Mediation by the Protein-tyrosine Kinase Tec of Signaling between the B Cell Antigen Receptor and Dok-1*

Koji Yoshida‡§, Yoshihiro Yamashita‡, Akira Miyazato‡‡, Ken-ichi Ohya‡‡, Akira Kitanaka**, Uichi Ikeda†, Kazuyuki Shimada†, Takeo Yamanaka††, Keiya Ozawa††, and Hiroyuki Mano§§

From the **Division of Functional Genomics, Departments of Hematology and Cardiology, Jichi Medical School, Kawachi-gun, Tochigi 329-0498, Japan, §Omiya Medical Center, Omiya-shi, Saitama 339-8503, Japan, and the ***First Department of Internal Medicine, Kagawa Medical University, Kagawa 761-0793, Japan

Received for publication, November 10, 1999, and in revised form, May 2, 2000
Published, JBC Papers in Press, May 22, 2000, DOI 10.1074/jbc.M909012199

A variety of growth factor receptors induce the tyrosine phosphorylation of a nonreceptor protein-tyrosine kinase Tec as well as that of a Tec-binding protein of 62 kDa. Given the similarity in properties between this 62-kDa protein and p62Tec, the possibility that these two proteins are identical was investigated. Overexpression of a constitutively active form of Tec in a pro-B cell line induced the hyperphosphorylation of endogenous Dok-1. Tec also associated with Dok-1 in a phosphorylation-dependent manner in 293 cells. Tec mediated marked phosphorylation of Dok-1 both in vivo and in vitro, and this effect required both the Tec homology and Src homology 2 domains of Tec in addition to its kinase activity. Expression of Dok-1 in 293 cells induced inhibition of Ras activity, suggesting that Dok-1 is a negative regulator of Ras. In the immature B cell line Ramos, cross-linking of the B cell antigen receptor (BCR) resulted in tyrosine phosphorylation of Dok-1, and this effect was markedly inhibited by expression of dominant negative mutants of Tec. Furthermore, overexpression of Dok-1 inhibited activation of the c-fos promoter induced by stimulation of the BCR. These results suggest that Tec is an important mediator of signaling from the BCR to Dok-1.

The protein-tyrosine kinase (PTK) Tec is initially isolated from mouse liver (1) and was subsequently shown to be expressed in many tissues, including spleen, lung, brain, and kidney (2). Four Tec-related PTKs, including Btk (3, 4), Itk (also known as Emt or Tsk) (5–7), Bmx (8), and Txk (or Rlk) (9, 10), have since been molecularly cloned. With the exception of Txk, Tec and the Tec-related PTKs possess a relatively long NH2-terminal region that consists of a pleckstrin homology (PH) domain (11) and a Tec homology (TH) domain (12). The PH domain is thought to mediate protein binding to various phospholipids or phospholipid-derived molecules; for example, the Tec PH domain binds to phosphatidylinositol (PI) 3,4,5-trisphosphate (13), and the Btk PH domain binds to inositol 1,3,4,5-tetrakisphosphate (14) and PI 3,4,5-trisphosphate (15). These PH domain-phospholipid interactions are thought to mediate the conditional tethering of Tec family kinases to the cell membrane, suggesting that these enzymes might act downstream of PI 3-kinase. The TH domain of Tec PTKs contains proline-rich sequences that interact with the Src homology (SH) 3 domain of these same proteins (16) and, probably, also with the SH3 domains of other proteins. The intramolecular interaction between the TH and SH3 domains results in the binding of Tec proteins, which likely serve to mask their catalytic centers and to inhibit kinase activity.

Several Tec proteins are abundant in hematopoietic tissues and are therefore thought to play important roles in the development or maintenance of the hematopoietic system. Indeed, Tec PTKs are activated in blood cells by stimulation of cytokine receptors, lymphocyte surface antigens, G protein-coupled receptors, receptor type PTKs, or integrins (17). Furthermore, a functional Btk is indispensable for the maturation of B lymphocytes and the subsequent production of immunoglobulins. However, the downstream effectors of Tec family kinases remain largely unknown. Tec, Btk, and Itk each phosphorylate and activate phospholipase C (PLC)-γ2 (18). Candidate substrates for Btk also include BAP-135 (or TFII-I) (19) and WASP (20) and those for Tec include Grb10 (21), BRDG1 (22), and Sak kinase.2

Stimulation of cell surface receptors often induces the tyrosine phosphorylation of two unidentified Tec-binding proteins in addition to that of Tec. One of these Tec-binding phosphoproteins, p62, gives rise to a broad blurred band of ~62–66 kDa on immunoblot analysis with antibodies to phosphotyrosine, suggesting that the protein is phosphorylated on multiple tyrosine residues. The other Tec-binding phosphoprotein migrates at a position corresponding to a molecular size of ~56 kDa. Tyrosine phosphorylation of p62 is induced by cross-linking of the B cell antigen receptor (BCR) in B lymphocytes (23) and by activation of c-Kit in myeloid cells (24). Immunoblot analysis indicates that p62 is not identical to Sam68 or SHC.3

