Functional Interaction of Caveolin-1 with Bruton’s Tyrosine Kinase and Bmx

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Bruton’s tyrosine kinase (Btk), a member of the Tec family of protein-tyrosine kinases, has been shown to be crucial for B cell development, differentiation, and signaling. Mutations in the Btk gene lead to X-linked agammaglobulinemia in humans and X-linked immunodeficiency in mice. Using a co-transfection approach, we present evidence here that Btk interacts physically with caveolin-1, a 22-kDa integral membrane protein, which is the principal structural and regulatory component of caveolae membranes. In addition, we found that native Bmx, another member of the Tec family kinases, is associated with endogenous caveolin-1 in primary human umbilical vein endothelial cells. Second, in transient transfection assays, expression of caveolin-1 leads to a substantial reduction in the in vivo tyrosine phosphorylation of both Btk and its constitutively active form, E41K. Furthermore, a caveolin-1 scaffolding peptide (amino acids 82–101) functionally suppressed the autokinase activity of purified recombinant Btk protein. Third, we demonstrate that mouse splenic B-lymphocytes express substantial amounts of caveolin-1. Interestingly, caveolin-1 was found to be constitutively phosphorylated on tyrosine 14 in these cells. The expression of caveolin-1 in B-lymphocytes and its interaction with Btk may have implications not only for B cell activation and signaling, but also for antigen presentation.

Bruton’s tyrosine kinase (Btk) is a member of the Tec family of nonreceptor protein-tyrosine kinases (PTKs) (1–4). Tec kinases belong to a subfamily of Src-like kinases, of which five members have been identified in mammals: Tec, Btk, Itk, Bmx, and Txk. Three other related members have been identified in Drosophila (Btk29A), zebrafish (Itk), and Raja elganteria (clearnose skate, skate Ptk) (3, 4). Tec family PTKs are characterized by an N-terminal pleckstrin homology (PH) domain followed by a Tec homology, a Src homology 3 (SH3) domain, an SH2 domain, and a C-terminal kinase domain. Mutations in various parts of the gene lead to X-linked agammaglobulinemia (XLA), a primary immunodeficiency in humans characterized by the absence of B-lymphocytes (5–7). A mutation in the corresponding gene in mice results in X-linked immunodeficiency (xid) with a milder phenotype (8, 9).

Btk is expressed predominantly in B-lymphocytes as well as in myeloid and erythroid progenitor cells. In B cells, the Btk gene is developmentally regulated with expression occurring in most stages of the B cell lineage, except in terminally differentiated plasma cells (10, 11). The pattern of expression, together with the defects observed in the context of the XLA phenotype, lends support to the notion that Btk plays critical signaling roles at various stages of B cell development (12). Btk phosphorylation and activation in response to engagement of the B cell receptor (BCR) by antigen is a dynamic process whereby a variety of proteins interact with each other and recruit signaling molecules, resulting in physiological responses such as B cell proliferation and antibody production. Several important players that participate in the intracellular downstream signaling of Btk have been identified in recent years (2, 13).

Recently, modern biochemical and cell biological techniques have allowed the identification of a wide variety of cellular signaling molecules, which are recruited to flask-shaped plasmalemmal vesicles named caveolae (14). Caveolae, or “small caves” as they are sometimes called, are invaginations of the plasma membrane, which serve as cell-surface microdomains and play an important role in many cellular signaling pathways (14–19). Resistance to detergents at 4 °C, a light buoyant density, and richness in glycosphingolipids, cholesterol, and lipid-anchored membrane proteins are some of the major characteristics of caveolae (14, 20). Caveolin, a 22–24-kDa integral membrane protein composed of cytoplasmic N and C termini and a central intramembrane domain, is thought to be a major structural component of caveolae (21), but is also found in the cytoplasm (22). A 20-amino acid juxtamembrane region, also called the scaffolding domain, in the N-terminal region of the protein (residues 82–101) has been shown to mediate the association of caveolin with the bulk of relevant signaling molecules (23). Among these are G-proteins (α and β subunits), Ha-Ras, Fyn, Erk-2, epidermal growth factor, platelet-derived growth factor, tumor necrosis factor receptor-associated factor 2, endothelial nitric-oxide synthase, protein kinase C isoforms, insulin and Neu (ε-RBB2) receptors, and Src family tyrosine kinases (24–34). Binding to caveolin through the scaffolding domain is sufficient for inhibition of the in vitro kinase activity of c-Src or...
maintenance of the inactive conformation of G-proteins (35). Thus, it is envisaged that caveolin may function as a negative regulator for many signaling proteins. For insulin receptor signaling, however, caveolin has an activating function instead (36).

