THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 53, Issue of December 31, pp. 55089–55096, 2004
Printed in U.S.A.

SHIP Family Inositol Phosphatases Interact with and Negatively Regulate the Tec Tyrosine Kinase*

Received for publication, July 19, 2004, and in revised form, October 12, 2004
Published, JBC Papers in Press, October 18, 2004, DOI 10.1074/jbc.M408141200

Michael G. Tomlinson‡§¶, Victoria L. Heath§‖, Chris W. Turck‡, Steve P. Watson§, and Arthur Weiss‡**

From the ‡Department of Medicine and Howard Hughes Medical Institute, University of California, San Francisco, California 94143 and the §Centre for Cardiovascular Sciences, Institute of Biomedical Research, University of Birmingham, Birmingham B15 2TT, United Kingdom

The Tec family of protein-tyrosine kinases (PTKs), that includes Tec, Itk, Btk, Bmx, and Txk, plays an essential role in phospholipase Cγ (PLCγ) activation following antigen receptor stimulation. This function requires activation of phosphatidylinositol 3-kinase (PI 3-kinase), which promotes Tec membrane localization through phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P_3) generation. The mechanism of negative regulation of Tec family PTKs is poorly understood. In this study, we show that the inositol 5′ phosphatases SHIP1 and SHIP2 interact preferentially with Tec, compared with other Tec family members. Four lines of evidence suggest that SHIP phosphatases are negative regulators of Tec. First, SHIP1 and SHIP2 are potent inhibitors of Tec activity. Second, inactivation of the Tec SH3 domain, which is necessary and sufficient for SHIP binding, generates a hyperactive form of Tec. Third, SHIP1 inhibits Tec membrane localization. Finally, constitutively targeting Tec to the membrane relieves SHIP1-mediated inhibition. These data suggest that SHIP phosphatases can interact with and functionally inactivate Tec by de-phosphorylation of local PtdIns 3,4,5-P_3 and inhibition of Tec membrane localization.

Antigen receptor signal transduction is central to the development and function of T and B lymphocytes in generating a productive immune response. Key features of antigen receptor signaling include rapid activation of non-receptor protein tyrosine kinases (PTKs),[1] of the Src, Syk, and Tec families, followed by their rapid inactivation to terminate the signal. The activation mechanisms for these PTKs have been well characterized in recent years (reviewed in Refs. 1–3). However, the mechanisms by which their actions are terminated are not well understood (4, 5). Since the inactivation process is rapid, it is likely that negative regulatory phosphatases are directly recruited to activated PTKs. Few such PTK-phosphatase interactions have been reported, suggesting that they may be relatively weak and transient.

The Tec family of PTKs, comprising Tec, Btk, Itk, Bmx, and Txk (Rlk in mouse), perform an essential role in antigen receptor signaling of T and B lymphocytes (reviewed in Refs. 6 and 7). Tec family PTKs consist of an N-terminal pleckstrin homology (PH) domain (not present in Txk), a proline-rich region (also termed the Tec homology domain), Src homology 3 (SH3) and SH2 domains, and a C-terminal tyrosine kinase domain. Tec PTKs are activated following antigen receptor cross-linking by a two-step mechanism involving phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P_3) generation by phosphatidylinositol 3-kinase (PI 3-kinase), which promotes membrane targeting via the PH domain, and *trans*-phosphorylation by a Src family PTK, which activates the kinase domain (6, 7). The importance of Tec family PTKs was initially realized when mutations in Btk were found to result in the human disease X-linked agammaglobulinemia (XLA), in which mature B cells fail to develop (8). Subsequent analyses of gene-targeted mice and cell lines have confirmed that the primary effector function for Btk and Itk is the phosphorylation and activation of phospholipase Cγ (PLCγ) (6–8). Btk also promotes the synthesis of the PLCγ substrate, phosphatidylinositol 4,5-bisphosphate, by recruiting phosphatidylinositol 4-phosphate 5-kinases to the membrane (9). PLCγ generates the second messenger inositol trisphosphate and diacylglycerol, which promote mobilization of Ca^{2+} and activation of protein kinase C/Ras, respectively. Together these signals induce the expression of genes that are regulated by transcription factors such as nuclear factor of activated T cells (NFAT).

The mechanisms by which Tec family PTKs are inactivated are relatively poorly understood. Structural studies have lead to the hypothesis that Tec, Btk, and Itk are negatively regulated by an intramolecular interaction between the adjacent SH3 domain and proline-rich region (10–12). However this mechanism has not been proved. A number of distinct mechanisms have been reported for Btk inactivation. These include direct inhibition of Btk activity by the PH domain-interacting protein IBtk (13) and the SH3 domain-binding protein Sab (14). In addition, Btk membrane localization can be inhibited by protein kinase C-β-mediated phosphorylation of the proline-rich region (15), or by the 5′-inositol phosphatase SHIP1, which hydrolyzes PtdIns 3,4,5-P_3 (16, 17).

The SHIP family of inositol phosphatases, comprising the hematopoietic cell-specific SHIP1, and the ubiquitous SHIP2, are characterized by an N-terminal SH2 domain, a central inositol phosphatase domain and a C-terminal tail containing

* This work was supported, in part, by the Rosalind Russell Medical Research Center for Arthritis and by Grant CA72531 from the NCI, National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Centre for Cardiovascular Sciences, Division of Medical Sciences, Institute of Biomedical Research, Wolfson Drive, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK. Tel.: 0121-414-8308; Fax: 0121-415-8817; E-mail: m.g.tomlinson@bham.ac.uk.

‖ Current address: School of Biosciences, University of Birmingham, Birmingham B15 2TT, UK.

