Molecular cloning of a docking protein, BRDG1, that acts downstream of the Tec tyrosine kinase

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Tec, Btk, Itk, Bmx, and Txk constitute the Tec family of protein tyrosine kinases (PTKs), a family with the distinct feature of containing a pleckstrin homology (PH) domain. Tec acts in signaling pathways triggered by the B cell antigen receptor (BCR), cytokine receptors, integrins, and receptor-type PTKs. Although upstream regulators of Tec family kinases are relatively well characterized, little is known of the downstream effectors of these enzymes. The yeast two-hybrid system has identified several proteins that interact with the kinase domain of Tec, one of which is now revealed to be a previously unknown docking protein termed BRDG1 (*BCR downstream signaling* 1). BRDG1 contains a proline-rich motif, a PH domain, and multiple tyrosine residues that are potential target sites for Src homology 2 domains. In 293 cells expressing recombinant BRDG1 and various PTKs, Tec and Pyk2, but not Btk, Bmx, Lyn, Syk, or c-Abl, induced marked phosphorylation of BRDG1 on tyrosine residues. BRDG1 was also phosphorylated by Tec directly *in vitro*. Efficient phosphorylation of BRDG1 by Tec required the PH and SH2 domains as well as the kinase domain of the latter. Furthermore, BRDG1 was shown to participate in a positive feedback loop by increasing the activity of Tec. BRDG1 transcripts are abundant in the human B cell line Ramos, and the endogenous protein underwent tyrosine phosphorylation in response to BCR stimulation. BRDG1 thus appears to function as a docking protein acting downstream of Tec in BCR signaling.

The Tec protein tyrosine kinase (PTK) was initially identified in mouse liver (1). The subsequent molecular cloning of four Tec-related kinases, Btk (2, 3), Itk (also known as Emt or Tsk) (4–6), Bmx (7), and Txk (or Rlk) (8, 9), revealed that these enzymes, together with Tec, constitute a distinct subfamily of nonreceptor PTKs. With the exception of Txk, the members of this subfamily possess a long NH_2-terminal region consisting of a pleckstrin homology (PH) domain (10) and a Tec homology (TH) domain (11). Because PH domains bind phosphoinositides with high affinity, the Tec family kinases have been proposed to act downstream of phosphatidylinositol 3-kinase (PI3-kinase) in signaling pathways. Indeed, an increase in PI3-kinase activity results in activation of Btk (12), and incubation of cells with PI3-kinase inhibitors markedly reduces the activity of intracellular Itk. Interaction of the PH domain with phosphoinositides is probably required for targeting of Tec family kinases to the cell membrane, given that PI3-kinase inhibitors no longer suppress the activity of Itk when it is fused to the extracellular and transmembrane domains of c-Kit and thereby constitutively targeted to the membrane fraction (13).

All members of the Tec family of kinases are abundant in hematopoietic tissues and have thus been proposed to play important roles in blood cell development. Consistent with this hypothesis, mutations in Btk have been shown to cause X chromosome-linked agammaglobulinemia in humans (2, 3), indicating that Btk is indispensable for the maturation of B lymphocytes. Other Tec family kinases have also been shown to act as signaling intermediaries for lymphocyte surface antigens. For example, Tec is activated in response to engagement either of the B cell antigen receptor (BCR) in B lymphocytes (14) or of CD28 in T lymphocytes (15). Cross-linking of CD28 also induces the tyrosine phosphorylation and activation of Itk (16). The NH_2-terminal region of Btk, encompassing the PH domain and a portion of the TH domain, is able to bind directly to the α subunit of heterotrimeric GTP-binding proteins (17), suggesting that Tec family kinases also might act downstream of such G proteins. Indeed, in human platelets, stimulation of the thrombin receptor, a transmembrane receptor coupled to G proteins, induces activation of Tec (18). Furthermore, integrin stimulation has been shown to regulate Tec activity (18).

Whereas substantial progress has been made in identifying the upstream regulators of Tec family kinases, much less is known of their downstream targets. Candidates for direct substrates of these kinases include BAP-135 (19), phospholipase C (PLC)-γ2 (20), Vav (21), and Grb10 (or GrbIR) (22). Tec phosphorylation of PLC-γ2 results in the generation of inositol trisphosphate and consequent mobilization of intracellular Ca^{2+} from inositol trisphosphate receptor-gated stores (23), which may play an important role in B cell development. Tec family kinases also contribute to the regulation of the small GTP-binding protein Rho, which directs the formation of stress fibers as well as the activation of serum response factor (24). In addition, Tec is a potent activator of the promoter of the c-*fos* protooncogene (25). However, the nature of the direct effectors responsible for the transmission of these signals from Tec remains unclear. It also remains to be determined which effectors are common to all Tec family kinases and which ones are enzyme-specific.

