Reconstitution of Btk Signaling by the Atypical Tec Family Tyrosine Kinases Bmx and Txk*

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Bruton’s tyrosine kinase (Btk) is mutated in X-linked agammaglobulinemia patients and plays an essential role in B cell receptor signal transduction. Btk is a member of the Tec family of nonreceptor protein-tyrosine kinases that includes Bmx, Itk, Tec, and Txk. Cell lines deficient for Btk are impaired in phospholipase C-γ2 (PLCγ2)-dependent signaling. Itk and Tec have recently been shown to reconstitute PLCγ2-dependent signaling in Btk-deficient human cells, but it is not known whether the atypical Tec family members, Bmx and Txk, can reconstitute function. Here we reconstitute Btk-deficient DT40 B cells with Bmx and Txk to compare their function with other Tec kinases. We show that in common with Itk and Tec, Bmx reconstituted PLCγ2-dependent responses including calcium mobilization, extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) activation, and apoptosis. Txk also restored PLCγ2 calcium signaling but, unlike other Tec kinases, functioned in a phosphatidylinositol 3-kinase (PI 3-kinase)-independent manner and failed to reconstitute apoptosis. These results are consistent with a common role for Tec kinases as amplifiers of PLCγ2-dependent signal transduction, but suggest that the pleckstrin homology domain of Tec kinases, absent in Txk, is essential for apoptosis.

Bruton’s tyrosine kinase (Btk)1 is a member of the Tec family of nonreceptor protein-tyrosine kinases (PTKs) that includes Bmx, Itk, Tec, and Txk (reviewed in Ref. 1). Tec family PTKs are characterized by an NH2-terminal pleckstrin homology (PH) domain followed by a Tec homology domain (containing a Zn2+-binding Btk motif and a proline-rich region), a Src-homology 3 (SH3) and SH2 domain, and a COOH-terminal kinase domain (1, 2) (Fig. 1). Tec PTKs share 50–60% amino acid sequence identity and are predominately expressed by cells of the immune system (1). Bmx and Txk are somewhat atypical family members: Bmx shares little sequence homology with other Tecs within the proline-rich and SH3 domains; Txk is particularly atypical at the NH2 terminus, lacking the PH and Tec homology domains (Fig. 1).

Btk, the prototypical Tec family PTK, was first identified in B cells but is also expressed in myelomonocytic and mast cells (1). An essential role for Btk in signal transduction through the B cell antigen receptor (BCR) was revealed when mutations in Btk were shown to be responsible for a human disease termed X-linked agammaglobulinemia and for X-linked immunodeficiency in the xid mouse (reviewed in Ref. 3). Agammaglobulinemia patients suffer an intrinsic defect in pre-B to B cell development, which results in a lack of mature B cells (4). xid and btk-null mice share a similar, though less severe, phenotype (5, 6). Btk was subsequently shown to be activated in B cells by a two-step mechanism involving Lyn and PI 3-kinase. After BCR cross-linking, Lyn activates Btk by transphosphorylation (7–9), which leads to Btk autophosphorylation (10, 11). The PI 3-kinase-dependent step involves Btk membrane-targeting by PH domain engagement with phosphatidylinositol 3,4,5-trisphosphate (PIP3) (12, 13). Btk activity can be down-regulated by the SH2-containing inositol phosphatase (SHIP), which dephosphorylates PIP3 (14, 15).

An effector function for Btk was identified in experiments that used the DT40 chicken B cell system for gene knockouts. Btk-deficient DT40 cells exhibited a reduction in the level of phosphatidylinositol C (PLC)-γ2 phosphorylation upon BCR cross-linking and a consequent failure to mobilize calcium and generate inositol 3,4,5-trisphosphate (16). In addition, the downstream apoptotic response of DT40 cells after BCR cross-linking was impaired in Btk-deficient cells (17). Analyses of Syk-deficient DT40 cells has shown this PTK also to be crucial in phospholipase-dependent signaling (18). A model has been proposed in which the concerted actions of Btk and Syk in phosphorylating PLCγ2 are essential for BCR signal transduction (reviewed in Refs. 3, 19–21).

