lanes 6–10 and 16–19) was incubated with 4 µl of [Me$^2$]$^+$ in 20 mM Tris-HCl (pH 7.2), 1 mM Na$_2$MoO$_4$, 10 µCi of [$\gamma$-$^{32}$P]ATP, and the indicated concentrations of 100:1 manganese/magnesium (lanes 1–10) or manganese alone (lanes 11–19). Protein was fractionated by SDS-PAGE, transferred to PVDF, and stained with Ponceau S. Radiolabeled protein was detected by PhosphorImager (left panel). The amount of radiolabel incorporated into BAP/TFII-I N-2R, as quantitated by scintillation counting, is plotted as a function of [Me$^2$]$^+$ (right panel).

N-3R (residues 1–503), BAP-N-2R (residues 1–398), BAP-N-NR2 (residues 1–303), BAP-N-1R (residues 1–189), BAP-4R-CT (residues 514–957), and BAP 5R-CT (residues 675–957). Wild-type BAP/TFII-I, tagged with polyhistidine and a c-Myc epitope, was expressed and purified in parallel. Expression and Purification of Btk—To define the sites at which Btk phosphorylates BAP/TFII-I in vitro, it was essential to obtain active Btk in a form free of contaminating tyrosine kinases. Btk immunoprecipitated from mammalian cells was unsatisfactory for this purpose, since pilot experiments with a kinase-inactive mutant Btk revealed the presence of additional tyrosine kinase activities in such preparations. Recovery of kinase-inactive mutant Btk revealed the presence of additional contaminating bacterial kinase. Btk phosphorylates BAP/TFII-I (Fig. 2A, lanes 1–3 and 7–9); in reactions containing similar amounts of the kinase-inactive mutant, the corresponding fusion protein was phosphorylated at levels at least 3.6-fold lower (Fig. 2A, lanes 4–6 and 10–12). A prominent 75-kDa phosphorylated species was observed in all reactions. This protein is of bacterial origin, since it is also present in Ni$_2$-nitrilotriacetic acid affinity preparations of BAP/TFII-I fragments (Fig. 2B, lane 4). Phosphorylation of this protein and the trace phosphorylation of BAP/TFII-I N-2R. BAP/TFII-I N-2R was phosphorylated in reactions containing wild-type Btk in reactions containing wild-type protein (Fig. 2A, lanes 1–3 and 7–9); in reactions containing similar amounts of the kinase-inactive mutant, the corresponding fusion protein was phosphorylated at levels at least 3.6-fold lower (Fig. 2A, lanes 4–6 and 10–12). A prominent 75-kDa phosphorylated species was observed in all reactions. This protein is of bacterial origin, since it is also present in Ni$_2$-nitrilotriacetic acid affinity preparations of BAP/TFII-I fragments (Fig. 2B, lane 4). Phosphorylation of this protein and the trace phosphorylation of BAP/TFII-I N-2R. BAP/TFII-I N-2R was phosphorylated in reactions containing wild-type protein (Fig. 2A, lanes 1–3 and 7–9); in reactions containing similar amounts of the kinase-inactive mutant, the corresponding fusion protein was phosphorylated at levels at least 3.6-fold lower (Fig. 2A, lanes 4–6 and 10–12). A prominent 75-kDa phosphorylated species was observed in all reactions. This protein is of bacterial origin, since it is also present in Ni$_2$-nitrilotriacetic acid affinity preparations of BAP/TFII-I fragments (Fig. 2B, lane 4). Phosphorylation of this protein and the trace phosphorylation of BAP/TFII-I N-2R.
the divalent cation dependence of MBP-Btk in vitro was optimized. Autophosphorylation and phosphorylation of the BAP N-2R fragment were assessed at various concentrations of Mg$^{2+}$ or Mn$^{2+}$, alone or in combination (Fig. 2, C). Reaction products were resolved by polyacrylamide gel electrophoresis, transferred to PVDF membrane, and visualized by PhosphorImager (Fig. 2C, left panel). As observed above, MBP-Btk exhibited autokinase activity as well as activity against BAP N-2R (Fig. 2C, lanes 1–5 and 11–15), while MBP-Btk(K430E) exhibited neither activity (Fig. 2C, lanes 6–10 and 15–19). Membrane slices containing BAP N-2R, visualized by staining with Ponceau S, were excised, and $^{32}$P was quantitated (Fig. 2C, right panel). MBP-Btk was most active in reactions containing 2 mM Mn$^{2+}$, and these conditions were used in all subsequent in vitro reactions.