To increase our understanding of the downstream signaling mechanisms of Tec family kinases, we have used the yeast two-hybrid system to identify Tec substrates. One of the positive clones obtained has now been shown to encode a previously unidentified docking protein, which we have termed BRDG1. In a human B cell line, BRDG1 was shown to be phosphorylated on tyrosine residues in response to stimulation of the BCR. Furthermore, we have shown that phosphorylation of BRDG1 results in a feedback action on Tec, leading to its activation.

Materials and Methods

Cell Lines and Antibodies. UT-7 (26) was cultured in RPMI 1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS and 1 ng/ml human granulocyte–macrophage colony-stimulating factor. All other hematopoietic cell lines (27) were

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Abbreviations: PTK, protein tyrosine kinase; PH, pleckstrin homology; TH, Tec homology; PI3-kinase, phosphatidylinositol 3-kinase; BCR, B cell antigen receptor; PLC, phospholipase C; GST, glutathione *S*-transferase; SH, Src homology; DGK, diacylglycerol kinase.

Data deposition: The nucleotide sequence reported in this paper has been deposited in the GenBank/EMBL/DDBJ databases (accession no. AB023483).

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maintained in RPMI 1640/10% FBS medium. For BCR stimulation, Ramos cells (American Type Culture Collection, ATCC; Manassas, VA) were first incubated for 12 h in Iscove's modified Dulbecco's medium (IMDM; Life Technologies) containing 1% FBS and then exposed for 5 min to anti-human IgM $F(ab')_2$ fragments (10 μg/ml) (Southern Biotechnology Associates, Birmingham, AL), as described (14). 293 cells (ATCC) were maintained in DMEM-F12 (Life Technologies) containing 10% FBS and 2 mM L-glutamine.

Antibodies to BRDG1 were generated in rabbits injected with a glutathione *S*-transferase (GST) fusion protein containing the COOH-terminal half (residues 172 to 295) of BRDG1. Preparation of anti-Tec antibodies was described before (28). Antibodies to phosphotyrosine (4G10) and the FLAG epitope tag (M2) were obtained from Upstate Biotechnology (Lake Placid, NY) and Eastman Kodak (New Haven, CT), respectively. Antibodies to other PTKs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Isolation of BRDG1 cDNA and Construction of Expression Plasmids. A cDNA corresponding to the kinase domain of human Tec (amino acids 357 to 630) was inserted into the pGBT9 vector (CLONTECH), which encodes the DNA-binding domain of yeast GAL4, thereby yielding pGAL4bd-TecKD. This plasmid was then used in the two-hybrid screen as described (22, 29). The TIP4 cDNA thus obtained was labeled with [α-^{32}P]dCTP and then used as a probe to screen a UT-7 cell cDNA library constructed in the λZAPII phage vector (Stratagene). After the second round of screening, positive phage clones were converted to pBlueScript II plasmids (Stratagene) by the *in vivo* excision protocol, and the cDNA inserts were subjected to nucleotide sequencing.

The coding region of BRDG1 was amplified by PCR from the corresponding cDNA and inserted into the pcDNA3-FLAG vector, thereby yielding pcDNA-BRDG-F, which encodes the BRDG1 protein with a COOH-terminal FLAG epitope tag.

The BRDG1 cDNA corresponding to amino acids 1–295 or 172–295 was PCR-amplified and subcloned into pGEX2T vector (Amersham Pharmacia Biotech) to produce the GST-fusion protein of the full length or COOH-terminal half of BRDG1, respectively.

Transfection and Protein Analysis. 293 cells (2×10^6) were transfected with 10 μg of each expression plasmid by the calcium phosphate method. After 2 days of incubation, cells were solubilized in lysis buffer [1% Nonidet P-40/50 mM Tris·HCl (pH 7.4)/150 mM NaCl/1 mM NaF/1 mM Na_3VO_4/aprotinin (200 units/ml)/1 mM PMSF]. Immunoprecipitation and immunoblot analysis were performed as described (30), and immune complexes were detected with the enhanced chemiluminescence system (Amersham).

