Pleckstrin Homology Domains Interact with Filamentous Actin*

(Received for publication, January 4, 1999, and in revised form, April 6, 1999)

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A fraction of Bruton’s tyrosine kinase (Btk) co-localizes with actin fibers upon stimulation of mast cells via the high affinity IgE receptor (FceRI). In this study, a molecular basis of the Btk co-localization with actin fibers is presented. Btk and other Tec family tyrosine kinases have a pleckstrin homology (PH) domain at their N termini. The PH domain is a short peptide module frequently found in signal-transducing proteins and cytoskeletal proteins. Filamentous actin (F-actin) is shown to be a novel ligand for a subset of PH domains, including that of Btk. The actin-binding site was mapped to a 10-residue region of the N-terminal region of Btk. Basic residues in this short stretch are demonstrated to be involved in actin binding. Isolated PH domains induced actin filament bundle formation. Consistent with these observations, Btk binds F-actin in vitro and in vivo. Wild-type Btk protein is in part translocated to the cytoskeleton upon FceRI cross-linking, whereas Btk containing a mutated PH domain is not. Phosphatidylinositol 3,4,5-trisphosphate-mediated membrane translocation of Btk was enhanced in cytochalasin D-pretreated, FceRI-stimulated mast cells. These data indicate that PH domain-mediated F-actin binding plays a role in Btk co-localization with actin filaments.

Btk, a cytoplasmic protein-tyrosine kinase (PTK), is implicated in signal transduction initiated by numerous immune cell receptors including FceRI and B cell antigen receptor (reviewed in Refs. 1 and 2). Similar to other signaling proteins (3, 4), Btk is composed of several functional domains as follows: pleckstrin homology (PH), Tec homology (TH), Src homology (SH) 3, SH2, and SH1 (=kinase) domains in this order from N to C termini. Unlike Src family PTKs, Btk lacks the N-terminal myristoylation site and the C-terminal negative regulatory tyrosine residue (corresponding to Tyr-527 in pp60c-src). Upon B cell receptor stimulation, Btk is recruited to the plasma membrane through the interaction between the PH domain and phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (5–7). Then it is activated by phosphorylation at the activation loop (Tyr-551) by Lyn or Syk (8, 9). The phosphorylated Btk exhibits increased kinase activity and auto-phosphorylates at Tyr-231 (10). Since the proline-rich sequence in the TH domain has the capacity to interact with the SH3 domains of Src family PTKs (11), this interaction may be involved in Lyn phosphorylation of Btk. Btk regulates tyrosine phosphorylation of phospholipase C (PLC)-γ2 (and probably PLC-γ1 as well) and the sustained phase of calcium response in B cells (6, 7, 12). Another candidate target of Btk is BAP-135 (13), a protein of unknown function, and Btk also regulates stress-activated protein kinases, JNK and p38, in mast cells (14).

The PH domain is a protein module composed of loosely conserved sequences of ~100 amino acid residues (15, 16), which are found in numerous signal-transducing and cytoskeletal proteins (reviewed in Refs. 17–19). Tertiary structures of several PH domains including that of Btk have been solved (20–27). The core of the compact domain structures shared by these PH domains is a β sandwich formed by two nearly orthogonal antiparallel β sheets of 4 and 3 strands, respectively. One corner of the β sandwich is capped by the C-terminal α-helix. Three classes of molecules have so far been shown to interact with these apparently multifunctional domains. First, studies by Lefkowitz and co-workers (28, 29) established that the C-terminal portion and their flanking sequences of the PH domains of several proteins bind to the βγ complexes of heterotrimeric G-proteins. Second, several studies (26, 30, 31) showed that various PH domains bind to phosphatidylinositol 4,5-bisphosphate (PIP₂) and related molecules through the positively charged residues in their N-terminal halves. Low affinity binding of PIP₂ (Kd = ~30 µM (pleckstrin N-terminal PH domain) and 1.7 µM (PLC-δ1)) and Gβγ led to the hypothesis that PH domains function as membrane-localizing surfaces for PH domain-containing proteins. More recently, the Btk PH domain was shown to interact with PIP₂ (32) and inositol polyphosphates (33). Third, our previous studies (34, 35) demonstrated that multiple isoforms of protein kinase C (PKC) interact with several PH domains, including that of Btk. Btk was shown to be phosphorylated and enzymatically down-regulated by PKC in vitro and in vivo. A subsequent mapping study revealed that the second and third β-strands of the Btk PH domain interact with the C1 regulatory region of PKC that...
encompasses the pseudosubstrate region and the diacylglycerol/phorbel ester-binding region (36).