The protein p62Tec was isolated as a major substrate for activated Abl tyrosine kinases (25, 26). Dok-1 contains a PH domain at its NH2 terminus as well as multiple tyrosine residues that are potential binding sites for SH2 domains. Dok-1 is identical to the protein previously known as the GTPase-acti-

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2 Y. Yamashita, S. Kajigaya, A. Miyazato, K. Ohya, K. Yoshida, K. Kogure, M. Urabe, T. Yamanaka, K. Ozawa, and H. Mano, manuscript in preparation.
3 K. Yoshida, unpublished data.
Role of Dok-1 as a Substrate of Tec in BCR Signaling

EXPERIMENTAL PROCEDURES

Cells and Reagents—Ramos cells (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). BA/F3 cells (30) were cultured in the same medium containing mouse interleukin-3 (35 units/ml). For the cross-linking of BCR, Ramos cells were incubated for 12 h in Iscove’s modified Dulbecco’s medium (IMDM) (Life Technologies, Inc.) supplemented with 1% FBS and then stimulated for 5 min with anti-human IgM antibody F(ab’)_2 fragment (10 μg/ml) (Southern Biotechnology Associates, Birmingham, AL) as described previously (23). 293 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium-F12 (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (FBS). BA/F3 cells (25) were cultured in the same medium containing mouse interleukin-3 (35 units/ml). For the cross-linking of BCR, Ramos cells were incubated for 12 h in Iscove’s modified Dulbecco’s medium (IMDM) (Life Technologies, Inc.) supplemented with 1% FBS and then stimulated for 5 min with anti-human IgM antibody F(ab’)_2 fragment (10 μg/ml) (Southern Biotechnology Associates, Birmingham, AL) as described previously (23). 293 cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium-F12 (Life Technologies, Inc.) supplemented with 10% FBS and 2 mM l-glutamine.

Antibodies to phosphotyrosine and PLC-γ1 were obtained from Upstate Biotechnology (Lake Placid, NY), and antibodies to the FLAG epitope were from Eastman Kodak. Anti-Dok-1 were generated in rabbits in response to a glutathione S-transferase (GST) fusion protein containing the central portion of Dok-1 (GST-M). Anti-Tec antibodies were as described previously (31).

Construction of Expression Plasmids—An oligonucleotide encoding the myristylation signal (amino acids 1–10) of mouse LynA was ligated to a polymerase chain reaction-amplified fragment of estrogen receptor cDNA encoding the hormone binding domain (HBD) (32). This hybrid cDNA was then ligated to a fragment of mouse Tec cDNA encoding amino acids 3–660, and the ligation product was subcloned into the bicistronic vector pMX-ires-FLAG by single transformation. Construction of this plasmid was as described previously (33). BA/F3 cells were infected with the recombinant retrovirus and then cultured in the presence of blasticidin S (10 μg/ml) (Funakoshi, Tokyo, Japan). The pSRαa expression vectors for wild-type Tec, Tecαα, and Tec proteins lacking each subdomain were described previously (34, 35). For expression of the GST-tagged subdomains of Tec, the cDNA encoding each subdomain was amplified by polymerase chain reaction and inserted into the pEBG vector (36).

The human Dok-1 cDNA was ligated into the pSRαa expression vector, thereby generating pSRαa-Dok-1. The coding sequence for human Dok-1 was also amplified by polymerase chain reaction from the Dok-1 cDNA and inserted into the pcDNA3-FLAG vector; the resulting construct, pcDNA-Dok-F, encodes Dok-1 with a COOH-terminal FLAG epitope tag (Dok-F). The Dok-1 cDNA sequences encoding amino acids 1–271 and 1–117 were also amplified by polymerase chain reaction and inserted into the same vector; the resulting constructs encode the FLAG-tagged deletion mutants Dok-ΔC-F and DokΔMC-F, respectively.

Protein Analysis with 293 Cells—293 cells (2 × 10^6) were transfected with 10 μg of each expression plasmid by the calcium phosphate method. After culture for 2 days, cells were solubilized in lysis buffer (1% (v/v) Nonidet P-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM NaF, 1 mM Na3VO4, aprotinin (200 units/ml), 1 mM phenylmethylsulfonyl fluoride). Immunoblot analysis was performed as described previously (37), and immune complexes were visualized with the ECL detection system (Amersham Pharmacia Biotech).