For *in vitro* assay of kinase activity, immune complexes formed with antibodies to PTKs were washed twice with lysis buffer and three times with kinase buffer [20 mM Tris·HCl (pH 7.4)/50 mM NaCl/10 mM $MgCl_2$/2 mM $MnCl_2$] and then incubated with 0.37 MBq of [γ-^{32}P]ATP. To analyze BRDG1 phosphorylation, anti-Tec immunoprecipitates were reacted with 0.1 mM ATP plus 1 μg of GST or GST-BRDG1 fusion protein at 37°C, and the resulting samples were subjected to immunoblot analysis with antibodies to phosphotyrosine or GST (AMRAD, Kew, Victoria, Australia).

Introduction of pcDNA-BRDG-F with pSRα or pSRα-TecΔKD (24) into Ramos cells (5×10^6) were conducted by electroporation as described (25). After 12 h of culture in RPMI/10% FBS, cells were treated for 1 h in IMDM/1% FBS at the concentration of 5×10^6/ml. BCR of the transfected cells were then cross-linked as described above.

Results and Discussion

Isolation of BRDG1 cDNA. With the kinase domain of human Tec (amino acids 357–630) as a "bait," we attempted to identify substrates of Tec by yeast two-hybrid screening. From a panel of human cDNA libraries, we identified six Tec-interacting proteins (TIP1–TIP6) (22, 29). The TIP4 cDNA was isolated from a cDNA library prepared from Epstein–Barr virus-transformed B cells (CLONTECH), and was found to encode a previously unidentified protein. To isolate a full length TIP4 cDNA, we first attempted to identify hematopoietic cell lines in which the TIP4 transcripts are abundant.

Total RNAs were prepared from a panel of human hematopoietic cell lines (27) including those of T cell lineage (CCRF-CEM, Jurkat, and PEER), B cell lineage (Ramos and Raji), and myeloid (HEL, KU812, KG1, K562, and UT-7). Northern blot analysis of these RNAs with a probe prepared from the TIP4 cDNA obtained in the two-hybrid screen revealed the presence of a major TIP4 transcript of 1.6 kb and a minor one of 2.2 kb in B cells and some myeloid cells, but not in T cells (Fig. 1*A*). The same membrane was rehybridized with the β-actin cDNA to compare the quantities of loaded RNAs (Fig. 1*A*, bottom row).

On the basis of these results, a conventional cDNA library of UT-7 cells was constructed in the λZAPII phage vector and screened with the TIP4 cDNA probe. A positive clone with an insert of 1,454 bp was obtained. The 5′ region of this TIP4 cDNA was extended by 5′ rapid amplification of cDNA ends (data not shown). Nucleotide sequencing of the assembled 1,507-bp cDNA revealed the presence of a single ORF encoding a previously unidentified protein of 295 amino acids and with a calculated molecular mass of 34,291 Da (Fig. 1*B*). Given that we have shown that TIP4 participates in the signaling downstream of BCR (see below), we renamed this protein BRDG1 (*BCR downstream* signaling 1).

Database analysis revealed that the NH_2-terminal half of BRDG1 shows sequence homology to the PH domains of various signaling proteins, including hamster diacylglycerol kinase (DGK)-η (32% identity, 58% similarity), human DGK-δ (29% identity, 54% similarity), and a *Dictyostelium discoideum* homolog of Akt (also known as Rac-PK or PKB) (20% identity, 44% similarity) (Fig. 1 *B* and *C*). In addition, BRDG1 contains a tryptophan residue (Trp-112) at a position corresponding to that of the hallmark tryptophan residue of PH domains. Thus, BRDG1 is a PH domain-containing protein. The COOH-terminal half of BRDG1 is distantly related to the Src homology (SH) 2 domain. This region of BRDG1 shares 25% and 22% sequence identity with the SH2 domains of human PLC-γ2 and *Hydra* Csk, respectively. In addition, a GST fusion protein containing the COOH-terminal half of BRDG1, but not one containing the PH domain, interacted with a number of tyrosine-phosphorylated proteins from lysates of 293 cells, as judged by coprecipitation experiments (data not shown). However, several characteristic features of SH2 domains are not present in BRDG1; the well conserved tryptophan residue in the βA1 loop and the Phe-Leu-Val/Ile-Arg sequence of SH2 domains (31) is replaced by an alanine and a Met-Ile-Leu-Arg sequence, respectively. It therefore remains unclear whether the COOH-terminal half of BRDG1 shares the ability of genuine SH2 domains to recognize specifically tyrosine-phosphorylated proteins. In addition to the PH domain, BRDG1 possesses a proline-rich sequence that constitutes a potential binding site for SH3 or WW domains (Fig. 1 *B* and *D*). Another characteristic feature of BRDG1 is that it is rich in tyrosine residues, many of which are located in sequences potentially capable of binding to SH2 domains (Table 1). The overall structure of BRDG1 therefore resembles those of docking proteins such as IRS-1 or IRS-2 (32, 33), Gab1 or Gab2 (34, 35), and Dok-1 or Dok-2 (36–38).