In the present study, we provide evidence that a subset of the tested PH domains including that of Btk bind to the filamentous form of actin (F-actin) but not the globular (or monomer) form of actin (G-actin). PH domains induce actin bundle formation in vitro. The actin-binding site was mapped within the N-terminal 10 residues of the Btk PH domain. As expected from the binding site mapping data, PIP2 competed with actin for binding to the PH domains, whereas neither PKC nor Gβγ competed with actin for the PH domain binding. Furthermore, the intact Btk molecule could interact with F-actin. Consistent with these in vitro data, a fraction of Btk was translocated from the cytosol to the cytoskeleton to co-localize with actin fibers upon FcεRI cross-linking.

EXPERIMENTAL PROCEDURES

Reagents

Monoclonal anti-actin antibodies were purchased from Sigma and Roche Molecular Biochemicals. Anti-Btk, anti-PKC(MC5), anti-PLC-γ2, and anti-GTPase antibodies were from Santa Cruz Biotechnology. Anti-phosphotyrosine mAb (4G10) was obtained from Upstate Biotechnology, Inc. Polyclonal anti-GST and mAb H902 against an epitope on the phosphotyrosine site of the Btk cytoplasmic domain were from the Jolla Institute for Allergy and Immunology, respectively. Pansorbin (ELIAS Entwicklungslabor, Freiburg, Germany) and T. Mustelin (La Jolla Institute for Allergy and Immunology), respectively. Polyethylene glycol (Poros), protein A-agarose (Sigma), or protein G-agarose (Sigma) were used for immunoprecipitation. Biotinylated goat anti-rabbit IgG (Calbiochem), protein A-agarose (Sigma), or protein G-agarose (Sigma) were used for immunoprecipitation. Biotinylated goat anti-rabbit IgG and streptavidin-fluorescein isothiocyanate were purchased from BIO-RAD. Coated mica squares were floated on the samples, rinsed with the same buffer, and then stained with 1% Triton X-100, 1% PAA, buffered with 50 mM Tris-HCl, pH 7.6, before observation in a JEM-1230 electron microscope at 100 kV.

In Vitro Binding Assays Using GST Fusion Proteins

Glutathione S-transferase (GST) fusion proteins were engineered by PCR-assisted cloning as described previously (34). Briefly, PCR was performed between a 5′ primer with a BamHI recognition sequence at the 5′ extension and a 3′ primer, corresponding to the N and C termini of individual PH domains, respectively, using cDNA clones. PCR products were cloned into the pcRII vector (Invitrogen), and the correct clone, confirmed by a limited sequence determination, was cloned into the pGEX-3T vector (37). Mutant PH domains were also engineered by PCR. Expression and purification of fusion proteins were described as described (38). Wild-type GST fusion proteins used in this study were as follows: GST-BtkPH (coding for residues 1–139 of Btk), GST-EmtPH (residues 1–109), GST-BtkPH (residues 1–105), GST-PLC-γ1PH (residues 241–350), GST-OSBPPH (residues 93–190), GST-PLC-γ2PH (residues 476–521), GST-YavPH (residues 348–459), GST-AFAP-110PH (residues 346–445), GST-SynCPh (residues 291–404), and GST-BtkSH3 (residues 216–274). In some experiments, the GST portion was removed from the oxyester-binding protein (OSBP) PH domain by digestion with thrombin (Sigma) followed by chromatography with benzamidine-Sepharose 6B (Amersham Pharmacia Biotech) and DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

In vitro solution binding was assayed as described (36). Briefly, glutathione-agarose-bound GST fusion proteins were incubated with MC-P murine mast cell lysates in Nonidet P-40 lysate buffer in the presence or absence of competing molecules, and bound proteins, after extensive washes, were detected by immunoblotting with anti-actin, anti-PKC, or anti-Gβγ antibodies. In some experiments, bound proteins were silver-stained using a kit from Bio-Rad. Filter binding was also done as described (34). GST fusion proteins were separated by SDS-PAGE and blotted. Blots were incubated with purified rabbit skeletal muscle actin (obtained from Sigma or freshly prepared according to Spudich and Watt (39) in Nonidet P-40 lysate buffer, and bound actin was detected by probing with anti-actin.