The considerable structural homology exhibited among Tec family members suggests that, similar to Src family kinases (22), these proteins may have overlapping functions. A recent report has shown that Itk and Tec can reconstitute Btk responses including BCR-dependent PLCγ2 phosphorylation and calcium mobilization (23). This study examined proximal signaling events and did not analyze the capacity of Tec kinases to restore Btk-induced downstream responses including ERK MAPK activation and apoptosis (23). Moreover, the roles of the atypical Tec family PTKs, Bmx and Txk, have not been examined. Here we reconstitute Btk-deficient DT40 cells with Bmx and Txk, in addition to Itk and Tec. We confirm the role of Tec
kinases in phospholipase-dependent signaling (23) and extend our study to show reconstitution of downstream ERK MAPK activation and apoptotic responses. We find that Bmx can reconstitute Btk signaling, whereas Txk, although capable of restoring PLCγ2 signaling, is unable to reconstitute apoptosis and functions independently of PI 3-kinase.

EXPERIMENTAL PROCEDURES

Cells, Antibodies, and Biochemical Analyses

Cells—DT40 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1% chicken serum (Sigma), penicillin, streptomycin, glutamine, and 50 μg/ml mercaptoethanol. DT40 cell lines rendered deficient for btk and plcγ2 by homologous recombination were described previously (16, 24).

Antibodies—Anti-chicken mAb M4 (18), anti-HA mAb 12CA5 (25), anti-phosphotyrosine mAb 5H1 (25), and antisera generated against Btk, and anti-HA mAb 12CA5 were from Becton Dickinson (Becton Dickinson). Anti-phosphotyrosine mAb 5H1 (25), and antisera generated against Btk, and anti-HA mAb 12CA5 were from Becton Dickinson (Becton Dickinson). Flow cytometry was performed using a FACSCalibur (Becton Dickinson) and analyzed using CellQuest software (Becton Dickinson).

Biochemical Analyses—ERK MAPK assays were as described (26). The PI 3-kinase inhibitors wortmannin and LY 294002 were from Calbiochem. Immunoprecipitations, immune complex protein kinase assays, and immunoblotting were as described previously (27).

Cloning and Transfection of Tec Family Kinases

Human Bmx (28), mouse Itk (29), mouse Tec (type IV) (30), and human Txk (31) were cloned by a polymerase chain reaction-based strategy using published sequences. The polymerase chain reaction utilized cDNA libraries (prepared by T. McClanahan, DNAX) from lung (Bmx) and from T cells (Itk, Tec, and Txk). The sequence was confirmed by DNA sequencing. Cloning of mouse Btk and production of HA-tagged versions of the Tec family PTKs were as described (25). For transfection into DT40 cells, Tec family PTKs were subcloned into the pApuroII vector, and cells were transfected by electroporation (18).

Calcium Flux Analyses

DT40 cells were labeled with 1 μM indo-1 AM (Molecular Probes, Eugene, OR) for 30 min at room temperature. After labeling, cells were washed and resuspended in RPMI 1640 supplemented with 1% fetal calf serum and 20 mM Hepes buffer. Measurement of calcium flux was performed using a FACS Vantage (Becton Dickinson, Mountain View, CA).

RESULTS

Reconstitution of Btk Function Is Dependent on Expression Levels—Human Btk was previously shown to restore BCR-induced PLCγ2 phosphorylation, calcium flux, and apoptosis in Btk-deficient DT40 cells (16, 17). Here we use mouse Btk to reconstitute Btk-deficient DT40 cells and show that the level of functional reconstitution is dependent on expression levels (Fig. 2). Btk-deficient DT40 cells were transfected with mouse Btk, and three stable clones were selected that expressed different levels of Btk, as measured by Western blotting of whole cell lysates followed by PhosphoImager quantitation (facilitated by the use of 125I-conjugated second Abs) (Fig. 2A). A direct comparison of endogenous chicken Btk in WT DT40 cells and transfected levels of mouse Btk was not possible because of poor cross-reactivity of the mouse Btk antisera with chicken Btk. Similar cell surface expression levels of the BCR on each clone was confirmed by FACS analysis using the M4 anti-chicken mAb (data not shown).

The three DT40 cell lines that expressed different levels of mouse Btk were compared with WT and Btk-deficient cells for their capacity to flux calcium and undergo apoptosis after BCR cross-linking (Figs. 2, B–C). The initial peak calcium flux and its sustained elevation was relatively poorly reconstituted in clone 2A2, which had the lowest level of Btk. In contrast, clones 1B4 and 1B2, which had greater Btk expression levels, showed reconstitution comparable with WT cells (Fig. 2B). BCR-induced apoptosis was similarly dependent on Btk expression levels. The level of apoptosis increased approximately in proportion to Btk expression (Fig. 2C). These results are consistent with data from Btk-transgenic mice that show Btk to be a limiting component of BCR signaling (32). Taken together, this suggests a role for Btk as a signal amplifier.