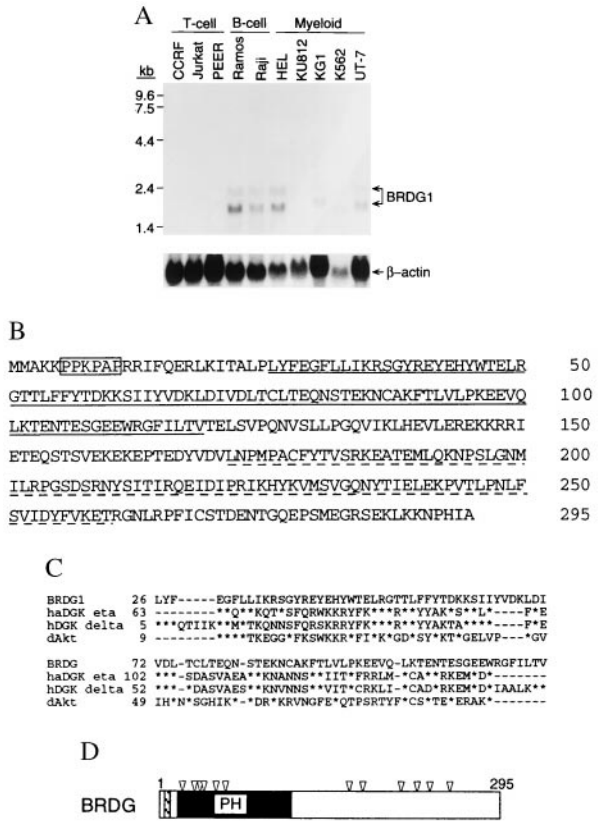

Fig. 1. (*A*) Northern blot analysis of total RNA (20 μg per lane) from CCRF-CEM (CCRF), Jurkat, PEER, Ramos, Raji, HEL, KU812, KG1, K562, and UT-7 cells with the ^{32}P-labeled BRDG1 cDNA (top row) or β-actin cDNA (bottom row). The positions of molecular size standards (in kilobases) are shown on the left and the hybridizing transcripts are shown on the right. (*B*) The deduced amino acid sequence of human BRDG1. The sequence is shown in single-letter notation, with the PH domain and the putative SH2 domain indicated by solid and broken underlines, respectively. The proline-rich motif is *boxed*. Residue numbers are on the right. (*C*) Comparison of the amino acid sequence of BRDG1 with those of golden hamster DGK-η (haDGK eta; GenBank accession no. Q64398), human DGK-δ (hDGK delta; accession no. D73409), and the *D. discoideum* Akt homolog (dAkt; accession no. P54644). Residues identical with those of BRDG1 are shown as asterisks; dashes represent gaps introduced to optimize alignment. Residue numbers are shown on the left. (*D*) Overall structure of BRDG1. The proline-rich motif and the PH domain are shown as hatched and solid boxes, respectively. The positions of tyrosine residues are indicated by arrowheads.

BRDG1 Is an *in Vivo* Substrate of Tec. To determine whether BRDG1 might function as a docking protein, we investigated whether it is phosphorylated at a high stoichiometry by PTKs in intact cells. We subjected 293 cells to transient transfection with an expression plasmid encoding BRDG1 with a COOH-terminal FLAG epitope tag either alone or together with a plasmid encoding Tec or a kinase-defective Tec mutant (Tec^{KM} in which Lys-397 in the putative ATP binding site is replaced with Met) (39). BRDG1 was immunoprecipitated from the various transfected cells with antibodies to FLAG and then subjected to immunoblot analysis with antibodies either to phosphotyrosine or to FLAG. FLAG-tagged BRDG1 was expressed in the cells as a protein of ≈37 kDa (Fig. 2*A*). When expressed alone, BRDG1 showed a very low level of tyrosine phosphorylation in 293 cells. However, coexpression with Tec resulted in a marked increase in the extent of tyrosine phosphorylation of BRDG1; coexpression with Tec^{KM} had no such effect. When expressed together with Tec, BRDG1 was associated with a tyrosine-phosphorylated protein of ≈70 kDa (Fig. 2*A*, lane 3), the same molecular size as that of Tec, suggestive of a physical interaction between BRDG1 and Tec. To confirm this hypothesis, we expressed BRDG1 in 293 cells either alone or together with Tec or Tec^{KM}, immunoprecipitated BRDG1 with antibodies to FLAG, and subjected the immunoprecipitates to the immunoblot analysis with antibodies to Tec (Fig. 2*B*). BRDG1 was associated with Tec protein only in the presence of Tec activity. Reprobing of the membrane with antibodies to FLAG demonstrated that the amount of BRDG1 immunoprecipitated from the various transfected cells was constant. Immunoblot analysis of total cell lysates with antibodies to Tec also confirmed that Tec and Tec^{KM} were expressed in equivalent amounts. Therefore, BRDG1 appears to be an efficient substrate of Tec in intact cells, and the BRDG1–Tec interaction is phosphorylation-dependent.