** Current address: Max Planck Institute of Psychiatry, Munich, Germany.

[1] The abbreviations used are: PTK, protein-tyrosine kinase; GST, glutathione *S*-transferase; mAb, monoclonal antibody; IL, interleukin; HA, hemagglutinin; PI and PtdIns, phosphatidylinositol; PLC, phospholipase C; NFAT, nuclear factor of activated T cells; SH, Src homology domain; β-gal, β-galactosidase; TCR, T cell receptor.

protein interaction motifs for SH3, SH2, and phosphotyrosine binding (PTB) domains (reviewed in Refs. 18 and 19). SHIP phosphatases de-phosphorylate the 5 position on inositol rings of PtdIns 3,4,5-P_3 and inositol-(1,3,4,5)-tetrakisphosphate (IP4). PtdIns 3,4,5-P_3 is a ligand for the PH domains of Tec PTKs and several other PH domain-containing signaling proteins, and is required for membrane localization and function of Btk (16, 17) and Itk (20, 21). SHIP phosphatases are themselves regulated by membrane targeting via binding of their SH2 domain to phosphorylated immunoreceptor tyrosine-based inhibitory motifs (ITIMs), the best characterized of which is present in the inhibitory low affinity receptor for IgG antibodies, FcγRIIB, expressed in B cells, mast cells and macrophages (18, 19). The phenotypes of SHIP-deficient mice are consistent with the role of SHIP phosphatases as important negative regulators. SHIP1$^{-/-}$ mice die at an early age due to myeloid cell infiltration into the lungs, which is caused by enhanced signaling through a number of receptors (22, 23), whereas SHIP2$^{-/-}$ mice die perinatally because of insulin hypersensitivity (24).

In the present study we have addressed the mechanism of negative regulation for Tec. Previous studies in cell lines have shown that Tec, like Btk and Itk, can function in antigen receptor signaling to activate PLCγ (25–27). Analyses of Tec$^{-/-}$ and Btk$^{-/-}$Tec$^{-/-}$ mice have suggested that Tec plays a relatively minor role alongside Btk in PLCγ activation in B cells (28) and in platelets (29), which may be caused by its relatively low level of expression (30). Tec is believed to play a more important role in activated and Th2 effector T cells, where Tec protein is substantially up-regulated (30). In a recent study, we demonstrated that Tec is unique among Tec family PTKs in its capacity to induce PLCγ1 phosphorylation and NFAT activation when overexpressed in T and B cell lines (30). These data suggest that Tec is regulated in a manner that is distinct from other Tec family PTKs. Indeed, we hypothesize that Tec overexpression overcomes the effects of an endogenous Tec-specific negative regulator. The Tec SH3 domain is a candidate binding partner for such a negative regulator, because a Tec SH3 point mutant is hyperactive, unlike point mutants of other Tec domains (30). In this study we have identified a novel interaction between SHIP family phosphatases and the SH3 domain of Tec, but not other family members. This interaction inhibits Tec membrane localization and effector function.

EXPERIMENTAL PROCEDURES

Cells—The Jurkat and HUT78 T cell lines were cultured in RPMI supplemented with 5% fetal bovine serum, penicillin, streptomycin, and glutamine. The human kidney 293T cell line was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and glutamine.

Plasmids—Glutathione *S*-transferase (GST)-tagged SH3 domain constructs were generated by PCR from mouse Tec, isoform IV (amino acids 178–245), Btk-(212–282), and Itk-(171–238), and human Bmx-(186–297) and Txk-(82–151) cDNA (26) and cloned into pGEX-2T (Amersham Biosciences, Piscataway, NJ). Tec family expression constructs, HA-tagged at the N terminus, have been described previously (30). Point mutants of the Tec proline-rich region (P158A, P161A, P168A, P169A, P170A, P171A) and SH3 domain (W215L, W216L) have also been described before (30). Tec family yeast two-hybrid constructs were generated by PCR amplification of inserts followed by cloning into the pACT2 vector (BD Biosciences), in-frame with the N-terminal GAL4 activation domain. Myr-Tec was generated by PCR to replace the HA tag with the 17 N-terminal amino acids of chicken Src. Tec-GFP, tagged with GFP at the C terminus, was described previously (30). The human SHIP1 and SHIP2 cDNAs were gifts from L. Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, WA) and S. Moodie (Metabolex Inc., Hayward, CA), respectively. SHIP1 and SHIP2 were PCR-amplified and subcloned into the pEF6 vector (Invitrogen, Carlsbad, CA), in-frame with the C-terminal Myc tag. Truncation mutants of full-length SHIP1, containing amino acids 1–173, 1–370, 364–1192, and 900–1192, were generated by PCR. The SHIP1 yeast two-hybrid construct was generated by PCR amplification of SHIP1 followed by cloning into the pGBT9 vector (BD Biosciences), in-frame with the N-terminal GAL4 DNA binding domain. The NFAT luciferase reporter contained three copies of the distal NFAT site from the *IL-2* promoter (31). The pEF6-*lacZ* expression construct was from Invitrogen.

Antibodies—The anti-SHIP1 rabbit antiserum was kindly provided by M. Coggeshall (University of Oklahoma, Oklahoma City, OK) and the anti-Tec rabbit antiserum was from Upstate Biotechnology (Charlottsville, VA). The anti-HA tag mAb was 16B12 (Covance Research Products, Berkeley, CA), the anti-Myc tag mAb was 9B11 (Cell Signaling Technology, Beverley, MA), and the anti-GST mAb was from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-TCRβ mAb C305, for Jurkat stimulations, was described previously (32).

Biochemical Analyses—Recombinant GST-SH3 fusion proteins were made in the BL21(DE3)pLysS strain of *Escherichia coli* (Invitrogen) according to the manufacturer's protocol (Amersham Biosciences). For GST pull-downs, Jurkat and HUT78 cell lysates were prepared by lysing 1×10^8 cells in 1 ml of 1% Nonidet P-40 lysis buffer containing protease inhibitors, precleared three times with 25 μg of GST coupled to 25 μl of glutathione-Sepharose (Amersham Biosciences), and centrifuged at 100,000 × *g* for 30 min. GST pull-downs were done from these lysates using 10 μg of recombinant GST-SH3 fusion protein coupled to 12.5 μl of glutathione-Sepharose. Pull-downs were washed twice with 1 ml of 1% Nonidet P-40 lysis buffer, twice by centrifuging through 1 ml of 1 M sucrose in 1% Nonidet P-40 lysis buffer, and twice more with 1 ml of 1% Nonidet P-40 lysis buffer. Proteins were separated by SDS-PAGE and visualized by SimplyBlue Coomassie staining (Invitrogen). Protein bands were excised, the gel sliced into 1-mm square pieces and subjected to an in-gel digestion with endoproteinase Lys-C (Roche Diagnostics, Indianapolis, IN) (33). Peptides were extracted from the gel pieces and cleaned up using a gel-loader pipette tip filled with 100 nl of POROS C_{18} resin (Applied Biosystems, Foster City, CA). The peptide mixture was eluted into a nanospray glass capillary (Protana, Odense, Denmark) using 500 nl of 60% methanol, 5% formic acid. After mounting the capillary onto the nanospray source (Protana), peptide solutions were infused into an LCQ Classic Iontrap mass spectrometer (ThermoFinnigan, San Jose, CA) at a flow rate of 10 nl/min. Individual peptide ions were isolated and subjected to MS/MS analysis using an automated "zoomscan walking" method (34). The acquired MS/MS spectra were subjected to a non-redundant protein data base search using the SEQUEST program (Jimmy Eng and John Yates III, University of Washington, Seattle, WA) (35). For GST pull-downs from SHIP-transfected 293T cells, several modifications were made to the above protocol; the preclearing and 100,000 × *g* centrifuge steps were omitted, the pull-down used 5 μg of recombinant GST-SH3 fusion protein, and samples were washed four times with 1 ml of 1% Nonidet P-40 lysis buffer. Immunoprecipitations and Western blotting were performed as previously described (36). Western blots were visualized using Western Lightning chemiluminescence reagents (PerkinElmer Life Sciences) in combination with either a Kodak Image Station (Kodak, Rochester, NY) or with Hyperfilm (Amersham Biosciences) developed using a Compact X4 film processor (Xograph Imaging Systems, Gloucestershire, UK).