Localization of Btk Phosphorylation Sites within BAP/TFII-I—Similar amounts of purified, epitope-tagged BAP/TFII-I truncation mutants, as assessed by immunoblotting with an anti-Myc antibody (Fig. 3A), or BAP/TFII-I by Site-directed Mutagenesis—Site-directed mutagenesis was used to identify the specific tyrosine residues targeted for phosphorylation by Btk in each of the three intervals identified in the previous experiments. There are four tyrosine residues, Tyr$^{248}$, Tyr$^{249}$, Tyr$^{251}$, and Tyr$^{257}$, within the second nonrepeat region (residues 190–303) of BAP/TFII-I. Simultaneous mutation of these tyrosine residues to phenylalanine in BAP N-2R and BAP N-3R (BAP N-2R(4F) and BAP N-3R(4F)) specifically eliminated phosphopeptides previously mapped to the second nonrepeat region (Fig. 4B, compare lanes 3 and 5 with lane 6). Thus, at least one of these four tyrosine residues is a target for phosphorylation by Btk in vitro. Tryptic phosphopeptides derived from outside of the second nonrepeat region of BAP/TFII-I were unaffected by these point mutations. Phosphorylation of other sites within BAP/TFII-I by Btk is therefore independent of phosphorylation in the 190–303 interval.

To determine which of the four tyrosines in the second nonrepeat region are phosphorylated by Btk in vitro, full-length, 
and their effects on tryptic phosphopeptide patterns were analyzed (Fig. 5B). In addition, the double Y346F, Y357F mutant was analyzed because these residues lie within a single tryptic fragment. The Y357F mutation was sufficient to eliminate the two predominant phosphopeptides present in the wild-type substrate (Fig. 5B, compare lanes 4 and 1). Mutation of Tyr246 had no additional effect (Fig. 5B, lane 5). The phosphopeptide pattern was unchanged by individual mutations at Tyr322, Tyr346, or Tyr373. We conclude that Tyr246 (Fig. 5D) is phosphorylated by Btk in vitro.

Similar mutational analysis revealed that residue Tyr462, which lies within the third HLH-like repeat, is phosphorylated in vitro by Btk, because a Y462F mutation, in the context of full-length BAP/TFII-I, eliminated both of the tryptic phosphopeptide fragments specific to this region (Fig. 5C, compare lane 3 with lanes 6 and 7; relevant peptides are marked by closed arrows). Mutation of residues Tyr419 and Tyr478 did not alter the tryptic phosphopeptide pattern, relative to that of the wild-type protein (Fig. 5C, lanes 2 and 5). Moreover, the phosphopeptide pattern of the Y451F, Y462F double mutant was identical to that of the Y462F single mutant. Thus Tyr462 (Fig. 5D) is the predominant site of in vitro phosphorylation by Btk within the third HLH-like region of BAP/TFII-I.

To determine the contribution of Btk phosphorylation of BAP/TFII-I at the identified sites relative to total phosphorylation by Btk in vitro, BAP/TFII-I full-length protein bearing phenylalanine mutations at residues Tyr246, Tyr357, and Tyr462 (BAP/TFII-I(3YF)) was expressed in E. coli and purified in parallel with the wild-type BAP/TFII-I protein (Fig. 5D). BAP/TFII-I(3YF) was phosphorylated to a substantially lesser extent than the wild-type protein in vitro kinase reactions performed in duplicate with MBP-Btk; control reactions were run in the presence of kinase-defective MBP-Btk(K430E) (Fig. 5E). Quantitation of phosphorylated products (Fig. 5F) indicated that the majority of Btk-specific phosphorylation was eliminated by the mutation of Tyr246, Tyr357, and Tyr462 to phenylalanine. The Btk-specific phosphorylation remaining in BAP/TFII-I(3YF) is consistent with data presented in Fig. 4A, which indicated that at least one site for Btk phosphorylation resides outside of the N-3R region of BAP/TFII-I.