The Dok-1 cDNA fragments encoding amino acids 1–118, 124–271, or 273–481 were inserted into the pGEX2T vector (Amersham Pharmacia Biotech), and the resulting constructs were introduced into *Escherichia coli* for the production of GST-PH, GST-M, and GST-C fusion proteins, respectively. For the *in vitro* kinase assay, immunoprecipitates prepared with anti-Tec were washed twice with lysis buffer and three times with kinase buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 10 mM MgCl₂, 2 mM MnCl₂) and were then incubated with 0.1 mM ATP and 1 μg of either GST or GST-Dok-1 fusion proteins in a total volume of 30 μl. The reaction mixtures were then subjected to immunoblot analysis with antiphosphotyrosine or anti-GST (AMRAD, Kew, Victoria, Australia).

Ha-Ras was expressed in 293 cells as described above, either alone or together with Dok-F, Tec, or both of these proteins. The GST-bound form of Ras was precipitated by glutathione-Sepharose beads (Amersham Pharmacia Biotech) conjugated with a GST fusion protein containing the Ras binding domain (RBD) of Raf-1. Bound proteins were eluted from the beads and probed with anti-Ras (Transduction Laboratories, Lexington, KY) as described previously (38).

Electroporation of Ramos Cells—Ramos cells (1 × 10⁶/experiment) were subjected to electroporation with 10 μg of pcDNA-Dok-F plus 20 μg of either pSRα or pSRα containing Tecκα or TecκΔD, as described previously (39). Six hours after transfection, the culture medium was changed to IMDM supplemented with 1% FBS, and the cells were incubated for an additional 12 h before being subjected to BCR cross-linking.

For luciferase assays, Ramos cells were subjected to electroporation with 2 μg of the fos/luc reporter plasmid (39) plus 10 μg of expression plasmids for Dok-F or its mutants. Five hours after transfection, cells were incubated for 5 h in the absence or presence of antibodies to human IgM (10 μg/ml). Luciferase activity was measured with the use of the luciferase assay system (Promega, Madison, WI) and is expressed as relative light units/min/microgram of protein.

RESULTS

Phosphoproteins Associated with Activated Tec—To gain insight into signaling downstream of Tec, we attempted to identify direct substrates of the kinase activity of this PTK. Proteins that are highly phosphorylated in cells expressing a constitutively active mutant of Tec would be expected to be candidates for such substrates. We therefore first prepared an expression plasmid that encodes a Tec construct (mHTec) containing an NH₂-terminal myristylation signal and the HBD of the estrogen receptor (Fig. 1A). Many growth factors induce the activation of intracellular PI 3-kinase, resulting in the production of phospholipids to which the PH domains of Tec PTKs bind; this targeting to the cell membrane induces Tec activation. The heterologous myristylation signal serves to constitutively target Tec to the cell membrane and thereby to render the activation of this PTK independent of growth factor stimulation. The HBD of the estrogen receptor was introduced into the chimeric Tec protein between the myristylation signal and the Tec sequence to serve as a dimerization motif. Exposure of cells expressing mHTec to β-estradiol is expected to induce the dimerization of mHTec molecules and thereby further increase kinase activity.

To obtain a stable cell line expressing mHTec, we infected the mouse pro-B cell line BA/F3 with a recombinant bicistronic retrovirus that encodes this protein as well as the product of the estrogen receptor (Fig. 1A). Many growth factors induce the activation of intracellular PI 3-kinase, resulting in the production of phospholipids to which the PH domains of Tec PTKs bind; this targeting to the cell membrane induces Tec activation. The heterologous myristylation signal serves to constitutively target Tec to the cell membrane and thereby to render the activation of this PTK independent of growth factor stimulation. The HBD of the estrogen receptor was introduced into the chimeric Tec protein between the myristylation signal and the Tec sequence to serve as a dimerization motif. Exposure of cells expressing mHTec to β-estradiol is expected to induce the dimerization of mHTec molecules and thereby further increase kinase activity.

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Role of Dok-1 as a Substrate of Tec in BCR Signaling

Fig. 1. Identification of PLC-γ and Dok-1 as substrates of Tec. A, schematic representation of the structure of mHTec. The myristylation signal (Myr) and the HBD of the estrogen receptor were fused with the NH2-terminal region of Tec. The PH, TH, SH3, SH2, and kinase domains of Tec are indicated. B, total cell lysates (TCL) (10 μg of protein/lane) of parental BA/F3 cells (P) and BA/F3-mHTec cells (T), as well as anti-Tec immunoprecipitates (Tec IP) prepared from these lysates, were fractionated by SDS-polyacrylamide gel electrophoresis on a 7.5% gel and subjected to immunoblot analysis with anti-Tec. The positions of mHTec and p70 Tec are indicated. C, the anti-Tec immunoprecipitates described in B were subjected to immunoblot analysis with anti-Tec. The positions of mHTec and p70Tec are indicated. D, total cell lysates (10 μg of protein/lane), anti-Dok-1 immunoprecipitates, and anti-PLC-γ immunoprecipitates prepared from BA/F3 and BA/F3-mHTec cells were subjected to immunoblot analysis with antiphosphotyrosine (upper panel). The positions of p150, p110, and p62 are shown on the left and those of Dok-1 and PLC-γ are shown on the right. The same membrane was reprobed with anti-Dok-1 (αDok) or anti-PLC-γ, as indicated (lower panel).