Yeast Two-hybrid—Yeast media and culture conditions were as previously described (37), except that in synthetic media, twice the level of amino acids and nucleotides were used. The strain PJ69–4A (*MAT**a** trp1-901, leu2-3,112, ura3-52, his3-200, gal4Δ, gal80Δ, GAL2-ADE2, LYS2::GAL1-HIS3, met2::GAL7-lacZ*) (38) was transformed with combinations of Gal4p activation and binding domain fusions. Yeast two-hybrid assays were performed as previously described (39). Briefly, cultures were grown in selective media to stationary phase, diluted to OD_{600} of 0.5 and spotted on to synthetic media either containing or lacking histidine. Plates were incubated for 5 days at room temperature.

Transfections—Jurkat and HUT78 cells were transfected in a volume of 0.4 ml of RPMI (without serum) by electroporation using a Gene Pulser Electroporator (Bio-Rad) set at 250 V, 960 μF (Jurkat) or 240 V, 960 μF (HUT78). 293T cells were transfected by the calcium phosphate method using the CalPhos mammalian transfection kit (BD Biosciences).

Luciferase Assays—Jurkat and HUT78 cells were transfected, as described above, with the expression construct of interest, in addition to 20 μg of the luciferase reporter construct and 2 μg of pEF6-*lacZ* to control for transfection efficiency. Sixteen hours after transfection, live cells were counted by trypan blue exclusion and samples divided for luciferase assay, β-gal assay, and Western blotting, as described (30). All luciferase assay data were normalized to β-gal values. Expression of each construct was confirmed by Western blotting.

Microscopy—Transfected Jurkat cells were sorted for Tec-GFP using a MoFlo cell sorter (DakoCytomation, Glostrup, Denmark) and pre-

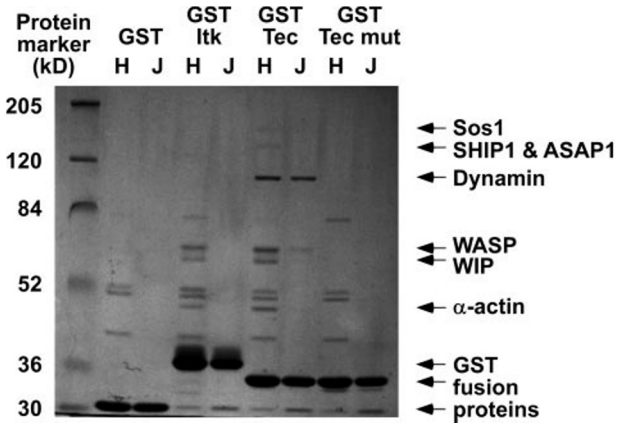

FIG. 1. **Identification of Tec and Itk SH3 domain-interacting proteins.** Lysates of HUT78 (*H*) and Jurkat (*J*) T cell lines were subjected to pull-down with GST or GST fusion proteins of the SH3 domains of Itk, Tec, or an inactive Tec point mutant. Interacting proteins were separated by SDS-PAGE and detected by Coomassie Blue staining. Six protein bands that were specific to intact Itk and/or Tec SH3 domains were excised and identified by mass spectrometry. Bands containing SOS1, SHIP1, and ASAP1, and dynamin appeared specific for Tec, whereas WASP, WIP, and α-actin interacted with both Tec and Itk SH3 domains.

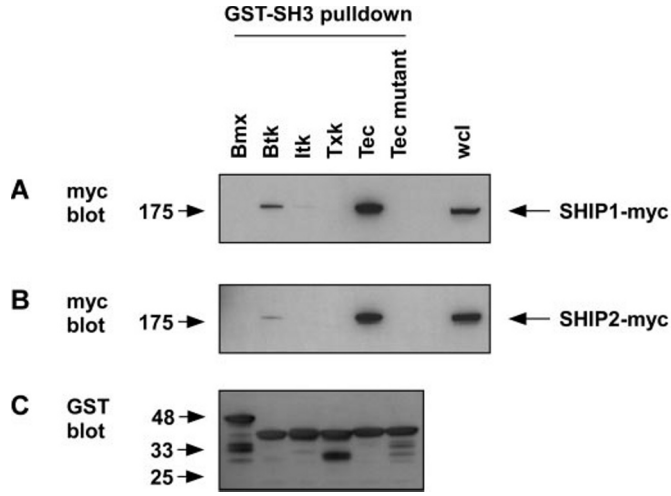

FIG. 2. **The Tec SH3 domain interacts with SHIP1 and SHIP2 with greater efficiency than other Tec family PTKs.** *A*, SH3 domain interaction with SHIP1 is robust for Tec, relatively weak for Btk, and undetectable for Bmx, Itk, and Txk. Lysates of 293T cells, transfected with a Myc-tagged form of SHIP1, were subjected to pull-down with GST fusion proteins of the SH3 domains of Bmx, Btk, Itk, Txk, and Tec. SHIP1 was detected in pull-downs and in whole cell lysates (*wcl*) by anti-Myc immunoblotting. *B*, SH3 domain interaction with SHIP2 is robust for Tec, relatively weak for Btk and undetectable for Bmx, Itk, and Txk. Lysates of 293T cells, transfected with a Myc-tagged form of SHIP2, were subjected to pull-down as described in *A*. *C*, immunoblots from *A* and *B* were stripped and re-analyzed by anti-GST immunoblotting. A representative blot confirms that the levels of GST fusion proteins were similar for each pull-down.