Asays for Detection of Actin-PH Domain Interactions

Co-sedimentation Assay—Freshly prepared G-actin was first polymerized in F buffer (G buffer (Ref. 52) plus 100 mM KCl and 2 mM MgCl₂) at room temperature for 20–30 min, incubated with GST fusion proteins or isolated PH domains for another 20 min, and centrifuged at 100,000 × g for 60 min. Both the supernatants and pellets recovered in SDS-PAGE sample buffer were analyzed by SDS-PAGE followed by Coomassie staining or immunoblotting.

Co-immunoprecipitation of Btk and actin. COS-7 cells were electroporated with 10 μg of the N-terminally epitope (H902)-tagged wild-type Btk cDNA or (R430B) btk cDNA and the pME18S vector (40). Cells were lysed in Nonidet P-40 lysis buffer 48 h after transfection. H902 mAb immunoprecipitates were analyzed by immunoblotting with anti-actin.

Actin Polymerization Assays

Fluorimetric Assay—Actin polymerization was measured by changes in fluorescence of pyrene-labeled actin based on the 25-fold increase in fluorescence of actin monomers when they incorporate into filaments. Actin polymerization was initiated by the addition of 2 mM MgCl₂ and 150 mM KCl to mixtures of 3 μM G-actin and various concentrations of PH domain proteins in buffer A (2 mM Tris, pH 7.6, 0.2 mM CaCl₂, 0.2 mM dithiothreitol, 0.5 mM ATP). The fluorescence intensity was measured with a Perkin-Elmer LS-50 instrument as a function of time using an excitation wavelength of 365 nm and measuring emission at 386 nm.

Dynamic Light Scattering (41)—The total light scattering intensity and the intensity of autocorrelation function of samples containing F-actin and various amounts of PH domains were measured using a Brookhaven Instruments BI30AT apparatus with a 128-channel autocorrelator. Samples were placed in a 6-mm inner diameter siliconized glass tube, and the autocorrelation function of quasielastically scattered light at 90° was determined by measurements of 5 min duration using a channel delay time of 10, 40, 160, and 640 μs in four sets of 321 channel-dead time spans to the range 0 to 1000.

Electron Microscopy—Samples containing 3 μM F-actin and either 0.8 μM OSBP PH domain or 0.8 μM GST in solutions containing 2 mM MgCl₂, 150 mM KCl, 5 mM Tris, pH 7.6, 0.2 mM CaCl₂, 0.3 mM dithiothreitol, 0.1 mM EDTA, and 0.5 mM ATP were negatively stained and observed by electron microscope using standard techniques. Carbon-coated microtubes were floated on the samples, rinsed with the same solution lacking proteins, and stained with 2% uranyl acetate in H₂O. Stained replicas were laid onto formic acid-washed carbon mesh grids, and the grids were observed in a JEOL electron microscope at 100 kV.

Mast Cell Stimulation and Btk Localization—RBL-2H3 rat mast cells were grown as a monolayer in RPMI 1640 supplemented with 20% fetal calf serum, 10 mM HEPES, 1 mM sodium pyruvate, and penicillin/streptomycin. Cells were sensitized with anti-DNP IgE and stimulated with DNP conjugates of bovine serum albumin for the indicated times. Cells were fixed with 3.7% formaldehyde, permeabilized, and incubated with anti-Btk followed by fluoresceinated anti-rabbit IgG as well as rhodamine-phalloidin. Stained cells were observed with a Bio-Rad MRC600 confocal laser scanner (Bio-Rad). Cells were visualized by light microscopy.

Expression and Partial Purification of Btk in Insect Cells—Non-tagged and human immunodeficiency virus gp120 epitope-tagged Btk proteins were expressed in Sf9 insect cells using a baculovirus expression vector, pVL1393, according to the manufacturer's instructions (Invitrogen). Tagging was done by inserting into the Nol site of the btk 1580–1690 coding region, and the fusion was a peptide Met-Ala-Ile-Gln-Ala-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Lys-Leu-Ser in front of the first codon. Btk proteins were partially purified by ion exchange chromatography with DEAE-Sepharose or Mono-Q (Amersham Pharmacia Biotech) or by affinity chromatography with H902 mAb immobilized onto Affi-Gel 10 (Bio-Rad).

RESULTS

Btk Is Co-localized with the Actin Fibers upon FcεRI Cross-linking—FcεRI cross-linking induces dramatic cytoskeletal changes accompanied by increased F-actin content, membrane ruffling, increased cell adhesion and spreading and the formation of actin-rich adhesion structures, termed actin plaques (44). In order to finely localize the Btk protein in mast cells undergoing these dynamic changes, RBL-2H3 rat mast cells were stained immunofluorescently with anti-Btk antibody, which gave a single Btk band in immunoblotting of RBL-2H3.
cell lysates (Fig. 1A). Confocal microscopy revealed that Btk has a diffuse distribution in the cytoplasm in resting cells. However, upon FcεRI cross-linking, co-staining of F-actin with rhodamine-phalloidin, and for Btk, using the above-mentioned rabbit anti-Btk followed by fluoresceinated anti-rabbit IgG. These images are from the dorsal surface of the cells. The right column shows the merged images with yellow areas indicating co-localization of Btk and F-actin.