The Tec-binding phosphoproteins p150 and p62 in BA/F3-mHTec cells remained candidates for in vivo substrates of Tec. Immunoblot analysis of BA/F3-mHTec cell lysates showed that p62 is not Sam68 (data not shown). In contrast, the extent of tyrosine phosphorylation of PLC-γ in BA/F3-mHTec cells was markedly greater than that in the parental BA/F3 cells (Fig. 1D), and the electrophoretic mobility of PLC-γ1 was identical to that of p150. The possibility that p150 is an isoform of PLC-γ may be consistent with the previous observation that PLC-γ2 contributes to signaling by Tec PTKs (40).

With regard to the identity of p62 in BA/F3-mHTec cells, we previously showed that activation of c-Kit in myeloid cells or of the BCR in B cells induces tyrosine phosphorylation of both Tec and a Tec-binding protein also termed p62. The phosphorylation profile and size of this p62 protein suggested that it might be identical to p62Dok-1. Indeed, immunoprecipitation and immunoblot analysis revealed that the extent of tyrosine phosphorylation of Dok-1 was markedly increased in BA/F3-mHTec cells compared with that in BA/F3 cells (Fig. 1D). The electrophoretic mobility of Dok-1 was also identical to that of the Tec-binding protein p62.

Dok-1 as an in vivo substrate of Tec—To investigate the possible interaction of Tec with Dok-1, we constructed expression plasmids for FLAG-tagged Dok-1 and Dok-1 deletion mutants (Fig. 2A). Immunoprecipitation with anti-FLAG and immunoblot analysis with anti-phosphotyrosine revealed that FLAG-tagged full-length Dok-1 (Dok-F) expressed in 293 cells exhibited a low level of tyrosine phosphorylation (the protein is apparent as a doublet in the top panel of Fig. 2B). Coexpression of Tec markedly increased the extent of Dok-F phosphorylation, suggesting that Dok-F is an in vivo substrate for Tec. A Dok-1 mutant (DokΔC-F) lacking the COOH-terminal region of the intact protein migrated as a single band and showed a reduced extent of Tec-induced tyrosine phosphorylation compared with Dok-F. Further deletion of the middle portion of Dok-1 containing the Dok homology region (distantly related to the phosphotyrosine binding domain) (41), yielding the mutant DokΔMC-F, prevented Tec-induced phosphorylation. These results suggested that both the middle and COOH-terminal regions of Dok-1, but not the PH domain, contain the residues targeted by Tec.

Reprobing of the anti-FLAG immunoprecipitates with anti-Tec revealed that Tec coprecipitated with Dok-F but not with DokΔC-F or DokΔMC-F (Fig. 2B). Further probing of the membrane with anti-FLAG demonstrated that Dok-F and its truncation mutants were expressed in similar amounts (Fig. 2B). Thus, the COOH-terminal region of Dok-1 appears to contain the major binding site for Tec.

With regard to the region of Tec that functions as the binding site for Dok-1, the Tec protein consists of PH, TH, SH3, SH2, and kinase domains (Fig. 1A). We therefore prepared expression plasmids that encode Tec mutants lacking each subdomain and assessed the ability of these mutants to phosphorylate Dok-1 in 293 cells (Fig. 2C). Unexpectedly, the PH domain of Tec was not required for efficient phosphorylation of Dok-1. In contrast, deletion of the TH domain of Tec markedly reduced the extent of phosphorylation of Dok-1, and deletion of the SH2 domain almost completely prevented Dok-1 phosphorylation. As expected, deletion of the kinase domain of Tec destroyed the ability of Tec to phosphorylate Dok-1. Reprobing of the same membrane with anti-FLAG revealed that the amounts of Dok-1 precipitated were similar among the cells expressing the various Tec proteins. The reduced extent of Dok-1 phosphorylation in cells expressing Tec mutants lacking the TH or SH2 domains was not attributable to impaired kinase activity of Tec, given than the autophosphorylation activities of these mutants were previously shown to be no less than that of the wild-type protein (22).

We next prepared eukaryotic expression plasmids for GST fusion proteins containing the various Tec domains. These plasmids were introduced into 293 cells together with pcDNA-Dok-F and in the absence or presence of a vector encoding full-length Tec, to examine which domains of Tec are able to associate with Dok-F in intact cells. GST or the GST
Fusion proteins were purified from the transfected cells with the use of glutathione-Sepharose beads, and the proteins bound to the beads were subjected to immunoblot analysis with anti-FLAG. In the absence of coexpression of full-length Tec, no substantial binding of Dok-1 to any of the Tec domains was apparent (Fig. 2D). In contrast, in the presence of full-length Tec, a large amount of Dok-1 bound to the SH2 domain of Tec.