pared for microscopy as described (30). Slides were examined using a Leica TCS SP2 confocal microscopy system with a Leica DMIRE2 inverted microscope and a 63×/1.4 NA oil objective (Leica Microsystems, Heidelberg, Germany) Approximately twenty fluorescence images were collected per stack (*z*-axis), at intervals of 0.5 microns, and calibrated to fall within the linear range of the camera. Data were presented as both mid-plane images of a single z-section and as two-dimensional projection images of all z-sections merged together.

RESULTS

The Tec SH3 Domain Interacts with SHIP1 and SHIP2—Structural studies have lead to the hypothesis that Tec, Btk, and Itk are negatively regulated by an intramolecular interaction between the adjacent SH3 domain and proline-rich region (10–12). A prediction of this model is that inactivating mutations of either the SH3 or proline-rich region would result in a hyperactive molecule. However, using a Jurkat T cell line model system to investigate this, we have found that a SH3 point mutant is more active than wild type, but that the proline mutant is not (30). This suggests that the initial model is incorrect for Tec, which instead may be regulated by a novel mechanism that involves its SH3 domain interacting with a negative regulator.

In order to identify a putative negative regulator, we used a proteomic approach to identify proteins that interact with recombinant GST fusion proteins of the SH3 domain of Tec. A GST fusion protein of the SH3 domain of Itk was included to enable identification of proteins that interact uniquely with Tec. GST-SH3 pull-downs were carried out using lysates of Jurkat and HUT78 T cell lines, and interacting proteins were detected by Coomassie Blue staining (Fig. 1). Six protein bands that were present in Tec pull-downs, but not in control GST and GST-Tec SH3 mutant (W235L/W236L) pull-downs, were excised and identified by mass spectrometry. The cytoskeleton-related proteins α-actin, Wiskott-Aldrich Syndrome protein (WASP), and WASP-interacting protein (WIP) were found to interact with both Tec and Itk SH3 domains (Fig. 1), indicating that they are unlikely to be Tec-specific negative regulators. The identification of WASP in Itk SH3 pull-downs confirms a previous report (40) and is consistent with a role for Itk in regulating actin polymerization (41). In contrast, dynamin (a GTPase), SOS1 (a guanine nucleotide exchange factor for Ras), ASAP1 (a GTPase-activating protein for Arf), and SHIP1 were observed to bind selectively to Tec (Fig. 1).

SHIP1 is known to negatively regulate Btk membrane localization by de-phosphorylating PtdIns 3,4,5-P_3 (16, 17). Therefore we focused on SHIP1 as a potential negative regulator of Tec. To confirm that the SH3 domain interaction with SHIP1 was specific to Tec, the samples shown in Fig. 1 were Western-blotted with a SHIP1 antiserum. SHIP1 was detected only in Tec SH3 pull-downs from HUT78 cell lysates (data not shown), which is consistent with SHIP1 expression in HUT78 but not Jurkat (42).

To determine whether the SH3 domains of the other Tec family PTKs could interact with SHIP1, GST-SH3 pull-downs were performed with lysates of 293T cells transiently transfected with Myc-tagged SHIP1, followed by anti-Myc Western blotting (Fig. 2*A*). The Tec SH3 domain interacted relatively strongly with SHIP1, confirming the observations made using HUT78 cells (Fig. 1). SHIP1 interacted relatively weakly with the Btk SH3 domain, but not with Bmx, Itk, Txk, or mutant Tec SH3 domains (Fig. 2*A*). To address whether a similar relationship holds for SHIP2, GST-SH3 pull-downs were performed using lysates from 293T cells transiently transfected with Myc-tagged SHIP2 (Fig. 2*B*). SHIP2 was detected relatively efficiently in Tec SH3 pull-downs, weakly in Btk SH3 pull-downs, and not at all in Bmx, Itk, Txk, or mutant Tec SH3 pull-downs (Fig. 2*B*). To confirm that similar quantities of each GST-SH3 fusion protein were used in these experiments, the pull-downs were Western-blotted with an anti-GST mAb (Fig. 2*C*).

These data show that the Tec SH3 domain interacts with SHIP1 and SHIP2 more efficiently than do other Tec family SH3 domains, supporting the possibility that SHIP1 and SHIP2 selectively regulate Tec.

The Tec SH3 Domain Can Interact with Two Distinct Regions of SHIP1—In order to further characterize the interaction between Tec and SHIP1, we attempted to map the SH3 binding site on SHIP1. Six canonical SH3-binding motifs with the se-