Table 1. Potential SH2-binding tyrosine in BRDG1

Flanking sequence	Binding proteins
LPL Y^{27} FEG	
RSG Y^{39} REY	
YRE Y^{42} EHY	Fps
YEH Y^{45} WTE	
LFF Y^{57} TDK	Csk
SII Y^{65} VDK	PLC-γ1,(SHP2)
TED Y^{168} VDV	PLC-γ1,(SHP2)
ACF Y^{180} TVS	Csk
SRN Y^{211} SIT	
IKH Y^{227} KVM	p85, (Shc)
GQN Y^{236} TIE	Csk
VID Y^{255} FVK	

To determine whether BRDG1 is a specific substrate of Tec or whether it is also phosphorylated by other PTKs, we expressed BRDG1 in 293 cells either alone or together with representatives of various cytoplasmic PTK subfamilies (Fig. 2*C*). Whereas Pyk2 induced the phosphorylation of BRDG1 to the same extent as did Tec, BRDG1 was not phosphorylated by Lyn, Syk, or c-Abl (Fig. 2*C*, top row). We further investigated the kinase specificity of BRDG1 phosphorylation among Tec family members. Neither Btk nor Bmx phosphorylated BRDG1 in 293 cells (Fig. 2*D*, top row). These data, however, left a possibility that only Tec and Pyk2 were highly active in 293 cells. Generally, it is difficult to quantitatively compare the kinase activities of distinct PTKs; for instance, Tec cannot efficiently phosphorylate poly(Glu, Tyr) or enolase (data not shown). Therefore, to measure the kinase activity, we conducted an *in vitro* kinase assay with [γ-^{32}P]ATP and demonstrated autophosphorylation activity of each PTK (bottom rows Fig. 2 *C* and *D*). Although there was a diversity in their autophosphorylation activities, it was apparent that BRDG1 phosphorylation did not parallel the intensity of PTK phosphorylation. These data suggest that BRDG1 receives inputs from a highly restricted group of cytoplasmic PTKs. However, stimulation with stem cell factor induced the tyrosine phosphorylation of BRDG1 in 293 cells expressing c-Kit (data not shown). It is therefore possible that BRDG1 acts downstream of receptor-type PTKs under certain conditions.

We also verified that BRDG1 is a direct substrate of Tec *in vitro*. GST or a GST-fusion protein containing full length BRDG1 was incubated with immunoprecipitated Tec and 0.1 mM ATP, separated by SDS/PAGE, and subjected to immunoblot analysis with antibodies to phosphotyrosine. Tec phosphorylated tyrosine residues of GST-BRDG1 but not of GST (Fig. 2*E*), indicating that BRDG1 is a direct substrate of Tec.

Although BRDG1 was initially isolated as a protein that binds to the kinase domain of Tec in yeast cells, we investigated