Given that coexpression of Tec should result in marked tyrosine phosphorylation of Dok-1 in 293 cells, these data indicate that the Tec-Dok-1 interaction is mediated predominantly by the SH2 domain of Tec and phosphotyrosine residues of Dok-1.

To confirm that Dok-1 is a direct substrate of Tec, we prepared recombinant GST fusion proteins containing various domains of Dok-1 (Fig. 2A) and performed an in vitro kinase assay. Recombinant Tec was immunoprecipitated from 293 cells and incubated with ATP and either GST or GST fusion proteins containing the PH domain (GST-PH), the central region (GST-M), or the COOH-terminal region (GST-C) of Dok-1. GST or the GST fusion proteins were purified with the use of glutathione-Sepharose beads, and bound proteins were eluted and subjected to immunoblot analysis with anti-FLAG to detect Dok-1.

FIG. 2. Identification of Dok-1 as a direct substrate of Tec. A, schematic representation of Dok-1-based proteins. A FLAG epitope tag (closed diamond) was added to the COOH-terminal end of proteins comprising amino acids 1–481 (Dok-F), 1–271 (DokΔC-F), or 1–117 (DokΔMC-F) of Dok-1. Recombinant GST (closed oval) fusion proteins containing amino acids 1–118 (GST-PH), 124–271 (GST-M), or 273–481 (GST-C) of Dok-1 were also constructed. The PH and Dok homology domains as well as the positions of tyrosine residues (arrowheads) of Dok-1 are indicated. B, the empty vector (V) or expression vectors for Dok-F (Dok), DokΔC-F (ΔC), DokΔMC-F (ΔMC), or Tec (T) were introduced into 293 cells in the indicated combinations. Dok proteins were immunoprecipitated from cell lysates with anti-FLAG and subjected to immunoblot analysis with antiphosphotyrosine (α-Tyr) (top panel), anti-Tec (middle panel), or anti-FLAG (bottom panel). The positions of Tec and of Dok mutants are indicated. Asterisks denote the positions of Ig heavy and light chains. C, the empty vector or the expression vector for Dok-F was introduced into 293 cells either alone (-) or together with vectors encoding wild-type Tec (WT) or Tec mutants lacking (Δ) the indicated domains. Dok-F was immunoprecipitated by anti-FLAG and subjected to immunoblot analysis with antiphosphotyrosine (upper panel) or anti-FLAG (lower panel). D, GST or GST fusion proteins containing the indicated Tec domains were expressed in 293 cells in the absence or presence of full-length Tec and Dok-F, as indicated. GST or the GST fusion proteins were purified with the use of glutathione-Sepharose beads, and bound proteins were eluted and subjected to immunoblot analysis with anti-phosphotyrosine (left panel) or anti-GST (right panel). The positions of molecular size standards (in kilodaltons), GST fusion proteins (asterisks), and Ig heavy chain (arrowhead) are indicated.

In the absence of coexpression of full-length Tec, no substantial binding of Dok-F to any of the Tec domains was apparent (Fig. 2D). In contrast, in the presence of full-length Tec, a large amount of Dok-1 bound to the SH2 domain of Tec.
Role of Dok-1 as a Substrate of Tec in BCR Signaling

The susceptibility of Dok-1 to phosphorylation by various PTKs—Dok-1 was molecularly cloned as a major substrate of c-Kit, a receptor-type PTK. Carpino et al. (25) also showed that Dok-1 may be phosphorylated by c-Kit, a receptor-type PTK. The susceptibility of Dok-1 to phosphorylation by various PTKs was further investigated by expressing Dok-1 in 293 cells together with representatives of a variety of nonreceptor PTK subfamilies. Immunoblot analysis of Dok-1 immunoprecipitates with antiphosphotyrosine revealed that marked phosphorylation of Dok-1 was induced by coexpression with Tec, c-Abl, Lyn, Syk, or Pyk2, but not with Jak2, Csk, or Fak (Fig. 3A). Rebinding of the membrane with anti-Dok-1 verified the presence of similar amounts of Dok-1 in the various immunoprecipitates. As repeatedly shown in subsequent figures, an increase in the extent of phosphorylation of Dok-1 was accompanied by a decrease in the electrophoretic mobility of the protein, as revealed by immunoblot analysis with our polyclonal antibodies to Dok-1, which were generated in response to the central region of the protein.