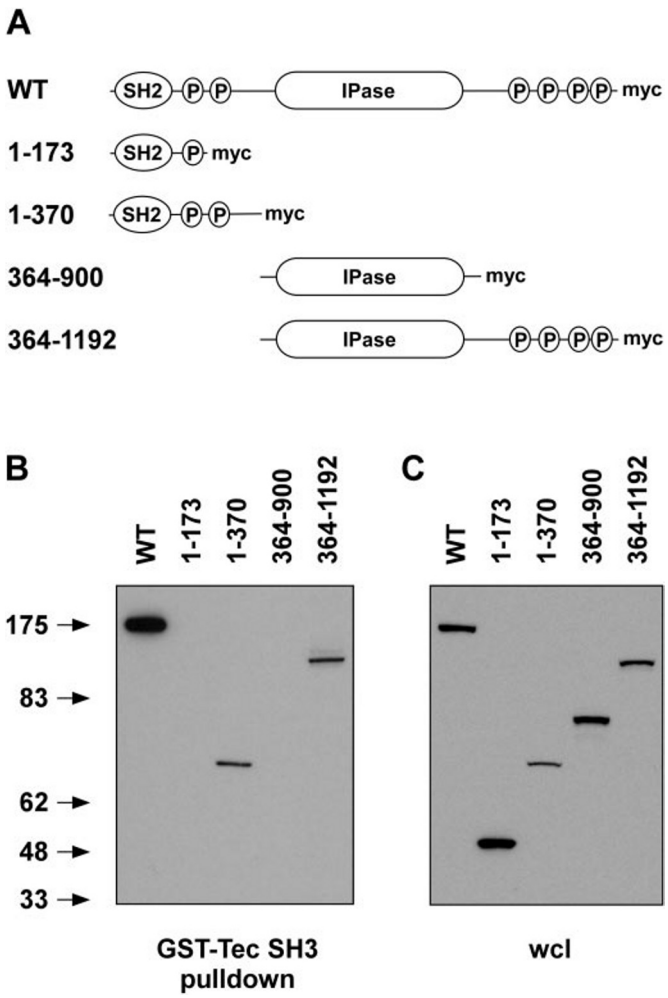

FIG. 3. **The Tec SH3 domain interacts with two distinct regions of SHIP1.** *A*, diagrammatic representation of SHIP1 wild-type (*WT*) and truncation mutants. SHIP1 constructs were Myc-tagged at the C terminus. Consensus (R/K)*XX*P*XX*P and P*XX*P*X*(R/K) SH3 binding motifs (*P*) and the inositol phosphatase domain (*IPase*) are indicated. *B*, the Tec SH3 domain interacts with the SHIP1 SH2-IPase interdomain region (amino acids 1–370 but not 1–173) and the C terminus (364–1192 but not 364–900). Lysates of 293T cells, transfected with Myc-tagged forms of SHIP1, were subjected to pull-down with a GST fusion protein of the Tec SH3 domain. SHIP1 was detected in pull-downs by anti-Myc immunoblotting. *C*, similar expression levels of the SHIP1 constructs used in *B* were confirmed by anti-Myc immunoblotting of whole cell lysates.

quence (R/K)*XX*P*XX*P or P*XX*P*X*(R/K) (where P is proline, R/K is arginine or lysine, and *X* is any amino acid) (43) are found in SHIP1 (Fig. 3*A*). To test whether one such site was responsible for Tec SH3 domain binding, four truncation mutants were generated (Fig. 3*A*) and expressed in 293T cells. Pull-downs using the Tec GST-SH3 domain fusion protein demonstrated that two distinct regions of SHIP1 were sufficient to interact with Tec (Fig. 3, *B* and *C*). One was located in the interdomain region between the SH2 and phosphatase domains, because amino acids 1–370 of SHIP1 interacted with Tec but 1–173 did not. The other region was located in the C-terminal tail, because amino acids 364–1192 interacted but 364–900 did not (Fig. 3, *B* and *C*). These mapping data suggest that the Tec-SHIP1 interaction is complex, with the potential for two Tec proteins to bind to a single SHIP1 molecule simultaneously. Further mutational studies are required to reveal the precise binding sites on SHIP1.

Tec, but Not Btk or Itk, Interacts with SHIP1 in an SH3-dependent Manner—In Figs. 1–3 we have shown that the SH3 domain of Tec is sufficient to interact with SHIP phosphatases. To determine whether full-length Tec interacts with SHIP1 in intact cells, we performed immunoprecipitation using antibodies that would precipitate endogenous proteins in cell lines, or epitope-tagged forms of Tec and SHIP1 in transfected cells. An interaction could not be detected (data not shown), which suggests that Tec and SHIP1 do not interact in intact cells or that the interaction is not maintained during cell lysis and immunoprecipitation. To distinguish between these possibilities we performed a yeast two-hybrid assay, as this is a more sensitive way to detect weak and transient interactions in living cells (44). The bait was SHIP1 fused to the GAL4 DNA-binding domain and the prey was Tec, or other family members, fused to the GAL4 activation domain. In this system, a SHIP1-Tec interaction would reconstitute the activity of the GAL4 transcription factor, leading to histidine (His) transcription and growth of the yeast on His-deficient media. In Fig. 4, each yeast strain grew on control His-sufficient media (*left panel*), but on His-deficient medium growth was only observed in the presence of both SHIP1 and Tec (*right panel*). An intact Tec SH3 domain was required for growth and, importantly, neither Itk nor Btk could rescue growth (Fig. 4). These data suggest that Tec, but not Itk or Btk, can interact with SHIP1 *in vivo* and that this interaction requires the Tec SH3 domain.

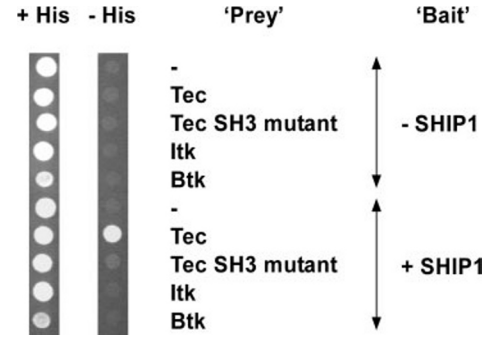

FIG. 4. **Tec, but not Itk, or Btk, interacts with SHIP1 in an SH3-dependent manner.** Yeast two-hybrid analyses were performed using a bait of SHIP1 fused to the GAL4 DNA-binding domain and a prey of Tec, Itk, or Btk fused to the GAL4 activation domain. On histidine-deficient media (−*His*, *right panel*), a SHIP1 interaction with Tec reconstituted the activity of the GAL4 transcription factor, leading to His transcription and growth of the yeast. No growth was observed for Tec with an inactivating mutation of the SH3 domain, for Itk or Btk, or in the absence of SHIP1. As a control, on His-sufficient media (+*His*, *left panel*), similar growth was observed for each yeast strain.