Correspondence between Phosphorylation Sites in Vitro and in Vivo—We next wished to determine whether the phosphorylation sites defined in vitro correspond to sites at which BAP/TFII-I is hyperphosphorylated in vivo upon cotransfection of Btk. Epitope-tagged BAP/TFII-I or the BAP N-3R fragment was expressed in 293 cells with cotransfected Btk or kinase-inactive Btk(K430E). Protein was labeled metabolically with 32P and recovered by immunoprecipitation with an anti-c-Myc antibody. Immunoprecipitates were fractionated by polyacrylamide gel electrophoresis, and radiolabeled BAP/TFII-I species were isolated. Phosphoamino acid analysis of gel-purified BAP/TFII-I revealed the presence of radiolabeled phosphoryrosine upon coexpression with active Btk but not with Btk(K430E) (data not shown). Tryptic digestion of BAP N-3R, labeled in vivo, revealed that a subset of peptides were preferentially phosphorylated in the presence of Btk, relative to Btk(K430E) (Fig. 6A, compare lanes 2 and 3). The majority of these phosphorylated peptides corresponded to tryptic fragments phosphorylated by Btk in vitro (Fig. 6A, lane 1; relevant peptides are marked by arrows), consistent with the interpretation that they contain target sites for direct phosphorylation by Btk in vivo.

The major sites at which BAP/TFII-I is hyperphosphorylated upon cotransfection with Btk were determined by site-directed mutagenesis (Figs. 6, B and C). A Btk-enhanced, in vivo labeled phosphopeptide from BAP N-3R was absent in the BAP N-3R(4F) mutant (Fig. 6B, compare lanes 1 and 3; the relevant
peptide is marked by the open arrow), consistent with identification of Tyr248 as a target site for Btk in vitro. A pair of Btk-dependent radiolabeled phosphopeptides (Fig. 6B, lanes 1 and 2, closed circles) are absent in the BAP N-3R(4F) mutant and its derivatives (Fig. 6B, lanes 3–6), indicating that one or more of the four tyrosines in the second nonrepeat region of BAP/TFII-I is a target for phosphorylation by an endogenous tyrosine kinase other than Btk. Additional mutation of Tyr451 and Tyr462 to phenylalanine eliminated a Btk-dependent phosphopeptide (Fig. 6B, closed arrow), consistent with the identification of Tyr462 as a target for Btk phosphorylation in vitro. Last, examination of the in vivo phosphopeptide pattern from full-length BAP/TFII-I revealed that a Btk-dependent species was absent from BAP/TFII-I(Y357F) but unperturbed by mutations at Tyr248, Tyr451, or Tyr462 (Fig. 6C). Thus, residue Tyr357 of BAP/TFII-I is hyperphosphorylated in the presence of cotransfected Btk in vivo.

If residues Tyr248, Tyr357, and Tyr462 are indeed major sites of Btk phosphorylation within BAP/TFII-I in vivo, then BAP/TFII-I(Y387F), in which these residues are mutated, should exhibit reduced Btk-dependent tyrosine phosphorylation in transfected cells. BAP/TFII-I N-3R became hyperphosphorylated on tyrosine when coexpressed with Btk in 293 cells (Fig. 6D, compare lanes 1 and 2). This is in contrast to the mutant BAP/TFII-I N-3R(3YF), which showed no increase in tyrosine phosphorylation when coexpressed with Btk (Fig. 6D, compare lanes 3 and 4). Quantitation indicated that tyrosine phosphorylation of unmutated BAP N-3R increased at least 2-fold in the presence of Btk, while there was no discernible Btk-dependent tyrosine phosphorylation of the 3YF mutant (Fig. 6E). We conclude that the major sites at which BAP/TFII-I is phosphorylated in mammalian cells upon cotransfection with Btk correspond to the predominant targets of phosphorylation by Btk in vitro.