Given that Tec phosphorylates Dok-1 at a high stoichiometry, we examined whether Dok-1 is also an effective substrate for other Tec family members. Dok-1 was expressed in 293 cells either alone or in combination with Tec, Btk, Bmx, or Itk, immunoprecipitated by the polyclonal antibodies to Dok-1, and probed with antiphosphotyrosine. None of the other members of the Tec family phosphorylated Dok-1 as efficiently as did Tec (Fig. 3B); no phosphorylation of Dok-1 was apparent with Btk or Bmx, and only a moderate level of phosphorylation was induced by Itk. Again, reprobing of the membrane with anti-Dok-1 revealed the presence of similar amounts of Dok-1 in the various immunoprecipitates.

Role of the Tec-Dok-1 Interaction in BCR Signaling—The potential role of the Tec-Dok-1 interaction in BCR signaling was investigated with the human immature B cell line Ramos. Cells were incubated for 12 h in IMDM supplemented with 1% FBS and were then stimulated for 5 min with anti-human IgM F(ab')2 fragments. As previously shown (23), BCR engagement resulted in the phosphorylation of Tec (Fig. 4A). In addition, several Tec-binding proteins became phosphorylated on tyrosine residues in response to BCR stimulation; these proteins included p150, p62, and p56. Analysis of anti-Dok-1 immunoprecipitates from Ramos cells revealed that BCR engagement also induced the tyrosine phosphorylation of Dok-1 and that the electrophoretic mobility of Dok-1 was identical to that of the Tec-binding protein p62. To examine whether this is the case, the total cell lysates of Ramos cells were precleared by normal rabbit serum or anti-Tec serum. The lysates were then subjected to the immunoprecipitation with the antibodies to Dok-1, followed by the immunoblot analysis with antiphosphotyrosine antibody or anti-Dok-1 antibody. As evident in Fig. 4B, the preclear treatment with anti-Tec serum significantly reduced the protein amount as well as tyrosine phosphorylation of Dok-1. These data suggested the idea that Tec physically associates with Dok-1 in Ramos cells.

It should be noted, however, that we could not directly detect Dok-1 in the anti-Tec immunoprecipitates by using the antibodies to Dok-1. Our efforts have been hampered by the low sensitivity of anti-Dok-1 antibodies for immunoblot analysis. The anti-Dok-1 serum used in this manuscript was most efficient among our antisera raised against different epitopes of Dok-1 and commercially available antibodies. As shown in Fig. 3, even this antibody could yield an only weak staining for Dok-1 in 293 cells overexpressing this protein (Fig. 3). Importantly, phosphorylation of Dok-1 partially inhibited the binding of these antibodies, probably because of steric hindrance by the phosphate moieties. Therefore, although our data collectively indicate that the Tec-binding p62 is Dok-1, we cannot yet rule out the possibility that the Tec-binding protein designated p62 actually comprises phosphorylated proteins in addition to Dok-1.

Our results have thus shown that (i) Tec phosphorylates Dok-1 both in vitro and in vivo and that (ii) BCR engagement induces the phosphorylation of both Tec and Dok-1. To verify that Tec is the kinase responsible for the phosphorylation of Dok-1 in BCR signaling, we introduced Dok-F into Ramos cells either alone or together with a kinase negative mutant (TecKM, in which Lys397 in the ATP binding site is replaced with Met) or a kinase domain-deleted mutant (TecKD) of Tec. The transfected cells were stimulated with anti-IgM, and Dok-F was immunoprecipitated by anti-FLAG and analyzed with antiphosphotyrosine. BCR cross-linking increased the extent of tyrosine phosphorylation of Dok-F in cells overexpressing this protein alone (Fig. 4C). Coexpression of TecKM or TecKD reduced the extent of Dok-F phosphorylation both in unstimulated cells and in those stimulated through the BCR, with the effect of TecKD being more pronounced than that of TecKM. Probing of the immunoblot membrane with anti-FLAG revealed that the amounts of Dok-F were similar in the various transfectants. Both Tec mutants therefore inhibited the BCR-induced phosphorylation of Dok-1 in a dominant negative manner, suggesting that Tec plays a prominent role in transmission of signals from the BCR to Dok-1.
The observation that diverse growth stimuli induce the tyrosine phosphorylation of Dok-1 suggests that this protein contributes to growth signaling. However, only a few proteins including RasGAP have been identified to date as the downstream effector of Dok-1. Phosphorylation of Dok-1 on tyrosine residues induces its binding to RasGAP (26); this binding is mediated through a phosphotyrosine-SH2 domain interaction, and likely results in the recruitment of RasGAP to the cell membrane and consequent facilitation of the conversion of the GTP-bound, active form of Ras to the GDP-bound, inactive form. Such a scenario, however, suggests that Dok-1 serves to inhibit, rather than to promote, cell growth.