Visual inspection of the Btk amino acid sequence shows the presence of a potential caveolin-binding motif between residues 581 and 588 (WXFFXXXW) in the catalytic domain. Thus, the functional significance of a bona fide caveolin-binding motif in this family of tyrosine kinases warrants functional characterization, particularly with respect to its role in signal transduction.

In the present study, therefore, we set out to investigate whether Btk is capable of interaction with caveolin-1. Our results demonstrate that caveolin-1 interacts with wild type Btk, as well as the constitutively active mutant E41K. In addition, we present evidence suggesting that this interaction leads to the down-regulation of the in vivo tyrosine phosphorylation of Btk. Altogether, these data may shed some light on the molecular mechanism(s) of Btk regulation, activation, and signaling.

MATERIALS AND METHODS

Reagents—The protease inhibitor mixture was obtained from Roche Molecular Biochemicals in tablet form. All phosphatase inhibitors were purchased from Sigma. Anti-Btk rabbit polyclonal and mouse monoclonal antibodies have been described previously (37). Monoclonal anti-V5 horseradish peroxidase antibody was from Invitrogen (Groningen, The Netherlands). Monoclonal and polyclonal antibodies against caveolin-1 and Bmx were purchased from Transduction Laboratories (Lexington, KY). The nonionic detergent n-octyl glucoside was purchased from Sigma-Aldrich.

Cell Culture and Transfections—COS-7 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) heat-inactivated fetal calf serum and 1000 units of penicillin-streptomycin (Invitrogen Life Technologies). HUVECs were maintained in RPMI 1640 supplemented with 20% (v/v) fetal calf serum and antibiotics. All HUVEC cultures were washed several times with ice-cold PBS before the cell suspension was incubated for 1 h at 4 °C in 2% paraformaldehyde for 10 min. The fixed cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked for 2 h in PBS containing 0.1% bovine serum albumin-C (Aurion, The Netherlands) and 0.1% Tween. Following incubation with a polyclonal antibody against caveolin-1, the cells were washed and stained further with a Cy3-conjugated donkey anti-rabbit IgG before processing of the slides for immunofluorescence.

For lipid raft visualization, rhodamine-conjugated cholera toxin B (CT-B) (List Biological Laboratories, Campbell, CA) was used for labeling ganglioside GM1, as previously described (40). Purified splenic B cells from CBA mice were washed in serum-free medium and attached to 3-aminopropyltriethoxy silane-coated coverslips (Sigma). Lipid raft aggregation or patching was induced following CT-B labeling by incubating the cells with anti-CT-B antibody (1/250 in PBS, 0.1% bovine serum albumin; Calbiochem-Novabiochem Corp) for 30 min on ice. After an additional 20-min incubation at 37 °C, cells were fixed, permeabilized, and decorated with anti-caveolin-1 antibody. As secondary antibody, fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG was used. Confocal microscopy analysis was performed as previously described (37).

Sequence Alignments—Sequence alignments of the Tec family kinases showing the conserved caveolin-binding motif were performed using a Macintosh version of the ClustalX program as previously described (3). The multiple sequence alignments have been refined using SeqVu (Garvan Institute of Medical Research, Darlinghurst, Sydney, New South Wales, Australia). Segments of the sequence alignments were chosen and viewed using FreeHand software (Macromedia).