Expression of Tec Family PTKs in Btk-deficient DT40 Cells and Quantitation of Their Relative Expression Levels—Btk-deficient DT40 cells were transfected with Bmx, Itk, Tec, or Txk, and stable clones were selected. Expression of each PTK was demonstrated by Western blotting of whole cell lysates with specific antisera (Fig. 3A). Expression of endogenous Bmx, Itk, Tec, or Txk was not detectable in the parental Btk-deficient cells (Fig. 3A). Similar cell surface expression levels of the BCR on each clone was confirmed by FACS analysis (data not shown).

Because reconstitution of Btk function is dependent on expression levels (Fig. 2), the relative levels of each Tec family PTK were quantitated using HA-tagged versions as a common reference (Fig. 3, B–D). Tagged kinases were not used in reconstitution experiments because tagging was found to disrupt Btk function (data not shown). To allow relative quantitation, HA-tagged Tec family PTKs were in vitro translated. Translated products were protein-tyrosine phosphatase-treated with YOP to remove potential tyrosine phosphorylation from the HA tag that could affect the epitope. Western blotting was then performed with anti-HA-tag mAb 12CA5 (Fig. 3B). Relative expression levels were quantitated by phosphorimaging analysis. In Fig. 3C, a second set of Western blot analyses were performed, this time with specific Tec family antisera to compare the relative amounts of the quantitated HA-tagged versions from Fig. 3B with whole cell lysates of the transfected clones expressing each Tec family PTK. Quantitation of the
data from Fig. 3C with reference to those from Fig. 3B allowed calculation of the relative efficacies of each specific antiserum followed by the relative expression levels of each PTK (Fig. 3D). Btk, Itk, Tec, and Txk were found to be expressed at similar expression levels in the clones tested (within a 4-fold range), but Bmx was expressed relatively strongly by DT40 cells (Fig. 3D). The high level of Bmx expression was a feature of all clones analyzed. In contrast, Txk was generally less well expressed, and clone 3H11 had the greatest level of expression among 15 clones analyzed (data not shown). The data shown in the following sections were generated using the clones shown in Fig. 3 but were representative of at least two clones analyzed in each assay.

Activation of Tec Kinases after Antigen Receptor Cross-linking—Btk is transiently activated after BCR engagement, as measured by anti-phosphotyrosine blotting and in vitro kinase activity toward an exogenous substrate (33). Btk activation is a multistep process involving transphosphorylation of Btk by Lyn and subsequent Btk autophosphorylation (7, 8, 10, 11). Experiments using DT40 cells have generated data that is consistent with this mechanism (9).

Here we show that Bmx, in addition to Btk, Itk, and Tec, were rapidly but transiently activated in DT40 cells after BCR cross-linking, as measured by anti-phosphotyrosine blotting (Fig. 4A). Interestingly, some differences in the kinetics of activation of each kinase were apparent after phosphorimaging quantitation of the data (Fig. 4B). In particular, Btk and Tec phosphorylation was more sustained than that for Bmx and Itk. The pattern of whole cell lysate protein phosphorylation in these experiments was not affected by the presence of Btk.