To assess the functional significance of phosphorylation at residues Tyr248, Tyr357, and Tyr462, we examined the transcriptional activity of wild-type and phenylalanine-substituted BAP/TFII-I on the c-fos promoter, which is responsive to BAP/TFII-I (32, 33). Transfection of COS-7 cells with wild-type BAP/TFII-I stimulated expression of the c-fos luciferase reporter gene relative to the vector-transfected control (Fig. 7A). Mutation of Tyr248, Tyr357, or Tyr462 individually to phenylalanine impaired the ability of BAP/TFII-I to stimulate c-fos activity. Interestingly, cotransfection of BAP/TFII-I(Y248F) suppressed expression from the c-fos promoter, relative to the samples cotransfected with vector alone, suggesting that this mutant may have repressive activity. The BAP/TFII-I(Y357F) triple mutant also displayed reduced activity relative to wild-
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FIG. 6. Btk phosphorylates BAP at similar positions in vitro and in vivo. A, comparison of tryptic phosphopeptides from BAP/TFII-I N-3R, phosphorylated in vitro by Btk or in vivo upon cotransfection with Btk. Lane 1, tryptic phosphopeptides from BAP/TFII-I N-3R, phosphorylated in vitro by MBP-Btk. Lanes 2 and 3, tryptic phosphopeptides from BAP/TFII-I, labeled metabolically in 293 cells cotransfected with Btk (lane 2) or Btk(K430E) (lane 3). Tryptic phosphopeptides from metabolically labeled BAP/TFII-I N-3R that are enhanced by co-expression of Btk (lane 2) relative to co-expression with Btk/K430E (lane 3) correspond to phosphopeptides derived from BAP/TFII-I phosphorylated by Btk in vitro (closed arrows). B, effects of point mutations within the second nonrepeat or third HLH-like region on phosphorylation of BAP/TFII-I N-3R in vitro. Wild-type (lanes 1 and 2) or mutant (lanes 3-6) BAP/TFII-I N-3R was co-expressed with Btk (lanes 1, 3, 4, 5, and 6) or Btk(K430E) (lane 2), labeled metabolically, and analyzed by one-dimensional tryptic phosphopeptide mapping. Mutations in the second nonrepeat region (NR2) or the third HLH-like domain (3R) are identified at the top. 4F, phenylalanine substitutions at Tyr248, Tyr357, and Tyr277. wt, wild-type. Btk-dependent phosphopeptides are marked by arrows. Closed circles, Btk-independent phosphopeptides eliminated by the 4F mutation; open arrow, Btk-dependent phosphopeptide eliminated by the 4F mutation; filled arrow, Btk-dependent phosphopeptide eliminated by the Y415F, Y462F mutation. C, tyrosine 357 of BAP/TFII-I is a target for Btk-dependent phosphorylation in vivo. Full-length, wild-type (lanes 5 and 6) or mutant (lanes 1-4 and 7-10) BAP/TFII-I was coexpressed with Btk (Btk, lanes 1-5) or Btk(K430E) (Bke, lanes 6-10) in 293 cells, labeled metabolically with 32P, and analyzed by one-dimensional tryptic phosphopeptide mapping. Mutants (Y248F, Y277F, Y357F, and Y462F) are identified. A Btk-dependent tryptic phosphopeptide is eliminated by the Y357F mutation (arrow). D, hyperphosphorylation of BAP/TFII-I N-3R on tyrosine in the presence of coexpressed Btk is eliminated in the BAP/TFII-I(Y248F, Y357F, Y462F) N-3R mutant. Myc-tagged, BAP/TFII-I N-3R fragment (lanes 1 and 2) or the mutant BAP/TFII-I(Y248F, Y357F, Y462F) N-3R fragment (lanes 3 and 4) were coexpressed in 293 cells with Btk (lanes 1 and 3) or Btk(K430E) (lanes 2 and 4). BAP/TFII-I N-3R protein was immunoprecipitated using the anti-Myc antibody 9E10. Following immunoprecipitation, gel fractionation, and transfer to membrane, immunoblotting was performed with the anti-phosphotyrosine antibody 4G10 (upper panel) or the anti-Myc antibody 9E10 (lower panel). E, densitometric quantitation of the anti-phosphotyrosine immunoblot of D.