RESULTS

Tec Family Tyrosine Kinases Contain a Caveolin-binding Motif—Caveolin-binding motifs are present in most Gα subunits and the kinase domain of many families of tyrosine and serine/threonine kinases (Ssrc family kinases, protein kinase Ce, MAP kinase, epidermal growth factor receptor, insulin receptor, and platelet-derived growth factor receptor) (20, 24, 29, 41, 42). Two related caveolin-binding motifs (ΔXAAXXXΔ and ΔXAAΔXXA, where Δ is an aromatic amino acid) are found in most caveolin-associated proteins (19). We find that Btk contains a potential candidate caveolin-binding motif (WXFFXXXW) within its catalytic domain (Fig. 1A). Sequence alignment among all Tec family kinases clearly indicates the presence of a highly conserved caveolin-binding motif (Fig. 1B).

Interestingly, mutations corresponding to the invariant aromatic residues in this region have been identified in several patients with classical XLA (Table I). Btk Interacts with Caveolin-1—To determine the role of the caveolin-binding motif in protein-protein interactions, we set out to co-express Btk and caveolin-1 in COS-7 cells. Results

were incubated with 1 μg of each recombinant Btk and BtkΔSH1 proteins at 4 °C for 1 h. A polyclonal antibody against caveolin-1 was then added to the mixture and incubated for 2 h at 4 °C on an end-over-end rotating shaker to allow association of Btk protein and caveolin-1. The immunoprecipitated complexes were collected using a slurry of protein G-Sepharose beads that wash extensively in 2× Laemmli sample buffer, fractionated on 10% SDS-PAGE.

Immunoblotting—Immunoprecipitated Btk was resolved on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were then incubated in blocking buffer (5% dried milk in PBS and 0.05% Tween 20), probed with specific antibodies followed by horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected using the Supersignal® West Femto Chemiluminescence Western blotting detection system (Pierce).

In Vitro Autokinase Activity—100 ng of purified recombinant Btk protein was incubated with the caveolin-1 scaffolding peptides using concentrations of 2.5, 5, and 10 μM in a standard kinase reaction buffer. Briefly, reactions were performed in a 20-μl kinase buffer containing 20 mM HEPES buffer (pH 7.4), 10 mM MgCl2, 5 mM MnCl2, 200 μM cold ATP, and 1 μCi of [γ-32P]ATP for 10 min at room temperature. The reactions were stopped by addition of 8 μl of 4× sample buffer (37).

Immunofluorescence, Patching, and Confocal Microscopy—HUVECs were seeded overnight at 60% confluence onto culture slides coated with human fibronectin (Becton Dickinson). The following day, cells were transfected with the Btk/GFP construct and grown additionally for 24 h. HUVECs were washed several times with ice-cold PBS before the cell suspension was added to the mixture and incubated for 2 h at 4 °C. Immunoprecipitates were detected using the Supersignal® West Femto Chemiluminescence Western blotting detection system (Pierce).

Association of Btk with Caveolin-1

Cells were cultured at 37 °C in 1640 supplemented with 20% (v/v) fetal calf serum and antibiotics. All HUVEC cultures were washed several times with ice-cold PBS before the cell suspension was incubated for 1 h at 4 °C in 2% paraformaldehyde for 10 min. The fixed cells were permeabilized with 0.1% Triton X-100 for 10 min and blocked for 2 h in PBS containing 0.1% bovine serum albumin-C (Aurion, The Netherlands) and 0.1% Tween. Following incubation with a polyclonal antibody against caveolin-1, the cells were washed and stained further with a Cy3-conjugated donkey anti-rabbit IgG before processing of the slides for immunofluorescence.