Reconstitution of Btk Signaling by Bmx and Txk

FIG. 2. Reconstitution of Btk function in DT40 cells is dependent on expression levels. A, Western blot analysis of Btk expression levels in three different Btk-deficient clones transfected with mouse Btk. Whole cell lysates were immunoblotted with anti-mouse Btk antisera. Relative expression levels were quantitated by phosphorimaging analysis. Endogenous levels of chicken Btk (cBtk) in WT DT40 cells could not be quantitated relative to transfected levels of mouse Btk (mBtk) because of poor cross-reactivity of the mouse Btk antisera with chicken. B, reconstitution of BCR-induced calcium flux in Btk-deficient cells is dependent on expression levels. Intracellular free calcium levels in indo-1-loaded cells were monitored over an 8-min period by FACS. Anti-μ mAb M4 (2 μg/ml) was added at the 1-min time point. C, reconstitution of BCR-induced apoptosis in Btk-deficient cells is dependent on expression levels. Cells were cultured for 24 h with or without anti-μ mAb M4 (2 μg/ml) or with phorbol 12-myristate 13-acetate (PMA, 100 ng/ml) and ionomycin (1 μM) as a positive control. Apoptosis was measured by TUNEL assay. The percentage of apoptotic TUNEL-positive cells was calculated with reference to a negative control TUNEL reaction in the absence of terminal transferase. Negative controls yielded less than 1% TUNEL positive cells in all cases.
Bmx, Itk, or Tec (data not shown). This agrees with earlier data showing that Btk has no detectable affect on whole cell protein tyrosine phosphorylation, for which Lyn and Syk are largely responsible (16). Taken together, these results suggest that Tec kinases may be specific for a relatively narrow range of substrates. Txk, which lacks the PH domain common to the other Tec PTKs, was not inducibly phosphorylated through the BCR and had a relatively high basal level of phosphorylation on tyrosine (Fig. 4, A–B and data not shown). Whole cell protein tyrosine phosphorylation showed a similar pattern to WT DT40 cells (data not shown). These data suggest that Bmx can be activated in a similar manner to Btk, Itk, and Tec but that Txk may be regulated differently.

PI 3-kinase activity plays a key role in Btk activation by generating PIP3, the membrane ligand for the Btk PH domain (12–15). Recently, antigen receptor-induced Itk and Tec activation were shown to be PI 3-kinase-dependent (15). To test whether Bmx and Txk are similarly dependent on PI 3-kinase, we analyzed their BCR-induced phosphorylation in the presence of PI 3-kinase inhibitors. In Fig. 5A, PLC-γ2 phosphorylation was partially inhibited by wortmannin in cells expressing Btk, Bmx, Itk, and Tec—Studies using Btk-deficient DT40 cells have identified PLC-γ2 as an important substrate for Btk (16). Moreover, reconstitution of Btk-deficient human cells has shown that Itk and Tec can replace Btk function in regulating PLC-γ2 phosphorylation (23). Here we demonstrate that Bmx and, surprisingly, Txk, can restore phospholipase-dependent signal transduction in Btk-deficient cells.

In Fig. 5A, PLC-γ2 tyrosine phosphorylation was measured over a time course after BCR cross-linking. In WT DT40 cells, PLC-γ2 was maximally phosphorylated after 1 min and retained tyrosine phosphorylation above resting levels for up to 1 h after stimulation. This pattern of inducible phosphorylation was markedly reduced in the absence of Btk. Expression of all Tec family PTKs, including Txk, restored PLC-γ2 phosphorylation to at least WT levels.

We previously demonstrated that BCR-induced Btk, Bmx, Itk, and Tec, but not Txk, phosphorylation was dependent on PI 3-kinase (Figs. 4, C–D). Here we have extended these observations by measuring the effect of PI 3-kinase inhibitors on PLC-γ2 phosphorylation, which is probably a more accurate measure of Tec PTK activity (Figs. 5, B–C). Cells were stimulated in the presence or absence of wortmannin with anti-BCR mAb for 60 min. This late time point was chosen to reflect Tec PTK activity in sustaining PLC-γ2 phosphorylation. Earlier time points reflect a relatively large component of Syk activity in phosphorylating PLC-γ2 (e.g. compare WT versus Btk– in Fig. 5A). We found that PLC-γ2 phosphorylation was partially inhibited by wortmannin in cells expressing Btk, Bmx, Itk, and Tec but not Txk. Similar results were obtained with the PI

**FIG. 3.** Expression of Tec family PTKs in Btk-deficient DT40 cells and quantitation of relative expression levels. A. Western blot analyses of Tec family expression in transfected Btk-deficient DT40 cell clones versus parental Btk-deficient cells. Whole cell lysates were immunoblotted with the indicated antisera. B, Western blot analysis of in vitro translated HA-tagged Tec PTKs. HA-tagged versions of Tec family members were in vitro translated, immunoprecipitated with specific antisera, and immunoblotted with anti-HA antibody. Relative expression levels were quantitated by phosphorimaging analysis (not shown). C, Western blot analyses of HA-tagged Tec PTKs versus Tec PTKs expressed in transfected DT40 cells. HA-tagged Tec PTKs (equivalent amounts to those used in panel B) and whole cell lysates of transfected DT40 cell clones were immunoblotted with the indicated antisera. Relative expression levels were quantitated by phosphorimaging analysis (not shown). D, relative expression levels of Tec family PTKs in the DT40 clones tested. Values shown were calculated based on quantitation of the data in panels B and C.
3-kinase inhibitor LY294002 (data not shown). An actual enhancement of PLC$_g$$_2$ phosphorylation in the presence of wortmannin was observed in Txk-expressing cells in Figs. 5, B–C, but this was not seen consistently. These data suggest that Txk is unique among Tec PTKs in not requiring PI 3-kinase activity for its normal function.