FIG. 7. Mutation of Btk phosphorylation sites impairs BAP/TFII-I activity at the c-fos promoter in vivo. A, involvement of BAP/TFII-I residues Tyr248, Tyr357, and Tyr462 in Btk-independent activation of the c-fos promoter. COS-7 cells were transfected with 0.1 μg of c-fos in PGL3, 0.1 μg of pRL-TK, and 0.6 μg of vector, wild-type BAP/TFII-I, or mutant BAP/TFII-I, as indicated. Following serum stimulation, cells were assayed for c-fos and TK transcriptional activity. The activity of the c-fos promoter was analyzed relative to that of TK. Means and S.E. of three independent transfections are plotted. B, evaluation of BAP/TFII-I expression in transfected COS-7 cells. Equal volumes of cell lysate from the experiment in A were fractionated by SDS-polyacylamide gel electrophoresis and immunoblotted with the anti-Myc antibody 9E10. C, BAP/TFII-I residues Tyr248 and Tyr357 are implicated in Btk-dependent activation of a c-fos reporter construct in vivo. COS-7 cells were transfected with 0.1 μg of c-fos in PGL3; 0.1 μg of pRL-TK; 0.3 μg of Btk (Bbk) or Btk(K430E) (Bke); and 0.4 μg of wild-type BAP/TFII-I, mutant BAP/TFII-I, or vector DNA. Following serum stimulation, cells were assayed for c-fos and TK promoter activities. The activity of the c-fos promoter was analyzed relative to that of TK. Means and S.E. of three independent transfections are plotted. D, alignment of amino acid sequences in the vicinity of BAP/TFII-I tyrosine residues phosphorylated in vitro by MBP-Btk and the corresponding amino acid sequence of a previously described Btk autophosphorylation site (22).
Btk/TFII-I activity in this assay.

Btk has been shown to enhance the transcriptional activity of BAP/TFII-I at the c-fos promoter in fibroblastoid cell lines (32). In experiments presented above, transfection was performed under conditions that maximized the effect of BAP/TFII-I on transcription from the c-fos promoter. Under these conditions, mutation of Btk phosphorylation sites in BAP/TFII-I reduced the stimulatory effect of BAP/TFII-I. We next wanted to determine the effects of the same mutations on enhancement of BAP/TFII-I activity by Btk. These experiments were performed using transfection conditions in which stimulation of c-fos activity was dependent on the presence of both BAP/TFII-I and Btk (see “Materials and Methods”); transfection of BAP/TFII-I in the absence of active Btk, or of Btk in the absence of BAP/TFII-I, had no effect on c-fos promoter activity (Fig. 7C). Mutation of Tyr248, Tyr357, and Tyr462 to phenylalanine, singly or in combination, impaired (Fig. 7C, Y462F and 3YF) or eliminated (Fig. 7C, Y248F and Y357F) stimulation of c-fos promoter activity by Btk and BAP/TFII-I. The BAP/TFII-I(Y248F) mutant, moreover, consistently suppressed basal expression of the c-fos reporter construct as well (Fig. 7C). These results are consistent with roles for residues Tyr248 and Tyr357, and perhaps also for Tyr462, in mediating synergistic effects of BAP/TFII-I and Btk at the c-fos promoter.

**DISCUSSION**

While the participation of Btk in signaling pathways essential to B cell development and activation is clear, relatively little is known concerning the targets of this kinase and the consequences of their phosphorylation. BAP/TFII-I, phospholipase Cγ, protein kinase Cβ, and Wiskott-Aldrich syndrome protein have all been identified as possible Btk substrates in vivo (26, 41–43). Until now, however, direct phosphorylation of these substrates by Btk has not been documented. Results presented in this communication suggest strongly that Btk is capable of phosphorylating BAP/TFII-I directly, thereby providing one means by which Btk activation may mediate cellular responses to antigen receptor engagement.

Several lines of evidence support this conclusion. First, BAP/TFII-I is a substrate for bacterially expressed, purified Btk in vitro. Second, there exists a correspondence between tryptic phosphopeptides from BAP/TFII-I phosphorylated by Btk in vitro and Btk-dependent tryptic phosphopeptides from BAP/TFII-I expressed in mammalian cells. Third, mutation of BAP/TFII-I at tyrosine residues phosphorylated by Btk in vitro eliminated Btk-dependent phosphopeptides from metabolically labeled BAP/TFII-I.