SHIP1 and SHIP2 Inhibit Tec Function—SHIP1 is known to negatively regulate Btk by indirectly preventing its membrane localization through de-phosphorylation of PtdIns 3,4,5-P_3 (16, 17). It is not known if SHIP1 can negatively regulate Tec in the same way, and possibly also through a novel mechanism mediated by direct interaction with the SH3 domain. To address this, Jurkat T cells, which do not express SHIP1 (42), were used as a model system to measure Tec function in the presence or absence of transfected SHIP phosphatases. In Fig. 5, Jurkat cells were co-transfected with HA-tagged Tec (5 μg) and two different doses (1.25 or 5 μg) of Myc-tagged forms of wild-type SHIP1, phosphatase inactive SHIP1, or wild-type SHIP2, and an NFAT-luciferase reporter construct. Expression of each construct was confirmed by Western blotting with anti-Myc and anti-HA mAbs (Fig. 5, *lower panel*). In unstimulated cells (Fig. 5, *upper panel*), Tec-induced NFAT activation was potently inhibited by SHIP1 and SHIP2 (94 and 92% inhibition with 5 μg of SHIP1 and SHIP2, respectively). This inhibitory effect required phosphatase activity, as the phosphatase inactive form of SHIP1 did not inhibit Tec. To address whether SHIP

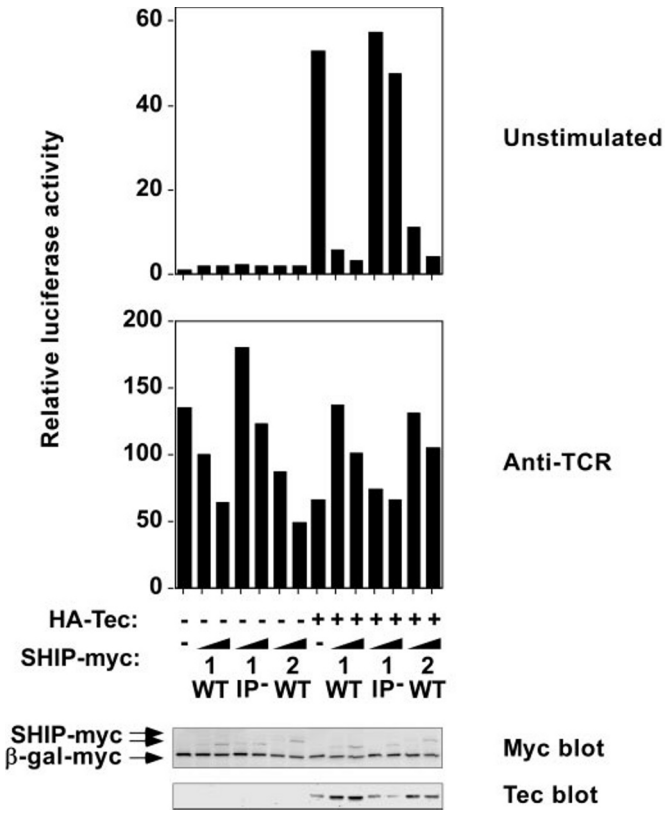

FIG. 5. **SHIP1 and SHIP2 inhibit Tec-induced NFAT activation.** Jurkat T cells were transfected with an NFAT-luciferase reporter construct, a β-gal construct, 0 or 5 μg of HA-tagged Tec, and 0, 1.25 or 5 μg of Myc-tagged SHIP1 (*WT*), phosphatase-inactive SHIP1 (*IP*$^-$), or SHIP2 (*WT*). Sixteen hours post-transfection, cells were either left unstimulated or were stimulated with anti-TCR mAb C305. Six hours later, cells were lysed, assayed for luciferase and β-gal, and the luciferase data was normalized for β-gal values (*upper panels*). The expression of SHIP and Tec constructs was confirmed by, respectively, anti-Myc and anti-Tec immunoblotting of whole cell lysates (*lower panels*). Anti-Myc immunoblotting also detected the Myc-tagged β-gal construct used for normalization. The data are representative of three independent experiments.

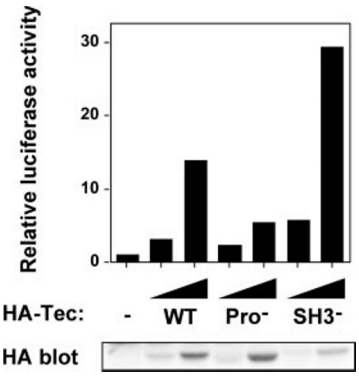

FIG. 6. **A mutant form of Tec with an inactivated SH3 domain is hyperactive.** HUT78 T cells were transfected with an NFAT-luciferase reporter construct, a β-gal construct, and HA-tagged Tec wild-type (*WT*), proline point mutant (Pro$^-$) or SH3 point mutant (SH3$^-$). Luciferase assays were performed on unstimulated cells and the data presented as described in the legend to Fig. 5 (*upper panel*). Control stimulations with PMA plus ionomycin gave similar relative luciferase activities for each sample (between 180 and 320, data not shown). Expression of each Tec construct was confirmed by anti-HA immunoblotting (*lower panel*). The data are representative of three independent experiments.

phosphatases inhibit Tec more specifically than other TCR signaling components, the cells were stimulated by TCR cross-linking. SHIP1 and SHIP2 did inhibit TCR-induced NFAT in cells that were not transfected with Tec (Fig. 5, *middle panel*), but this was substantially weaker than inhibition of Tec signaling. For example, 5 μg of SHIP1 and SHIP2 inhibited TCR-induced NFAT by only 53 and 64%, respectively. These data show that SHIP phosphatases are potent and relatively specific inhibitors of Tec function.

Inactivation of the SH3 Domain Generates a Hyperactive Form of Tec—Since SHIP phosphatases can negatively regulate Tec (Fig. 5) and the Tec SH3 domain is both necessary (Fig. 4) and sufficient (Figs. 1–3) for interaction with SHIP1, a form of Tec with a non-functional SH3 domain would be predicted to be hyperactive in SHIP1-expressing cells, relative to wild-type Tec. To test this hypothesis, Tec function was measured using NFAT reporter assays in HUT78 cells, which are known to express SHIP1 (42). In Fig. 6, HUT78 cells were transfected with two different doses of HA-tagged Tec wild-type, proline point mutant or SH3 point mutant, plus an NFAT-luciferase reporter construct. Wild-type Tec induced NFAT activation in a dose-dependent manner, and the proline mutant induced a slightly weaker response. The SH3 mutant induced substantially greater NFAT activation, despite its relatively low level of expression as detected by anti-HA Western blotting (Fig. 6). As a positive control, PMA and ionomycin stimulation induced NFAT activation in all samples, to a level between 180- and 320-fold (data not shown). These data show that inactivation of the SH3 domain, but not the proline-rich region, generates a hyperactive form of Tec in SHIP1-expressing cells.