The immunofluorescence results suggest that Btk may interact with actin in vivo. This possibility was further tested by immunoprecipitation from heterologous cells expressing Btk proteins. Actin was co-immunoprecipitated with anti-epitope antibody (H902) from lysates of COS-7 cells expressing epitope-

Fig. 1. Btk is co-localized with actin fibers. A, specificity of anti-Btk antibody used in this study. RBL-2H3 cells were sensitized by incubating overnight with anti-DNP IgE monoclonal antibody and left unstimulated or stimulated by DNP conjugates of human serum albumin for 3 min. Total cell lysates were separated by SDS-PAGE (8% gel) followed by immunoblotting with anti-Btk antibody. Positions of Btk and molecular mass markers are indicated. B, IgE-sensitized RBL-2H3 cells were stimulated by antigen for the indicated intervals before fixation and permeabilization. Cells were double-stained for F-actin, using rhodamine-phalloidin, and for Btk, using the above-mentioned rabbit anti-Btk followed by fluoresceinated anti-rabbit IgG. These images are from the dorsal surface of the cells. The right column shows the merged images with yellow areas indicating co-localization of Btk and F-actin.

tagged wild-type or kinase-dead (K430R) Btk (Fig. 2). These data suggest that actin interacts directly or indirectly with Btk and that the kinase activity of Btk is not required for this interaction.

PH Domains Including That of Btk Directly Bind Actin—In order to find the molecular basis for the Btk-actin interaction, we examined whether individual domains of Btk and other proteins bind to actin. GST fusion proteins containing the PH domains of Btk or OSBP immobilized on glutathione-agarose beads were incubated with MCP-5 mast cell lysates. Proteins bound to these fusion proteins were resolved by SDS-PAGE and stained with silver (data not shown). The GST fusion protein of the OSBP PH domain (GST-OSBPH) specifically bound a major protein of 43 kDa. The binding of the 43-kDa protein to GST-OSBPH was resistant to a high salt (1.2 M NaCl) wash. The closeness of this protein in size prevented GST-BtkPH from being separated from the 43-kDa protein by SDS-PAGE. The identity of this protein as actin was demonstrated by immunoblotting of proteins bound to GST-BtkPH or GST-OSBPH with anti-actin antibody (Fig. 3A). GST-BtkPH bound slightly more actin than GST-OSBPH in repeated experiments. In similar experiments using several other PH domains (Fig. 3B), binding of GST-PLCγ2PH to actin was very weak, whereas GST-PLCγ2PH containing the N-terminal portion of the split PH domain of rat PLC-γ2 did not bind to actin. In contrast, GST, GST-BtkSH3, or GST-BtkSH2 did not bind to actin in the same assay (Fig. 3B and data not shown). These results indicate that only a subset of PH domains bind to actin.

Next, we tested whether the interaction between actin and PH domains is direct or not. Purified actin (>98% pure) was used in place of mast cell lysates in a co-immunoprecipitation experiment. When affinity purified GST or GST-OSBPH released from glutathione-agarose beads was incubated with actin and precipitated with anti-GST antibodies and Pansorbin, actin was co-precipitated with GST-OSBPH but not with GST (data not shown), suggesting that the interaction between actin and PH domains takes place without the involvement of other proteins.

Another approach to demonstrate the direct interaction between PH domains and actin was used to test the actin-binding capacity of various PH domains. Affinity purified GST fusion proteins derived from various signaling proteins were resolved by SDS-PAGE and blotted onto polyvinylidene difluoride membranes. Blots were incubated with actin, and bound actin was immunologically detected. In this filter binding assay (Fig. 3C and the data summarized in Fig. 4B), actin binding capacities were observed with the PH domains from Btk, Emt, OSBP, and pleckstrin (both N- and C-terminal PH domains). However, PH domains derived from PLC-γ2, AFAP-110 (C-terminal PH domain), or syntrophin (C-terminal PH domain) did not bind to
actin. The Vav PH domain had a weak binding capacity.