We have relied on deletion and point mutagenesis to locate sites of protein phosphorylation. In pursuing this approach, we considered the possibility that truncation might permit phosphorylation at sites that are inaccessible in the intact protein or that a point mutation might indirectly impair phosphorylation at a different residue. In the studies presented here, however, the predominant patterns of phosphorylation within individual BAP/TFII-I fragments were recapitulated in the full-length protein, arguing that truncation did not generate artifactual phosphorylation sites. Moreover, within each phosphorylated region of BAP/TFII-I, as defined by nested truncations, mutation of a single tyrosine residue affected phosphorylation, eliminating only phosphopeptides mapping to that region. Taken together, these observations are consistent with the interpretation that tyrosine residues 248, 357, and 462 of BAP/TFII-I represent sites of direct phosphorylation by Btk. We note, however, that identification of phosphorylation sites by the methods employed here is limited by the stoichiometry of phosphorylation and, in the case of metabolic labeling, by the flux of phosphate through tyrosine. Thus, we cannot exclude the existence of additional Btk target sites within BAP/TFII-I.

The substrate specificities of Btk and other tyrosine kinases of the Tec family are as yet poorly defined. Of the three residues of BAP/TFII-I shown here to be phosphorylated by Btk (Fig. 7D), two (Tyr357 and Tyr462) occur in similar sequence contexts (nine identities among the 11 residues from the −5-position through the +5-position) within the loop regions of consecutive HLH-like domains. The context of Tyr248 is distinct, differing from the corresponding sequence surrounding Tyr357 in all of the 11 residues from −5 through +5, except the target tyrosine. The contexts of all three Btk phosphorylation sites in BAP/TFII-I differ substantially from that of the Btk autophosphorylation site at Tyr225 (22). Thus, any features of primary sequence that might confer specificity for Btk remain obscure.

BAP/TFII-I is able to associate with Btk and other kinases in vivo (26, 44), and Btk has a modest stimulatory effect on transcriptional activation by BAP/TFII-I in transfected cells (32). These observations as well as an apparent requirement for phosphorylation of BAP/TFII-I in transcriptional assays in vitro (31) have suggested that Btk and perhaps other kinases may regulate the activity of BAP/TFII-I. In support of this possibility, a BAP/TFII-I mutant carrying phenylalanine substitutions at both Tyr248 and Tyr249 exhibited impaired transcriptional activity in transfected cells (32). Although the Btk dependence of BAP/TFII-I activity was not addressed in that study, one interpretation is suggested by our identification of Tyr248 as a target site for phosphorylation of BAP/TFII-I. The effect of the mutations at Tyr248 and Tyr249 on transcription, however, would also be consistent with phosphorylation at Tyr248 by a kinase other than Btk, including members of the Tec family that are more broadly expressed. Consistent with this notion, we have observed that overexpression of the BAP/TFII-I(Y248F) mutant consistently impairs basal expression from a transfected c-fos reporter construct (Fig. 7A). Our findings suggest that two other Btk targets within BAP/TFII-I, residues Tyr357 and Tyr462, may be phosphorylated by more broadly expressed kinases as well, because phenylalanine substitutions at these sites also impair Btk-independent transcription from the c-fos promoter (Fig. 7A).

Our studies implicate phosphorylation of BAP/TFII-I at residues Tyr248 and Tyr357, and perhaps at Tyr462, in Btk-dependent stimulation of transcription from the c-fos promoter. Interestingly, the debilitating effects of these mutations were not additive. This suggests the existence of one or more functional interactions among these three sites; an understanding of these interactions may warrant further study.

Two of the Btk phosphorylation sites within BAP/TFII-I occur within loop regions of helix-loop-helix-like domains. This suggests that these loop domains are available for modification by phosphorylation and perhaps for interactions with other proteins. BAP/TFII-I promotes the formation of a ternary complex involving serum response factor, Phox1 and the c-fos promoter (28). On this basis, BAP/TFII-I has been proposed to act as a scaffold that assists in the assembly of multicomponent transcription complexes (28, 33). Phosphorylation of BAP/TFII-I, in particular within the loop regions of HLH-like domains, may facilitate the construction of such complexes, perhaps through the creation of binding sites for specific proteins.

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Ann Marie Egloff and Stephen Desiderio

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