SHIP1 Inhibits Tec Membrane Localization—SHIP1 inhibits membrane localization of Btk (16, 17), but it is not known if SHIP1 also affects Tec in this manner. To address this issue, GFP-tagged Tec was expressed in Jurkat T cells in the presence or absence of SHIP1, and the cells were visualized by fluorescence confocal microscopy. In the absence of SHIP1, Tec was largely membrane-localized (Fig. 7*A*), as previously reported (27, 30), which is consistent with the relatively high level of PtdIns 3,4,5-P_3 in Jurkat (42). As we have shown before (30), Tec exhibited a punctate localization pattern at the cell surface, which was most apparent when the z-sections for each image were merged to generate a two-dimensional projection image (Fig. 7*A*). In the presence of SHIP1, however, Tec was predominantly localized to the cytoplasm and the punctate localization pattern largely disappeared (Fig. 7*B*). These data show that SHIP1 inhibits membrane localization of Tec.

A Constitutively Membrane-targeted Form of Tec Is Refractory to Inhibition by SHIP1—Since SHIP1 inhibits both Tec membrane localization (Fig. 7) and Tec function in a phosphatase-dependent manner (Fig. 5), these effects are potentially mediated by de-phosphorylation of PtdIns 3,4,5-P_3. A prediction of this model is that artificially targeting Tec to the membrane should relieve SHIP1 inhibition. To test this hypothesis, we examined the effect of SHIP1 on the function of HA-tagged *versus* membrane-targeted Tec (Myr-Tec, containing the Src myristylation sequence at the N terminus). In Fig. 8, Jurkat cells were transfected with HA-Tec or Myr-Tec in the presence or absence of Myc-tagged SHIP1 and NFAT-luciferase activation was used as a readout for Tec activity. As shown previously (Fig. 5), SHIP1 potently inhibited NFAT activation by HA-Tec. In contrast, SHIP1 did not substantially inhibit NFAT activation by Myr-Tec. The slightly lower level of NFAT activity induced by Myr-Tec relative to HA-Tec is likely caused by lower expression levels, as measured by Tec Western blotting. These data suggest that SHIP phosphatases interact with and inhibit Tec by impairing membrane targeting to PtdIns 3,4,5-P_3.

DISCUSSION

We have previously found that Tec is unique among Tec family PTKs in its capacity to signal constitutively when over-

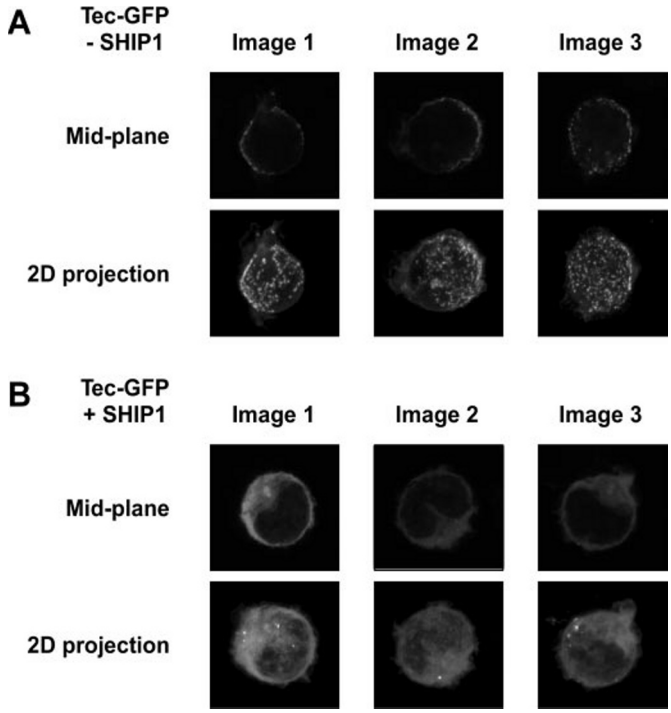

FIG. 7. **SHIP1 inhibits Tec membrane localization.** Jurkat cells were transfected with GFP-tagged Tec in the absence (*A*) or presence (*B*) of SHIP1. Twenty hours post-transfection, cells were fixed on glass slides and imaged by fluorescence confocal microscopy. Three representative cells are presented as mid-plane images of a single, central z-section and as two-dimensional projection images of all z-sections. Tec-GFP is displayed in *white*.

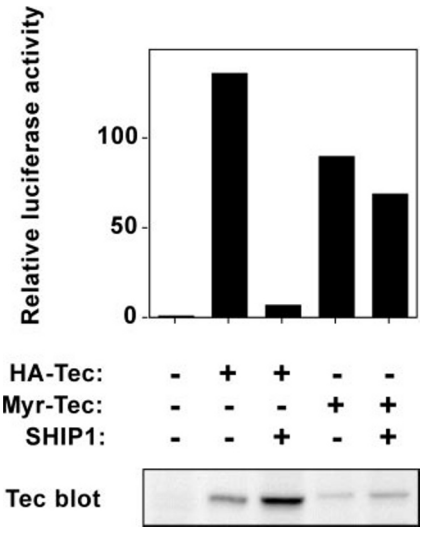

FIG. 8. **A constitutively membrane-targeted form of Tec is refractory to inhibition by SHIP1.** Jurkat T cells were transfected with an NFAT-luciferase reporter construct, a β-gal construct, 5 μg of HA-Tec or a membrane-targeted form of Tec (Myr-Tec), and 0 or 2 μg of Myc-tagged SHIP1. Luciferase assays were performed on unstimulated cells and the data presented as described in Fig. 5 (*upper panel*). Expression of Tec constructs was confirmed by anti-Tec immunoblotting (*lower panel*). The data are representative of three independent experiments.

expressed in lymphocyte cell lines (30). Therefore it is likely that regulatory mechanisms have evolved to prevent unwanted activation of endogenous Tec in resting cells. One such mechanism appears to operate at the level of Tec protein expression, since Tec is expressed at substantially lower levels than Itk and Btk in primary lymphocytes, but is up-regulated upon T cell activation and in effector T cells (30). However, additional mechanisms are probable and the hypothesis that underlies this study is that Tec is negatively regulated by a novel Tec-interacting protein.