In addition to defective PLC$_g$$_2$ phosphorylation, Btk-deficient cells are impaired in BCR-induced calcium mobilization (16, 23). This function of Btk in calcium signaling could be restored by Itk and Tec (23). Here we have tested whether Bmx and Txk can reconstitute calcium flux. In Fig. 6, DT40 intracellular calcium flux was measured over an 8-min period, with the addition of anti-BCR mAb after 1 min. WT DT40 cells showed an immediate rapid increase in intracellular calcium concentration after BCR engagement, which was sustained above basal levels over the course of the experiment. The initial peak of calcium was reduced in Btk-deficient cells, and no sustained elevation of calcium was observed. Consistent with the PLC$_g$$_2$ data (Fig. 5A), all Tec family PTKs restored both the initial peak and later sustained phase of the calcium flux to WT levels (Fig. 6). No BCR-induced calcium flux was observed in PLC$_g$$_2$-deficient DT40 cells, as previously shown (24). Taken together, these data indicate that all Tec family PTKs can reconstitute calcium flux in Btk-deficient DT40 cells (34). Moreover, full ERK activation appears to be dependent on protein kinase C, which is itself activated downstream of PLC$_g$$_2$ (35). Here we have tested Tec PTKs for their capacity to reconstitute the ERK MAPK signaling pathway in Btk-deficient DT40 cells (Fig. 7).

Cells were stimulated with anti-BCR mAb, and cells were lysed over a 2-h time course. ERK1 and ERK2 MAPK activity was measured by immune complex kinase assay using myelin basic protein as an exogenous substrate (Fig. 7). WT DT40 cells exhibited sustained ERK MAPK activation, whereas in the absence of Btk, ERK activation was transient and delayed. The kinetics of ERK activation in Btk- and PLC$_g$$_2$-deficient cells were strikingly similar (Fig. 7). These data are in agreement with recent studies (34, 35) and suggest that the rapid and sustained activation of ERK is dependent on Btk and PLC$_g$$_2$.

Bmx, Itk, Tec, and Txk were different in their capacities to fully restore ERK MAPK activation. Btk- and Bmx-expressing DT40 cells showed sustained ERK MAPK activation. The other Tec family expressing cells exhibited a more transient ERK activation. Nevertheless, ERK activity was sustained above basal levels throughout the 2-h time course in each case (Fig. 7). The data suggest that Tec kinases, including Txk, can play an important role in the rapid and sustained activation of ERK MAPK in antigen receptor signaling.

**The Role of Tec Kinases in Antigen Receptor-induced Apoptosis—**DT40 cells are programmed to die by apoptosis after BCR engagement (17). This apoptotic response was impaired in Btk-deficient cells but was restored by re-expression of Btk.
We therefore examined the capacity of other Tec family kinases to restore BCR-induced apoptosis in DT40 cells (Fig. 8). Cells were treated with anti-BCR mAb, and the extent of apoptosis was measured after 24 h by the TUNEL method. TUNEL provides a quantitative measure of the DNA fragmentation, which is a hallmark of apoptosis. In Btk-deficient cells, apoptosis was barely detectable after BCR cross-linking (Fig. 8). In contrast, a relatively high proportion of WT DT40 cells were apoptotic. Apoptosis was efficiently restored in Btk-deficient cells by expression of Btk, Bmx, and Itk. Reconstitution by Tec was relatively poor, but apoptosis at levels above those of Btk-deficient cells was seen consistently in several Tec-expressing clones (Fig. 8 and data not shown). In contrast, Txk failed entirely to reconstitute BCR-induced apoptosis (Fig. 8).