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from this experiment showed unequivocally that Btk interacts with caveolin-1 because immunoprecipitates of Btk contained caveolin-1, as detected by immunoblotting (Fig. 2A, top panel). Furthermore, co-expression of caveolin-1 and the constitutively active form of Btk, E41K, also leads to a strong association between these two proteins (Fig. 2A, top panel). Although it may appear from Fig. 2A as if the E41K mutant of Btk is less efficient in binding caveolin-1, in other experiments this difference was not apparent (data not shown). Antibodies directed against Bmx and detected with anti-caveolin-1 monoclonal antibodies. We demonstrate here that caveolin-1 is present in the anti-Bmx immune complex (Fig. 3C), but not in the immune complex precipitated with an irrelevant antibody or a
preimmune serum. These results are consistent with our earlier findings involving transiently transfected COS-7 cells co-expressing Btk and caveolin-1 (this work). Although Bmx has been implicated in a number of intracellular signaling pathways including cytokine, heterotrimeric G-protein and integrin receptors (4, 44), the functional significance of the interactions between Bmx and caveolin-1 may shed some light on the overall molecular mechanism(s) behind signaling pathways involving Tec family kinases.

\textbf{Btk Co-localizes with Caveolin-1}—To determine whether Btk can also co-localize with caveolin-1 in intact cells and confirm our biochemical findings, we performed confocal microscopic analysis on HUVECs transfected with Btk-GFP. Because these cells are known to abundantly express caveolin-1, which according to the current study interacts spontaneously with the endogenous Tec family member Bmx, they should represent an excellent model system for studies that, in particular, address translocation and targeting of Tec family kinases into caveolae microdomains. Indeed, as shown in Fig. 4C, Btk was found to co-localize with caveolin-1 with a complete overlap at the leading edge of the cells, specifically in areas corresponding to membrane ruffles. This result is consistent with our previous data showing translocation of Btk to similar regions following fibronectin stimulation of COS-7 cells transiently expressing Btk (data not shown).

\textbf{Caveolin-1 Down-regulates the Tyrosine Phosphorylation of Btk}—The experiments described above show that members of the Tec family kinases physically interact with caveolin-1. To examine the functional consequences of this interaction, we transiently co-expressed Btk or E41K with caveolin-1 and assessed their phosphorylation status. Immunoprecipitation with anti-Btk antibodies followed by Western blotting with anti-phosphotyrosine antibodies revealed that the in vivo tyrosine phosphorylation of wild type Btk and E41K was reduced to 31 and 46%, respectively (Fig. 5A, \textit{top panel}).

To determine whether the scaffolding domain of caveolin-1 is responsible for the observed down-regulation regarding the enzymatic activity of Btk, we utilized a synthetic peptide (peptide 1) derived from this region in an \textit{in vitro} autokinase assay. This caveolin scaffolding peptide inhibited completely the autokinase activity of Btk by caveolin-1 scaffolding peptide. Peptide 1 but not a scrambled control peptide (peptide 2) suppresses the catalytic activity of Btk.

\textbf{Caveolin-1 Is Expressed in Splenic B Cells}—To examine whether caveolin-1 is expressed in splenic cells and, in particular, purified splenic B-lymphocytes, we immunoblotted cell lysates with caveolin-1-specific antibodies (Fig. 5B, \textit{middle panel}), and anti-V5 antibodies for detecting caveolin-1 (\textit{bottom panel}). Btk was found to co-localize with caveolin-1 in splenic B cells and purified splenic B-lymphocytes. To our knowledge, this is the first report documenting expression of caveolin-1 in B-lymphocytes. The molecular mass of this protein, however,
seems to be slightly larger than 24 kDa, suggesting that it is altered because of posttranslational modification. Nevertheless, we show here that these cells preferentially express the α isoform of caveolin-1. This is consistent with recent data from macrophages (45), which have been shown to express caveolin-1α predominantly. In addition, using a highly specific phospho-caveolin-1 antibody, we demonstrate here that, under steady state conditions, caveolin-1 is phosphorylated on tyrosine 14 in spleen cells (Fig. 6B). Immunofluorescence analysis using purified spleen B cells reveals that caveolin-1 is predominately localized in the plasma membrane. Furthermore, caveolin-1 is concentrated in the area of cell-cell contact (Fig. 6C). These data are consistent with an earlier report showing caveolin-1 concentration in the cleavage furrow of mitotic cells (46).