Involvement of Basic Amino Acid Residues toward N Termini of PH Domains in Interactions with Actin—The actin-binding site was mapped within the PH domain of Btk. Filter binding assays were performed using various truncated and mutated GST fusion proteins and the data summarized in Fig. 4A. Since some actin-binding proteins interact with actin via their positively charged residues, mutants with substitutions of basic residues with alanine were tested. The results demonstrate that the amino acid sequence encompassing residues 11–20 is a minimal actin-binding stretch in which the basic residues play critical roles in the actin-PH domain interaction. The importance of the basic residues in this stretch is also reflected in the lack of actin binding capacity in the PH domains that lack the corresponding basic residues, e.g. those derived from PLC-γ2, AFAP-110, and sytophin (Fig. 4B). Furthermore, we examined several mutant PH domains of PLC-γ2 in which basic residues were introduced within the first β sheet. As shown in Fig. 4C, the PLC-γ2 mutant PH domain with triple substitutions, T477K/M483K/W484K, was positive for actin binding, whereas the other mutants with single substitutions at position 477 or 483 or with double substitutions at positions 483 and 484 failed to show any actin binding capacity. The creation of a gain-of-function mutant with the PLC-γ2 PH domain further underscores the involvement of the basic residues in binding to actin.

F-actin, but Not G-actin, Binds to the PH Domains—The above data did not specify or exclude either the monomeric G-actin or the polymerized F-actin as the PH domain ligand. Therefore, we took two approaches to determine which form of actin binds PH domains. Co-sedimentation experiments were carried out on a mixture of freshly purified actin and either GST, GST-OSBPPH, or GST-BtkPH(1/76) in F buffer. Affinity purified PH domain fusion proteins specifically co-sedimented with F-actin, whereas negligible amounts of these proteins co-sedimented with bovine serum albumin (Fig. 5A). In accordance with the in vitro binding results (Fig. 3), GST-BtkPH(1/76) co-sedimented with F-actin more efficiently than GST-OSBPPH, whereas GST did not. Purified OSBPPH and BtkPH(1/76) proteins that were released from GST by thrombin digestion also co-sedimented with F-actin (data not shown). In another type of experiment, mixtures of actin with either GST or GST-PH fusion proteins in G buffer were analyzed by native polyacrylamide gel electrophoresis followed by Coomassie Brilliant Blue staining. None of the GST-PH proteins made complexes with G-actin (data not shown).

Binding characteristics of PH domain with F-actin were analyzed using GST-OSBPPH. Co-sedimentation experiments were done on a fixed amount of freshly purified actin and increasing concentrations of GST-OSBPPH. When saturating amounts of GST-OSBPPH were bound to F-actin, the binding stoichiometry was one molecule of GST-OSBPPH to 5 molecules of actin (Fig. 5B, upper). Although similar analysis on GST-BtkPH was hampered by limited yields of soluble protein released from glutathione beads, the affinity of GST-BtkPH would be higher than that of GST-OSBPPH given the better in vitro binding results. These characteristics are similar to those of some actin-binding proteins (45).

Actin Competes for PH Domain Binding with PIP2, but Not with PKC or with G Protein Subunit—Since the PKC-, PIP2-, and inositol polyphosphate-binding sites were also mapped within the N-terminal portion of PH domains (26, 27, 30, 33, 36), we examined whether binding to these molecules interferes with the capacity of the PH domain to bind to actin, and vice versa. The presence of PIP2 in the incubation mixture of actin and GST-OSBPPH (or GST-BtkPH(1/76)) inhibited the actin co-sedimentation with GST-OSBPPH (or GST-BtkPH(1/76)) in a PIP2 concentration-dependent manner with an IC50 of ~2 μM (Fig. 5B, lower). This is consistent with the presumption that Lys-12 of Btk interacts with inositol 1,3,4,5-tetrakisphosphate (27). Phosphatidylinositol 4-phosphate also showed a similar, but less potent, inhibitory effect on the actin-GST-OSBPPH co-sedimentation (Fig. 6A). Phosphatidylycerine showed a slightly inhibitory effect at the highest tested concentration (50 μM). However, the other phospholipids tested, including phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol, failed to affect the actin binding of GST-OSBPPH.