**FIG. 5.** Reconstitution of PLCγ2 phosphorylation by Tec family PTKs and sensitivity to PI 3-kinase inhibitors in DT40 cells. A, time course for BCR-induced reconstitution of PLCγ2 phosphorylation by Tec family PTKs. Cells were lysed as a function of the indicated times after BCR cross-linking with anti-μ mAb M4 (4 μg/ml). PLCγ2 was immunoprecipitated with anti-PLCγ2 antisera and immunoblotted with anti-phosphotyrosine (APT, top panel) and with anti-PLCγ2 antisera (bottom panel). B, sustained PLCγ2 phosphorylation is sensitive to PI 3-kinase inhibition in Btk-, Bmx-, Itk-, and Tec-expressing cells but not in Txk-expressing cells. Cells were incubated for 30 min in vehicle control or 100 nm wortmannin and were unstimulated or stimulated with anti-μ mAb M4 (4 μg/ml) and lysed after 60 min. PLCγ2 was immunoprecipitated with specific antisera and immunoblotted with anti-phosphotyrosine and with specific antisera. C, the results from B presented as quantitated data as measured by phosphorimaging analyses.

**FIG. 6.** Reconstitution of calcium mobilization by Tec family PTKs in DT40 cells. Calcium measurements were as described in the legend for Fig. 2B.
Interestingly, PH domain mutants of Btk also failed to restore BCR-induced apoptosis (data not shown), implying a role for the PH domain in connecting Tec kinases to apoptosis induction. The effect of antigen receptor engagement can be mimicked by phorbol 12-myristate 13-acetate and ionomycin, which can activate protein kinase C and induce calcium influx, respectively. In Fig. 8, we show that all of the DT40 cell lines tested apoptose to a similar extent when treated with phorbol 12-myristate 13-acetate and ionomycin. These data suggest that Txk cannot restore the apoptotic pathway in DT40 cells despite effective reconstitution of PLC\(_{\gamma 2}\)/calcium/ERK MAPK signaling.

**DISCUSSION**

Agammaglobulinemia patients, the *xid* mouse, and the Btk knockout mouse show that Btk plays a nonredundant role in B cell function and development (reviewed in Ref. 3). Analyses of Btk-deficient B cell lines have revealed a role for Btk in BCR-induced PLC\(_{\gamma 2}\) phosphorylation and sustained calcium mobilization (16, 23). Reconstitution of human agammaglobulinemia-transformed B cell lines with Itk and Tec have shown these PTKs to be analogous in function to Btk in PLC\(_{\gamma 2}\) signaling (23). Here we have focused on the atypical Tec family PTKs, Bmx and Txk, whose roles are unclear. Bmx fully restored signaling in Btk-deficient chicken DT40 cells, in common with Btk, Itk, and Tec. Txk could also reconstitute PLC\(_{\gamma 2}\) signaling despite a different mechanism of activation and a failure to restore downstream apoptosis, suggesting an essential role for the PH domain and/or membrane association in the apoptosis response.

Txk is predominantly expressed in T cells and mast cells (36, 37). The mechanism of activation and functions of Txk are unknown. Txk shares 50–60% sequence identity with other Tec kinases but is atypical in that it lacks a PH domain (Fig. 1). Our data suggest that Txk, in common with other Tec kinases, can function in antigen receptor signal transduction and that PLC\(_{\gamma 2}\) is a downstream target. In Btk-deficient cells, Txk effectively restored BCR-induced calcium mobilization and partially restored ERK MAPK activation, which are both downstream of PLC\(_{\gamma 2}\) activation in DT40 cells (35). We found that expression levels of Txk and other Tec kinases correlated with the extent of functional reconstitution (Fig. 2 and data not shown). This has been noted previously (15, 23, 32) and implies a role for Tec kinases as modulators or amplifiers of phospholipase dependent signaling.

Functional differences were observed between Txk and the other Tec PTKs. First, no induction of Txk tyrosine phosphorylation, which is a good measure of activation for other Tec family PTKs (25, 38–40), was detected after BCR cross-linking.
(Fig. 4, A–B) despite the apparent induction of Txk activity as measured by PLCγ2 phosphorylation (Fig. 5A). Second, Txk phosphorylation (Fig. 4C), PLCγ2 phosphorylation (Fig. 5B), and calcium mobilization (data not shown) were not dependent on PI 3-kinase activity in Txk-expressing cells. Third, Txk could not reconstitute apoptosis (Fig. 8), the functional response of DT40 cells to BCR signaling.