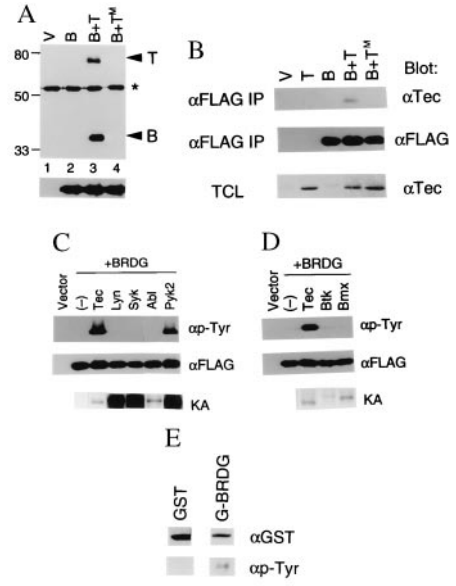

Fig. 2. (*A*) Tec-induced phosphorylation of BRDG1 *in vivo*. Ten micrograms of pcDNA3-FLAG vector (V) or of pcDNA-BRDG-F (*B*) were introduced into 293 cells (2×10^6) by the calcium phosphate method either alone or together with expression plasmids encoding either Tec (T) or a kinase-defective mutant of Tec (T^M). After 48 h of culture, cells were lysed and BRDG1 was immunoprecipitated with antibodies to FLAG. The resulting precipitates were fractionated by SDS/PAGE on a 7.5% gel and subjected to immunoblot analysis with antibodies to either phosphotyrosine or FLAG. The positions of Tec and BRDG1 are indicated at the bottom, and positions of molecular size standards (in kilodaltons) are on the left. The asterisk denotes the position of IgH. (*B*) Physical interaction of BRDG1 with Tec in intact 293 cells. Cells transfected with the empty vector (V) or with vectors encoding BRDG1 (B), Tec (T), or Tec^{KM} (T^M) as indicated at the top were subjected to immunoprecipitation (IP) with antibodies to FLAG (αFLAG), and the resulting precipitates were then subjected to immunoblot analysis either with antibodies to Tec (αTec) or FLAG. Total cell lysates (TCL) (10 μg of protein) of each set were also subjected to immunoblot analysis with anti-Tec antibody. (*C*) Effects of various PTKs on phosphorylation of BRDG1 in 293 cells. Cells were transfected with empty vector (Vector) or with pcDNA-BRDG-F (+BRDG) in the absence (−) or presence of expression plasmids encoding Tec, Lyn, Syk, c-Abl, or Pyk2. BRDG1 was immunoprecipitated from the various transfected cells with anti-FLAG antibody and then subjected to immunoblot analysis with antibodies to phosphotyrosine (αp-Tyr) or to FLAG (αFLAG). The PTKs were also immunoprecipitated from the same set of cells and subjected to an *in vitro* kinase assay without exogenous substrates. Autophosphorylation of each PTK (KA) is shown at the bottom. (*D*) Effects of various Tec family kinases on BRDG1 phosphorylation in 293 cells. Cells were transfected with empty vector (Vector) or with pcDNA-BRDG-F (+BRDG) in the absence (−) or presence of expression plasmids encoding Tec, Btk, or Bmx. BRDG1 was immunoprecipitated from the transfected cells and probed with antibodies to phosphotyrosine or FLAG. Autophosphorylation activity of each PTK (KA) is shown at the bottom. (*E*) Phosphorylation of BRDG1 by Tec *in vitro*. Immunoprecipitates prepared from 293 cells expressing Tec with anti-Tec antibody were washed and then incubated at 37°C for 15 min with 0.1 mM ATP plus 1 μg of GST or GST-BRDG1 (G-BRDG), as indicated at the top. The samples were then subjected to the immunoblot analysis with antibodies to GST (αGST) or to phosphotyrosine (αp-Tyr).

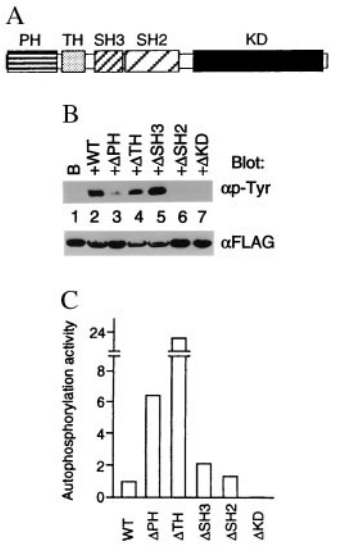

Fig. 3. (*A*) Organization of the Tec protein into PH, TH, SH3, SH2, and kinase (KD) domains. (*B*) Effect of various deletion mutants of Tec on BRDG1 phosphorylation in 293 cells. Recombinant BRDG1 was immunoprecipitated with anti-FLAG antibody from 293 cells expressing BRDG1 (B) either alone or together with wild-type Tec (WT) or Tec mutants lacking (Δ) the indicated domains. The resulting precipitates were then subjected to immunoblot analysis with antibodies to phosphotyrosine (αp-Tyr) or FLAG (αFLAG). (*C*) Autophosphorylation activity of various Tec mutants. Wild-type Tec and the various Tec mutants used in *B* were immunoprecipitated from transfected 293 cells and subjected to immunoblot analysis with either anti-phosphotyrosine antibody or anti-Tec antibody. Autophosphorylation activity of Tec and its mutants were shown in arbitrary units as tyrosine phosphorylation intensity per protein amount, both measured by a densitometer.