When GST-BtkPH was incubated with mast cell lysates in the presence of increasing concentrations of actin, no changes in the level of bound PKC were found (data not shown). The addition of various concentrations of rat brain PKC to the mixture of GST-BtkPH beads and actin did not change the level of bound actin (data not shown). These data showed that the interaction of PH domains with actin is not significantly affected by PKC, although mapping studies have demonstrated physically adjacent regions in the PH domain as the binding sites, i.e. residues 11–20 of Btk for actin (Fig. 4A) versus residues 28–45 for PKC (36). Furthermore, actin did not have effects on the binding of GST-BtkPH to Gβ, as expected from the previous observation that Gβ binds to the C-terminal and further downstream sequences (Fig. 6B). Of note, GST-OSBPPH did not bind to Gβ. Therefore, we have concluded that PH domains have distinct binding sites for these binding molecules in a relatively short stretch of the sequence.

PH Domains Induce Actin Filament Bundle Formation—Effects of PH domains on actin polymerization were examined by fluorometric assays using pyrene-labeled actin, dynamic light scattering, and electron microscopic techniques. Since we...
could easily obtain a large quantity of GST-OSBP PH protein, we used purified OSBP PH domain preparations that were released from GST by digestion with thrombin. Fig. 7A shows that the OSBP PH domain alters slightly the fluorescence of pyrene-labeled actin monomers but does not perturb the kinetics of polymerization unless it is present at a relatively high molar ratio to actin of 1:4. The decrease in polymerization rate is consistent with the occlusion of filament ends that would occur if the PH domain caused actin filament bundle formation, as shown below. The data shown in Fig. 7A also rule out the possibility that the PH domain either sequesters actin monomers or blocks the ends of actin filaments. The final fluorescence of pyrene-labeled actin was also not significantly altered by the PH domain, again consistent with a lack of monomer binding or filament barbed end blocking. An assay (46) in which rhodamine-phalloidin was added to F-actin polymerized with or without PH domains confirmed that approximately equal amounts of actin polymerized in both cases (data not shown). The similar rates of rhodamine-phalloidin binding also indicated that the binding of the PH domain does not perturb the filament structure in a way that alters its interaction with phalloidin.
Fig. 5. PH domains co-pellet with F-actin in co-sedimentation assays. A, freshly purified actin (1.6 μM) was polymerized in F buffer (200 μl) in the presence of ~6 μM each of GST, GST-OSBP, or GST-BtkPH(1/76). Bovine serum albumin (3 μM) was used to check for nonspecific precipitation of GST or GST fusion proteins. Precipitated (upper, the entire precipitates analyzed) and non-precipitated (lower, one-tenth of the whole volume) proteins were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. Positions of actin, bovine serum albumin, GST, and GST fusion proteins are indicated. B, binding kinetics of GST-OSBP with F-actin (upper panel). Co-sedimentation experiments were carried out using increasing concentrations of GST-OSBP and a fixed concentration (1 μM) of freshly purified actin. Amounts of GST-OSBP bound or unbound to F-actin were estimated by densitometry using the standard bands with known amounts. Amounts of the non-specifically precipitated GST-OSBP were subtracted. Scatchard analysis was also done to obtain the dissociation constant (lower panel). B/F denotes the ratio of the bound to free GST-OSBP proteins.

Fig. 6. PIP2, but not Gβ, competes with F-actin for binding to PH domains. A, co-sedimentation experiments were done in the presence of 0 (lane 1 of each group), 0.04 (lane 2), 0.2 (lane 3), 1 (lane 4), 5 (lane 5), or 50 μM (lane 6) phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylserine (PS). F-actin-bound or precipitated proteins were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining. Positions of actin and GST-OSBP are indicated. B, in vitro solution binding experiments were done to detect Gβ binding to PH domains. GST or GST fusion protein beads were incubated with MCP-5 mast cell lysates in the presence of various concentrations of exogenous actin, and bound Gβ was detected by immunoblotting.

Light scattering assays show that the size of the structures formed by polymerizing actin in the presence of the OSBP PH domain increases at increasing concentrations of the PH domain (Fig. 7B). This large increase in light scattering intensity caused by the PH domain at molar ratios to actin as low as 1:30 suggests that the PH domain promotes actin filament bundle formation, and these bundles of actin were observed by electron microscopy (Fig. 7E). The fact that light scattering is increased by the PH domain in solutions of both low and high ionic strengths shows that the bundling activity of the PH domain appears to be specific and not simply a feature of the polycationic nature of the PH domain, since bundling by polycations is generally inhibited (but not prevented) by 150 mM KCl. Fig. 7C shows the intensity autocorrelation function derived from dynamic light scattering. The slower decay of the autocorrelation function at increasing concentrations of the PH domain confirms that large bundles of filaments form in the presence of the PH domain.