Interestingly, Txk can be palmitylated and may be constitutively membrane-associated (47). This would potentially position Txk in close proximity to activating Src family PTKs (47). This provides an explanation for its relatively high basal phosphorylation state, which could mask subtle changes in tyrosine phosphorylation that may occur upon Txk activation. The PI 3-kinase-dependent activity of Txk is not surprising given the phosphoamino acid state, which could mask subtle changes in tyrosine phosphorylation. This provides an explanation for its relatively high basal phosphorylation state, which could mask subtle changes in tyrosine phosphorylation. PI 3-kinase and may function irrespective of CD28 costimulation. Such a model is consistent with reports that Txk expression levels in T cells are regulated, being down-regulated in T cell activation (37) and preferentially expressed in Th2 relative to Th2 T cell clones (36), perhaps suggesting a role in the differential regulation of cytokine expression.

Txk failed to restore BCR-induced apoptosis in Btk-deficient DT40 cells despite reconstituting PLCγ2 signaling and at least partially reconstituting ERK MAPK activation. ERK MAPK plays a key role in transducing signals from the cytoplasm to the nucleus (41). ERK reconstitution by Txk and other Tec kinases was not surprising because sustained ERK activation in DT40 cells requires a Btk/PLCγ2/protein kinase C signaling pathway (34, 35). This is consistent with our kinetic analyses of ERK activation that show strikingly similar profiles between Btk- and PLCγ2-deficient cells (Fig. 7). In contrast to ERK activation, the mechanism of BCR-induced DT40 cell apoptosis is not known, but correlative data suggests that c-Jun NH2-terminal kinase 1 (JNK1) activation may be required (34). It is possible that Txk cannot activate this signaling pathway, whereas other Tec family kinases can, perhaps by virtue of interactions mediated by the PH domain. For example, the PH domain of Btk has been shown to bind not only to PI(3,4,5)P3 (12) but also to the α subunits of heterotrimeric G proteins (42, 43) and to BAP-135 (44), a protein of unknown function.

In addition to BCR-induced apoptosis, Btk was reported to play a PLCγ2-independent role in radiation-induced apoptosis of DT40 cells (17). We have been unable to repeat these data. Instead we find no difference between WT and Btk-deficient DT40 cells in their apoptotic responses to ionizing radiation (data not shown).

In common with Txk, Bmx is an atypical Tec family PTK but for different reasons. Bmx has the same domain organization as Btk, Itk, and Tec (Fig. 1), but the proline-rich Tec homology and SH3 domains are poorly conserved (39, 45). These domains are proposed to interact intramolecularly and function in negative regulation of Tec PTKs (46). It is possible that these domains in Bmx have co-evolved divergently from other Tec PTKs yet perform the same negative regulatory function. Our data is consistent with this hypothesis, because Bmx is activated in a PI 3-kinase-dependent manner in common with Btk, Itk, and Tec and fully reconstitutes signaling in Btk-deficient B cells.

We have shown that Bmx is able to regulate PLCγ2 activity in antigen receptor signaling, but this is unlikely to be an in vivo function because Bmx is not found in lymphocytes and is predominantly expressed in epithelial, endothelial, and granulocytic cells (39, 45). Our data suggest that Bmx may be activated in these cells by stimuli that induce both Src family and PI 3-kinase activity. Bmx may function in concert with receptor tyrosine kinases to regulate PLCγ activity, calcium mobilization, and ERK activity. This idea is supported by the observation that in prostate cancer cells, Bmx is activated by interleukin 6 (IL-6) in a PI 3-kinase-dependent manner and functions in neuroendocrine differentiation (39).

Reconstitution of Btk-deficient human cells demonstrated a role for Itk and Tec in PLCγ2 activation and sustained calcium signaling (23). We have performed a similar study in Btk-deficient DT40 chicken B cells. We have confirmed the findings of Fluckiger et al. (23) and have extended our study to show that the atypical Tec family PTKs, Bmx and Txk, can also function in phospholipidase-dependent signaling. We further show that Tec family PTKs can play a role in sustained ERK MAPK activation. Interestingly, Txk is unique among Tec PTKs as it functions independently of PI 3-kinase and fails to restore apoptosis, the biological response of DT40 cells to antigen receptor signaling. These data indicate that Tec PTKs provide at least two different signals, namely to the PLCγ2/ERK MAPK pathway that is not sufficient for apoptosis and to a PH domain-dependent pathway that is necessary for apoptosis.

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