whether other domains of Tec contribute to the interaction between the two full length proteins. The Tec protein is composed of five domains: a PH domain, a TH domain, an SH3 domain, an SH2 domain, and a kinase domain (Fig. 3*A*). We prepared expression plasmids that encode Tec mutants lacking each of these domains (24) and introduced them into 293 cells together with the BRDG1 vector. Immunoblot analysis of BRDG1 immunoprecipitates with antibodies to phosphotyrosine revealed that deletion of the PH domain of Tec markedly attenuated the tyrosine phosphorylation of BRDG1 (Fig. 3*B,* lane 3). Deletion of the SH3 domain or TH domain did not affect phosphorylation of BRDG1, but removal of the SH2 domain or kinase domain completely abolished BRDG1 phosphorylation (Fig. 3*B,* lanes 6 and 7). The reduced efficiency of BRDG1 phosphorylation by the PH domain and SH2 domain mutants was not attributable to a reduction in Tec activity of these mutants, because the autophosphorylation activity of these mutants were not inferior to that of wild-type Tec (Fig. 3*C*). Despite the high autophosphorylation activity of TecΔTH, it could phosphorylate BRDG1 to a level similar to that of wild-type Tec. Therefore, the TH domain of Tec may also play a role in the interaction between the two molecules.

Given that the PH domain is a binding site for phospholipids, this domain is probably required for physical tethering of Tec and BRDG1 to the cell membrane, resulting in an increase in the local concentrations of and in the interaction between the two molecules. The requirement of the Tec SH2 domain for BRDG1 phosphorylation suggests that both the SH2 and kinase domains of Tec recognize the same target. This conclusion is consistent with the previous observation of Songyang *et al.* (40) that cytoplasmic PTKs preferentially phosphorylate peptides that are also recognized by their own SH2 domains, providing a molecular basis for the "processive phosphorylation" model (41) that explains how hyperphosphorylation of docking proteins is achieved.

BRDG1 Regulation of Tec Activity. We next investigated whether the interaction of BRDG1 with Tec affects Tec activity. Cell lysates were prepared from 293 cells expressing Tec or Tec^{KM} either alone or together with BRDG1 and were divided into two portions. Tec was immunoprecipitated from both portions of each lysate; one set of precipitates was then subjected to immunoblot analysis with antibodies to phosphotyrosine, and

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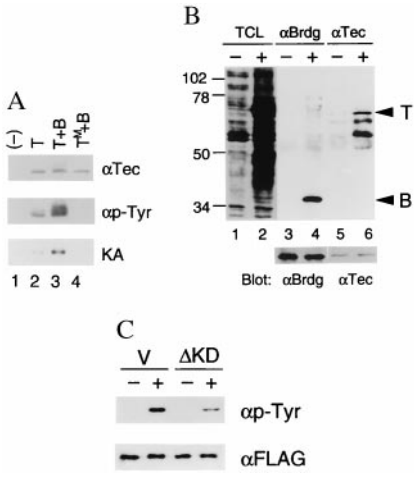

Fig. 4. (*A*) Activation of Tec by BRDG1. 293 cells were transfected with empty vector (−) or with expression vectors encoding Tec (T), Tec^{KM} (Tec^{M}), or BRDG1 (B) as indicated at the top. Tec immunoprecipitates prepared from the various transfected cells were then either subjected to immunoblot analysis with antibodies to Tec (αTec) or to phosphotyrosine (αp-Tyr) or assayed for *in vitro* kinase activity (KA) with [γ-^{32}P]ATP. (*B*) Ramos cells were left unstimulated (−) or stimulated (+) with anti-human IgM F(ab′)$_2$ fragments (10 μg/ml) for 5 min. Total cell lysates (TCL) (10 μg of protein per lane) and immunoprecipitates prepared with antibodies to either BRDG1 (αBrdg) or Tec (αTec) were then subjected to immunoblot analysis with antibodies to phosphotyrosine (top lane). The positions of Tec (T) and BRDG1 (B) are indicated on the right. The same membrane was reprobed with anti-BRDG1 antibody or anti-Tec antibody as indicated at the bottom. (*C*) Ramos cells (5×10^6) were electroporated with pcDNA-BRDG-F (10 μg) plus 15 μg of pSRα (V) or pSRα-TecΔKD (ΔKD). After 12 h of culture, cells were treated for 1 h in IMDM/1% FBS at the concentration of 5×10^6/ml and then left unstimulated (−) or stimulated (+) with anti-IgM antibody for 10 min. From each set, FLAG-tagged BRDG1 was immunoprecipitated and probed with either anti-phosphotyrosine antibody or anti-FLAG antibody.

the other set was subjected to an *in vitro* kinase assay (Fig. 4*A*). The extents of both the tyrosine phosphorylation and kinase activity of Tec were markedly increased by coexpression with BRDG1. Given that no phosphorylation of Tec^{KM} was apparent by either assay (Fig. 4*A*, lane 4, middle and bottom rows), the phosphorylation of the wild-type enzyme likely reflects its own kinase activity. Reprobing of the anti-phosphotyrosine immunoblot with antibodies to Tec revealed that the amounts of Tec and Tec^{KM} proteins precipitated were similar (Fig. 4*A*, top row). These results suggested that BRDG1 regulates the upstream kinase Tec.