Effects of the Btk PH domain on actin bundle formation were also measured by light scattering. As shown in Fig. 7D, addition of submicromolar amounts of the wild-type PH domain as a GST fusion protein caused a concentration-dependent increase in scattering from 3 μM F-actin, whereas no effect was observed with GST. This suggests that the actin filament bundle formation is a general property of actin-binding PH domains. A smaller effect on actin bundle formation was seen with the xid (R28C) mutant PH domain, indicating that a mutation outside the minimal actin-binding site can interfere with the actin binding capacity. This was further confirmed by the in vitro binding assay. Thus, approximately three times more GST-BtkPH(xid) protein than the wild-type fusion protein was required for binding to the same amount of actin in MCP-5 mast cell lysates (Fig. 8A).

Btk Co-localization with Actin and Membrane Translocation Are Dependent on the PH Domain—We examined whether the intact Btk molecule can bind actin in the setting of purified proteins. N-terminally epitope-tagged Btk, affinity purified to a purity of >80% with Sepharose-conjugated anti-epitope (H902) mAb, was incubated with freshly prepared actin and centrifuged. Sedimented proteins detected by immunoblotting showed that intact Btk molecules co-sedimented with actin, not with bovine serum albumin (Fig. 8B). These data, together with the association of Btk with actin in COS cells (Fig. 2), indicates...
FIG. 7. Effects of PH domains on actin polymerization. A, effect of isolated, purified OSBP PH domain polypeptide on actin polymerization was evaluated by the measurement of fluorescence of pyrene-labeled actin. 3 μM pyrene-labeled actin was polymerized in the presence of 0 (open}

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FIG. 8. Btk interacts with actin in vitro and in vivo. A, the indicated amounts of GST or GST fusion protein beads were incubated with MCP-5 mast cell lysates. The bead-bound actin was detected by immunoblotting with anti-actin antibody. B, the whole molecule Btk binds F-actin. The epitope-tagged Btk (200 ng) expressed in baculovirus immunoblotting with anti-actin antibody. Positions of Btk and actin that was nonspecifically stained due to its excess amount are indicated. C, Btk was translocated to the cytoskeleton upon FcεRI cross-linking. BMMC from CBA/J (wild-type) or CBA/CaH-NxidJ (xid) male mice were sensitized by an overnight incubation with anti-DNP IgE and stimulated by DNP-human serum albumin conjugates for the indicated intervals. Triton X-100-insoluble proteins (=cytoskeleton) and SDS-PAGE sample buffer soluble proteins (=total cell lysate) were analyzed by immunoblotting with anti-Btk that Btk can associate with F-actin in vitro and in vivo.

Furthermore, we determined whether Btk is present in the detergent-insoluble compartment (=the cytoskeleton) of BMMC. The amount of Btk protein present in the cytoskeleton increased significantly upon FcεRI cross-linking (Fig. 8C). Importantly, the xid mutant Btk did not translocate to the cytoskeleton upon stimulation, finding consistent with a decreased binding activity of the xid PH domain (Figs. 7D and 8A). Together with Btk co-localization with actin fibers in FcεRI-stimulated RBL-2H3 cells (Fig. 1B), these findings support a role of the Btk PH domain in the cytoskeletal localization of Btk protein.

FcεRI cross-linking also induces the membrane translocation of Btk (43) probably through the interaction of the Btk PH domain withPIP3 (5–7). We examined the effects of an actin filament-disrupting agent, cytochalasin D, and a phosphatidylinositol 3-kinase inhibitor, wortmannin, on the Btk localization in BMMC. Wortmannin, as expected, ablated the membrane translocation of Btk while cytochalasin D increased Btk levels in the membrane fraction in both unstimulated and FcεRI-stimulated mast cells (Fig. 9). In contrast, membrane translocation of PLC-γ2 which does not have the ability to bind to actin was not affected by either wortmannin or cytochalasin D. Confocal microscopy confirmed that wortmannin treatment did not prevent the Btk-actin filament co-localization, but cytochalasin D ablated the co-localization (data not shown). These observations suggest that a reduction in F-actin content favors the PH domain interaction with membrane-bound PIP3 and vice versa.