Caveolin-1 Co-localizes with Ganglioside GM1 in Splenic B Cells—To investigate whether caveolin-1 translocates to a specific plasma membrane microdomain, purified spleen B cells were stained with a rhodamine-conjugated cholera toxin β subunit (CT-B). This compound binds primarily to the ganglioside GM1 and has been used as a marker for lipid rafts (40). Although a homogeneous subcellular distribution has been observed for CT-B, staining became concentrated to distinct patches within the plasma membrane following cross-linking with an anti-CT-B antibody (Fig. 7A and data not shown). Remarkably, however, a substantial fraction of caveolin-1 was found to be associated with the CT-B-stained patches, indicating that caveolin-1 may be a principal component of the lipid raft microdomain in splenic B-lymphocytes (Fig. 7C).

**DISCUSSION**

In the present report, we describe the in vivo interaction of two members of the Tec family kinases, Btk and Bmx, with the membrane-organizing coat protein, caveolin-1. Using co-immunoprecipitation analysis involving both transiently transfected and native cells, we demonstrate here that antibodies directed against each of these proteins bring down the other in a reciprocal way, indicating a strong physical association between Btk and caveolin-1. In addition, we present clear evidence that this interaction is mediated by the catalytic domain of Btk.

The role of Tec kinases in signal transduction, however, has been characterized best for Btk in the context of BCR signaling. Btk is the only member of the Tec family kinases in which mutations have been found to be associated with a disease (7, 47, 48). Intriguingly, point mutations of the highly conserved aromatic residues in the caveolin-binding motif within the catalytic domain of Btk have been detected in patients with classical XLA (Table I) (7). At present, however, we do not know whether B cells from these patients express a stable functional protein at all. It would, therefore, be of considerable interest to investigate whether the functional interaction of Btk with caveolin-1 is maintained in these patients. Nevertheless, we can speculate that mutations within the caveolin-binding motif might compromise the stability, function, and/or signaling of Btk. This finding, however, is not without precedence. In fact, point mutations involving the same aromatic residues have also been found in the caveolin-binding domain of the insulin receptor in patients suffering from severe insulin resistance (49, 50).

Our findings raise a number of questions regarding the interaction of the Tec family kinases and caveolin-1. We have shown here that caveolin-1 down-regulates tyrosine phosphorylation of Btk. Furthermore, a caveolin-1 scaffolding peptide but not a control peptide was sufficient to entirely inhibit the in vitro autokinase kinase activity of Btk. These data are consistent with the general idea that caveolins may function as negative regulators inhibiting the activity of many signaling proteins (18). Although caveolin-1 has been shown to interact specifically with wild-type c-Src and Ha-Ras, it fails to form a stable complex with their constitutively active counterpart (19). Taken together, these results suggest that caveolin-1 prefers to associate with the inactive conformation of signaling molecules. Nevertheless, the high affinity of caveolin-1 for the inactive conformation of caveolin-interacting proteins is not reflected in Btk, because the constitutively active Btk mutant, E41K, could bind readily to caveolin-1. Importantly, the phosphorylation as well as kinase activity of Btk were neither necessary nor required for its functional interaction with caveolin-1.

Further experiments will be necessary to understand the biological implications regarding the high degree of conservation of a well defined caveolin-binding domain in all members of the Tec family kinases. In addition, it will be tempting to find out whether the tyrosine phosphorylation of caveolin-1 is required for the activation and/or regulation of Btk.