We investigated which domains of Tec are required for its activation by BRDG1. In addition to the PH and SH2 domains of Tec required for BRDG1 phosphorylation, the TH domain of Tec was also necessary for the regulation of Tec activity by BRDG1 (data not shown). A regulatory function similar to that of BRDG1 has been described for the docking protein Sin (also known as Efs) (42, 43). Binding of the proline-rich motif of Sin to the SH3 domain of c-Src has been proposed to change the conformation of the latter to an "open" state and thereby induce its activation. The TH domain of Tec family kinases is thought to contribute to the regulation of the conformation and activity of these enzymes (44). Therefore, by analogy with the Sin-c-Src interaction, BRDG1 may activate Tec by disrupting an intramolecular interaction.

BRDG1 Is Involved in the BCR Signaling. Given that the BCR is expressed on the surface of Ramos cells and that Tec is activated by BCR stimulation (14), we therefore investigated whether BRDG1 participates in BCR signaling in these cells. After culture for 12 h in 1% FBS/IMDM medium, Ramos cells were stimulated with antibodies to IgM, lysed, and subjected to immunoprecipitation with antibodies to BRDG1 or to Tec. Immunoblot analysis of the resulting precipitates with antibodies to phosphotyrosine showed that both BRDG1 and Tec became phosphorylated on tyrosine residues in response to BCR stimulation (Fig. 4*B*). Furthermore, BCR engagement induced the binding of several phosphoproteins to BRDG1, consistent with a role for BRDG1 as a docking protein. Two prominent phosphoproteins (p62 and p56) also became associated with Tec in response to BCR stimulation. The same membrane was reprobed with the antibodies to BRDG1 or Tec to demonstrate that the amount of precipitated each protein was constant throughout the BCR engagement (Fig. 4*B*, bottom row).

However, we could not clearly observe the physical interaction between Tec and BRDG1 in Ramos cells. This may be owing to the low sensitivity of our anti-Tec antibody in immunoblot analysis, because the binding was proved only weakly even in the high expression system in 293 cells (Fig. 2*B*). To further support the physiologic relevance of Tec-BRDG1 relationship, we then tested whether a dominant-interfering mutant of Tec (TecΔKD) can suppress the BCR-driven phosphorylation of BRDG1 in Ramos cells. A FLAG-tagged BRDG1 was introduced into Ramos cells either alone or together with TecΔKD. After 12 h of culture, cells were stimulated with anti-IgM antibody, and the introduced BRDG1 was immunoprecipitated. Probing the precipitates with anti-phosphotyrosine antibody has revealed that BCR engagement induced the phosphorylation of FLAG-tagged BRDG1 in Ramos cells (Fig. 4*C*, V lanes). Coexpression of TecΔKD significantly decreased the phosphorylation level of BRDG1 (Fig. 4*C*, ΔKD lanes), indicating that Tec is an intermediate between BCR and BRDG1.

In this study, we describe the molecular cloning of a previously unidentified PTK substrate, BRDG1, that exhibits a strict specificity for upstream kinases. BRDG1 is phosphorylated on tyrosine residues and binds to several phosphoproteins in B cells in response to BCR stimulation. The observation that only Tec among the Tec family kinases was able to phosphorylate BRDG1 suggests that these enzymes probably possess overlapping but distinct substrate specificities. This conclusion is consistent with the observation that p62^{Dok} is an efficient substrate for Tec but not for Itk or Btk (15) (K.Y., Y.Y., A.M., K-i.O., A.K., U.I., K.S., T.Y., K.O., and H.M., unpublished work). In addition, the fact that B lymphocytes from individuals with X-linked agammaglobulinemia express substantial amounts of Tec protein (14) suggests that Tec cannot fully compensate for the loss of Btk function in these cells. Together, these data indicate that the members of the Tec family of kinases fulfill distinct roles *in vivo*. Identification of substrates such as BRDG1 that are specific for individual Tec family kinases should help to clarify these roles.

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