Using a proteomic approach we identified the inositol phosphatases SHIP1 and SHIP2 as Tec SH3 domain-interacting proteins. We further demonstrated that the Tec SH3 domain is necessary for the SHIP1-Tec interaction in intact cells and that other Tec family PTKs do not interact with SHIP1. Since SHIP1 can negatively regulate Btk membrane localization by de-phosphorylation of PtdIns 3,4,5-P_3 (16, 17), SHIP phosphatases are attractive candidate negative regulators of Tec. A number of predictions of this hypothesis were tested in a T cell line model system for Tec function. The predictions were that SHIP phosphatases would inhibit Tec membrane localization and Tec function, an SH3 domain-mutated form of Tec would be hyperactive, and that artificially targeting Tec to the membrane would bypass the inhibitory effect of SHIP1. Each of these predictions was confirmed experimentally, thereby confirming that SHIP phosphatases interact with and negatively regulate Tec *in vivo*.

We speculate that the interaction between Tec and SHIP phosphatases is regulated by auto-phosphorylation of a tyrosine residue in the Tec SH3 domain, which is thought to occur upon Tec activation (6, 7). Importantly, the predicted auto-phosphorylation site is within the ligand-binding pocket of the SH3 domain (12, 45) and phosphorylation is proposed to modulate its binding specificity (46). Thus upon activation, phosphorylated Tec may exhibit reduced binding affinity for SHIP1, and the proteins may dissociate, allowing Tec translocation to PtdIns 3,4,5-P_3 in the membrane. Tec may subsequently be down-regulated by de-phosphorylation of its SH3 domain by a phosphatase such as PTP20, which interacts with the Tec SH2 domain and can de-phosphorylate Tec (47). This would allow re-association of Tec with SHIP phosphatases and their de-phosphorylation of local PtdIns 3,4,5-P_3, thus inhibiting Tec membrane localization to complete the cycle of regulation. This is unlikely to be the only mechanism of Tec regulation. Indeed, proteins that regulate Btk, such as IBtk (13), Sab (14), and PKCβ (15), may also regulate Tec. Furthermore, negative regulation is not the sole function of the SH3 domain of Tec, since it can also mediate recruitment to activated CD28 during T cell signaling (48, 49).

A striking feature of the Tec-SHIP1 interaction is that two distinct regions of SHIP1 are sufficient to interact with the Tec SH3 domain. These are the SH2-phosphatase interdomain region and the C terminus. We did not map the precise SH3-binding motifs, although multiple P*XX*P motifs are present within these two regions of both SHIP1 and SHIP2, some of which are canonical K/R*XXPXX*P or P*XXPX*(K/R) sequences (43). We cannot rule out the possibility that non-P*XX*P motifs are involved (43). This may be relevant to the SH2-phosphatase interdomain region, since neither of the two canonical P*XX*P motifs were sufficient for SH3 binding (data not shown). Regardless of the precise binding motifs, our data suggest that one SHIP1 molecule has the potential to interact with two Tec molecules. Such an interaction is not without precedent in cell signaling. For example, the guanine nucleotide exchange factor SOS interacts with two Ras molecules, one of which (Ras-GTP) activates SOS and the other of which (Ras-GDP) is a SOS substrate (50). Thus it is possible that an interaction of two Tec molecules with SHIP1 is functionally relevant. Indeed, structural studies have suggested that Tec family PTKs can form homodimers (10–12), and although the functional consequences of dimerization are not known, the interaction with SHIP1 may promote Tec dimerization.

Interestingly, Tec and SHIP1 are both known to interact

with Dok family adapter proteins. The Tec SH2 domain interacts with phosphotyrosine motifs within Dok-1 and Dok-2 and Tec is functionally inhibited by these adapters (51, 52), whereas NPXY motifs within the SHIP1 C terminus interact with the phosphotyrosine binding domains of Dok-1, Dok-2, and Dok-3 (53, 54). These findings suggest the existence of a Dok/SHIP/Tec negative regulatory complex, or signalosome, that is regulated by tyrosine phosphorylation in a comparable manner to the LAT/Gads/SLP76/PLCγ1 signalosome in TCR signaling (reviewed in Ref. 55). Recently a Dok-3/SHIP1 complex was shown to inhibit BCR signaling in a manner that did not involve inhibition of Btk (54). The possibility that Tec is the target of inhibition in this system has yet to be examined.

The interaction with Tec may provide answers to key questions concerning the mechanisms of SHIP1 inhibitory function. For example, SHIP1 can negatively regulate BCR signaling even in the absence of FcγRIIB co-ligation with the BCR (56, 57), and SHIP1 inhibits signaling of cytokine receptors for IL-3 (22) and stem cell factor (SCF) (58) in myeloid cells. For each of these signaling responses the mechanism of SHIP1 recruitment to the receptors is not clear. However, Tec is proposed to play a role in signaling from the BCR (28, 59), IL-3 (60), and SCF (61) receptors, suggesting that SHIP1 may inhibit signaling by these receptors via an effect on Tec. A second question concerns the role of the relatively poorly characterized regions of SHIP1, namely the SH2-phosphatase interdomain region and the C-terminal tail. In particular, the C terminus is required for the inhibition of SCF-induced mast cell activation (58) and for FcγRIIB-mediated inhibition of BCR-induced calcium mobilization (62), but the mechanism is unclear. Since Tec is thought to positively regulate these signaling pathways (28, 59, 61), the Tec interaction with the SHIP1 C terminus may, in part, explain these findings.

In summary, we have shown that SHIP1 and SHIP2 preferentially interact with Tec, compared with other Tec family PTKs, and that SHIP phosphatases negatively regulate Tec by inhibiting membrane localization. A prediction of this model is that Tec would be hyperactive in cells that are deficient in SHIP family phosphatases. Interestingly, the SHIP1$^{-/-}$ mouse dies at a young age because of myeloid cell infiltration of the lungs (22, 23). Since Tec is expressed in myeloid cells (59, 60, 63), hyperactive Tec may be partly responsible for this aberrant myeloid activation. The future generation of SHIP1$^{-/-}$ Tec$^{-/-}$ mice will help to address this issue.

Acknowledgments—We thank the Weiss and Watson Laboratories for helpful advice and comments. We thank Larry Rohrschneider, Shonna Moodie, and Mark Coggeshall for providing SHIP reagents, Hyewon Phee and Neetu Gupta for SHIP advice, Sascha Hughan for help with confocal microscopy and Roger Bird for cell sorting.

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