The existence of caveolae and expression of caveolin in lym-
phocytes has been controversial for years. Although caveolin-1 and 2 are ubiquitously expressed in most cell types, and in great abundance, in terminally differentiated cells such as adipocytes and endothelial cells, neither caveolin protein nor caveolea structures have been detected in blood cells (51). On the other hand, caveolin-3 expression is limited to muscle and neuronal cells (52). More recently, however, caveolin-1 expression has been detected in some T cell leukemia cell lines, suggesting that caveolea may also exist in some T cells (53). Furthermore, expression of caveolin has been observed in thymus, spleen, mast cells, and macrophages (22, 45, 54). To this end, although caveolin-1 expression has been confirmed in splenic-resident lymphocytes, we were unable to detect it in peripheral blood lymphocytes as well as in B cell lines (data not shown).

Here we describe our unexpected finding regarding expression of caveolin-1 in splenocytes, purified splenic B-lymphocytes, and B cell-depleted splenic cells also (Fig. 6A). Moreover, in these cells, caveolin-1 was unexpectedly found to be constitutively tyrosine-phosphorylated. Presumably, posttranslational modification as a result of phosphorylation may explain the higher mobility shift displayed by the protein in the splenic B cells in comparison with endothelial or fibroblast cells (Fig. 6B). As far as we know, in most cell types, caveolin-1 could only be phosphorylated following growth factor stimulation (55). Caveolin-1, however, has been shown to be an excellent substrate for c-Src and v-Abl tyrosine kinases (56). In contrast, we found no evidence that Btk, under steady state conditions, can phosphorylate caveolin-1 on tyrosine 14 (data not shown). Notably, because in resting splenic B cells Btk is not detectably phosphorylated, it may not necessarily be responsible for the tyrosine phosphorylation of caveolin-1. In addition, caveolin-1 is tyrosine-phosphorylated in splenic B-lymphocytes from xid and Btk knock-out mice, indicating that another tyrosine kinase, possibly a Src family member, may do so (data not shown). Finally, it is intriguing to learn that caveolin-1 is present and tyrosine-phosphorylated in splenic B-lymphocytes. In light of the recent report describing immunologic synapses in B cells (57), it will be of interest to determine whether caveolea structures exist in resting B cells and change form following antigen presentation.

Localization of Btk and Itk within microdomains or lipid rafts has recently been demonstrated (58, 59). Lipid rafts are the major functional compartments for signaling in human pre-B cell activation. Recruitment and activation of Btk/Tec family kinases occur in response to pre-BCR engagement and require physical association with rafts. However, the existing confusion regarding the relationship between caveolea and rafts has yet to be resolved. We speculate that rafts and/or caveolea binding recruits Btk, where its phosphorylation states can be dictated by nearby resident kinases and phosphatases, resulting in a cascade of downstream signaling events. Localization to microdomains, whether it be caveolea or rafts, could itself be a prerequisite for proper signaling of Tec family kinases. Indeed, this is substantiated by the lack of signaling manifested in some of the most common XLA mutations that affect the PH domain of Btk. Accordingly, an earlier report shows that a PH-deleted mutant of Itk, although readily phosphorylated, is excluded from the rafts (59). However, the question of whether XLA-causing mutations in the PH domain may compromise targeting of Btk to the caveolea microdomain is under intensive investigation in our laboratory.

Although there is no information regarding the regulation of other Tec family kinases by caveolin-1 at present, mice deficient in this protein have recently been shown to suffer primarily from vascular dysfunction and pulmonary defects (60, 61). In light of this, it is possible that the vascular abnormality observed in these mice might reflect an up-regulation of the kinase activity of Bmx, an endothelial and epithelial specific tyrosine kinase that has been shown to be involved in various signaling events of angiogenesis and vascular disease (62, 63).

In conclusion, in the present study we have identified caveolin-1 as a new partner for the Tec family kinases Btk and Bmx. Although the functional significance of this interaction is not presently understood, the negative regulation of Btk activity by caveolin-1 may represent a relevant consequence of the different signaling pathways where Btk is involved. Most importantly, it will be of considerable interest to understand the biological as well as functional implication(s) of Btk and caveolin-1 interaction. Additional studies will therefore be necessary to obtain a deeper insight into the overall function of caveolea in the biology of B cells. In particular, it is of profound interest to understand their specific role in B cell activation and antigen presentation.

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