The promoter of the c-fos proto-oncogene is activated in response to BCR cross-linking in Ramos cells, and this effect is almost completely inhibited by expression of a dominant negative mutant of Ras (data not shown). These results suggest that Ras mediates transmission of the activation signal from the BCR to the c-fos promoter. We examined the effect of exogenous Dok-1 on this signaling pathway with the use of the pfos/luc reporter plasmid, in which expression of the fruit fly luciferase gene is controlled by a fragment of the c-fos promoter. This plasmid was introduced into Ramos cells together with the expression plasmids for Dok-F or its deletion mutants (Fig. 2A). BCR cross-linking induced marked activation of the c-fos promoter in cells not expressing exogenous Dok-1 (Fig. 4D). Expression of Dok-F inhibited BCR-induced activation of the c-fos promoter, suggesting that Dok-1 negatively regulates BCR-Ras-c-fos signaling, as predicted from its interaction with RasGAP. Truncation of Dok-1 from the COOH terminus resulted in a stepwise decrease in this inhibitory effect.

Finally, we directly examined whether expression of Dok-1 down-regulates Ras activity in cells. An expression plasmid for Ha-Ras was introduced into 293 cells either alone or together with vectors encoding Tec, Dok-F, or both of these proteins. Given that only the GTP-bound form of Ras interacts with the RBD of Raf-1 (38), we assessed Ras activity by mixing lysates of transfected cells with a GST-RafRBD fusion protein immobilized on glutathione-Sepharose beads. GTP-bound Ras was then eluted from the beads and subjected to immunoblot analysis with anti-Ras (Fig. 4E). Consistent with the results of the c-fos promoter assay, expression of Dok-1 markedly inhibited Ras activity, again suggesting that Dok-1 functions as a negative regulator of Ras. Coexpression of Tec had no effect on Ras activity.

**DISCUSSION**

We have shown that Dok-1 is a direct substrate of the PTK Tec and that, at least in BCR signaling, Tec is an important regulator of Ras. Coexpression of Tec and Dok-1 almost completely inhibited Ras activity in transfected cells. This effect was due to the inhibition of Ras activity by Dok-1 rather than to the inhibition of Tec activity by Dok-1, as determined by the competition of Ras activity with the use of vectors encoding Tec, Dok-F, or both of these proteins. These results suggest that Dok-1 negatively regulates Ras in BCR signaling, as supported by the observation that Dok-1 expression inhibited BCR-induced activation of the c-fos promoter. These data are consistent with the proposal that Dok-1 is a negative regulator of Ras in BCR signaling.

**Fig. 4. Role of Dok-1 in BCR signaling.** A, Ramos cells (1 x 10^7) were cultured for 12 h in IMDM supplemented with 1% FBS and then incubated for 5 min in the absence (-) or presence (+) of anti-human IgM F(ab')2 fragments (10 μg/ml). Cell lysates were then subjected to immunoprecipitation with normal rabbit serum (NRS) or anti-Dok or anti-Dok-1. The resulting precipitates were fractionated by SDS-PAGE on a 7.5% gel and subjected to immunoblot analysis with antiphosphotyrosine (upper panel) or with anti-Tec and anti-Dok-1 (lower panel). The positions of molecular size standards (in kilodaltons) are shown on the left, and those of Tec, Dok-1, and p56 are indicated on the right. B, cell lysates of Ramos (1 x 10^7) stimulated with anti-IgM were incubated for 2 h with protein A-Sepharose beads plus normal rabbit serum (NRS) or anti-Tec serum (αTec). The lysates were then subjected to immunoprecipitation with anti-Dok-1 serum and immunoblotted with either antiphosphotyrosine (α-p-Tyr) or anti-Dok-1 (αDok). The position of Dok-1 is indicated at the right. C, Ramos cells (1 x 10^7) were subjected to electroporation with 10 μg of pcDNA-Dok-F plus 20 μg of pcDNA3 vectors encoding the FLAG tag, Dok-F, or Dok-F and Tec. D, Ramos cells (1 x 10^7) were subjected to electroporation with 2 μg of the pfos/luc reporter plasmid together with 10 μg of pcDNA3 vectors encoding the FLAG tag, Dok-F, or Dok-F and Tec. E, cell lysates were mixed with GST-RafRBD fusion protein immobilized on glutathione-Sepharose beads and then assayed for luciferase activity, which was expressed in relative light units/min/microgram of protein. Data are mean ± S.D. of triplicate determinations from a representative experiment. F, expression plasmids for Ras, Tec, and Dok-1 were introduced into 293 cells in the indicated combinations, and cells were subsequently lysed and mixed with GST-RafRBD fusion protein immobilized on glutathione-Sepharose beads. Cellular proteins that bound to the beads were then eluted and subjected to immunoblot analysis with anti-Ras. The position of active Ras is indicated on the right.
mediator of Dok-1 phosphorylation in vivo. Given that Dok-1 is hyperphosphorylated in cells expressing the Bcr-Abl fusion protein or v-Abl (25, 26), it is likely that Dok-1 also serves as a substrate for these activated Abl proteins. In addition, c-Abl also efficiently phosphorylates Dok-1. Our data showing that a wide spectrum of nonreceptor PTKs phosphorylates Dok-1 suggest that this protein receives input from various such enzymes under different conditions. Integrin activation has recently been shown to regulate Dok-1 phosphorylation (42), and evidence suggests that Dok-1 is also a substrate for receptor type PTKs. The function of Dok-1 in vivo has, however, remained unclear, although a recent study implicated this protein in insulin-induced cell migration (42).