**DISCUSSION**

The present study provides morphological evidence that Btk is translocated to the actin fibers beneath ruffled membranes upon FcεRI cross-linking. As a molecular basis for Btk co-localization with the actin fibers, PH domains including that of Btk were demonstrated to bind F-actin but not G-actin. In support of this notion, we observed lower actin-binding ability of the PH domain with xid mutation as a basis of the defective FcεRI-induced translocation of xid Btk. **PH Domains Bind F-actin**—Several PH domains in the context of GST fusion proteins or as isolated polypeptides were shown to bind to F-actin and induce an actin bundle formation. The short sequence around the first β sheet of the Btk PH domain was sufficient for binding to actin. Importantly, the basic residues toward both ends in this short stretch (10 residues) were critical determinants for actin binding. There are several precedents for the involvement of basic residues in actin binding (47, 48). Accordingly, some PH domains devoid of the corresponding basic residues did not exhibit this actin binding capacity. More directly, loss-of-function mutants of the Btk PH domain were obtained by substituting for the basic residues with Ala, and a gain-of-function mutant of the N-terminal half of the PLC-γ2 PH domain was made by substituting with Lys for the three residues in the first β sheet region. However, actin binding does not seem to be defined solely due to these basic residues 1) because the OSBP PH domain and the Vav PH domain have very different actin-binding ability despite the fact that both PH domains have equivalent numbers of basic residues in the actin-binding region. 2) The conformation around the actin-binding region must be important for the interaction because the xid mutation affects the actin-binding ability of the Btk PH domain.

**Actin Filament Bundling Activity of PH Domains**—Optical and electron microscopic observations demonstrated an actin filament bundle-forming capability of PH domains as isolated or GST fusion proteins. This activity and the use of a basic residue-rich stretch for actin binding are shared by the well characterized actin-bundling protein, MARCKS (48). MARCKS is a major PKC substrate. Dephospho-MARCKS induces actin bundle formation in the presence of concentrations of GST (open circle), GST-BtkPH(wild-type) (closed circle), or GST-BtkPHxid (inverted triangle). E, electron micrograph of actin filaments formed in the presence of GST (left) or OSBP PH domain protein (right). Scale bar, 0.5 μm.
filament bundling, whereas phospho-MARCKS, which has phosphorylation sites within the actin-binding region, does not. PKC-dependent phosphorylation displaces MARCKS from the plasma membrane to the cytoplasm. Filament bundling would require molecules either to have two (or more) actin-binding sites or to dimerize. Since an isolated PH domain has this activity and since the minimal actin-binding site is mapped within a 10-residue region of the Btk PH domain, we reason that PH domains have a dimerizing activity with a single actin-binding site. Indeed, Datta et al. (49) reported that the PH domain of Rac/Akt protein serine/threonine kinase has a dimerizing activity. Binding of phosphatidylinositol 3,4-bisphosphate to this PH domain facilitates dimerization of Rac/Akt (50). Alternatively, micromolar concentrations of multivalent cations such as the MARCKS peptide may induce actin filament bundling by condensation on the acidic actin filaments, thereby either reducing the electrostatic repulsion between filaments or inducing an attractive interaction (51). A recent report suggests that some F-actin bundling activities may involve polyelectrolyte effects in the absence of multifunctional actin-binding proteins (52). In any case Btk as a whole may involve polyelectrolyte effects in the absence of multifunctional actin-binding proteins (52). In any case Btk as a whole may involve polyelectrolyte effects in the absence of multifunctional actin-binding proteins (52). In any case Btk as a whole may involve polyelectrolyte effects in the absence of multifunctional actin-binding proteins (52).

Concluding Remarks—Btk regulates cytokine production by regulating the stress-activated protein kinases, JNK/SAPKs, upon FcRI cross-linking (14). Therefore, it is likely that Rho family GTases, which are known to be involved in FcRI-induced degranulation (56), are involved in signaling pathways downstream of Btk and upstream of JNK/SAPKs that regulates the activity of c-Jun and other transcription factors. It is an intriguing possibility that Btk is translocated to the actin cytoskeleton which Btk and its candidate downstream signal transducers, i.e. Rho family GTPases and their targets, work coordinately to reorganize. This is particularly interesting because actin polymerization is regulated via uncapping of actin polymers by binding of PIP_{2} as a PH domain ligand, to capping proteins such as gelsolin and profilin. Actin filament remodeling is a dynamic, orchestrated phenomenon involving numerous actin-mediated protein-protein and protein-lipid interactions may play key roles.

Acknowledgments—We thank Louise Wang for outstanding technical advice and Drs. Veila M. Fowler and Philip Kuhlen for their advice and some actin preparations.

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Libo Yao, Paul Janmey, Luciano G. Frigeri, Wei Han, Jun Fujita, Yuko Kawakami, John R. Apgar and Toshiaki Kawakami

J. Biol. Chem. 1999, 274:19752-19761.
doi: 10.1074/jbc.274.28.19752

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