Although Dok-1 was previously identified as a binding protein of RasGAP, it has been unclear whether Dok-1 activates or inhibits the activity of Ras. Our data support the latter of these two possibilities, consistent with the ability of Dok-1 to recruit RasGAP to the cell membrane in a phosphorylation-dependent manner (42). However, despite such Dok-1-mediated recruitment of RasGAP, Noguchi et al. (42) failed to detect inhibition of p44 and p42 mitogen-activated protein kinases in response to insulin in Chinese hamster ovary cells that express human insulin receptors (IR) (Chinese hamster ovary-IR cells). These observations are not necessarily incompatible with our data. With the use of transfected 293 cells, we showed that Dok-1 appears to transmit a negative signal to Ras through RasGAP. In the Chinese hamster ovary-IR cells, however, activated insulin receptors also likely send a positive signal to mitogen-activated protein kinases (or Ras) independent of their signaling to Dok-1. IRS proteins undergo marked tyrosine phosphorylation in response to stimulation of insulin receptors and thereby provide docking sites for PI 3-kinase and the protein-tyrosine phosphatase SHP-2, the latter of which regulates Ras activity (43). Furthermore, insulin receptors phosphorylate the adapter molecule SHC (44), which is a potent activator of the Ras-mitogen-activated protein kinase pathway. These insulin receptor-evoked positive signals to Ras may therefore have masked Dok-1 regulation of Ras activity in the study of Noguchi et al. (42). The phosphorylation of Dok-1 on multiple tyrosine residues, one of which may become a binding site for Nck (42), further suggests that Dok-1 may be functionally connected to a variety of signaling molecules in addition to RasGAP. Identification of cellular proteins that are recruited to these multiple phosphorylation sites of Dok-1 should clarify the in vivo roles of this protein (Fig. 5).

We showed that the extent of tyrosine phosphorylation of Dok-1 in BA/F3 cells expressing an active form of Tec was >50-fold that apparent in the parental BA/F3 cells. Tec also associated with, and phosphorylated multiple tyrosine residues of, Dok-1 in 293 cells. Furthermore, BCR-induced phosphorylation of Dok-1 in Ramos cells was markedly inhibited by expression of dominant negative mutants of Tec. Together, these data support the occurrence of a physical and functional interaction between Tec and Dok-1. At least in Ramos cells, Tec appears to be the predominant PTK responsible for the activation of Dok-1 (Fig. 5). However, the observation that a low level of tyrosine phosphorylation of Dok-1 remained apparent in Ramos cells expressing dominant negative mutants of Tec suggests that other PTKs also contribute to phosphorylation of Dok-1 in these cells.

Our data indicate that the SH2 domain of Tec binds to tyrosine residues of Dok-1 that are phosphorylated by Tec itself. Thus, the SH2 domain and the kinase domain of Tec appear to target the same tyrosine residues. This conclusion is consistent with the “progressive phosphorylation” model proposed to account for the hyperphosphorylation of docking proteins by nonreceptor PTKs (36). According to this scenario, Tec first phosphorylates a tyrosine residue of Dok-1 that is then recognized and bound with high affinity by the SH2 domain of Tec. The interaction of this phosphotyrosine residue with the SH2 domain of Tec then allows the kinase domain of Tec to phosphorylate the next target tyrosine of Dok-1. Repetition of this cycle would result in the hyperphosphorylation of Dok-1 by Tec.

The Tec family member Btk was not able to phosphorylate Dok-1 in 293 cells. Btk is abundant in Ramos cells and is activated in response to BCR stimulation (data not shown). However, in contrast to the situation with Tec, we were not able to detect tyrosine-phosphorylated p62 in anti-Btk immunoprecipitates prepared from BCR-stimulated Ramos cells (data not shown). A similar discrepancy between the abilities of Tec and Itk to phosphorylate Dok-1 was recently described (45). We have also previously identified a docking protein, BRDG1, that is an effective substrate for Tec but not for Btk or Itk (22). Members of the Tec family of PTKs therefore appear to possess distinct, but probably overlapping, target specificities, with Dok-1 being one example of such a member-specific substrate.

Acknowledgments—We thank B. Clarkson for the human Dok-1 cDNA, M. Kawabata for the pcDNA3-FLAG vector, B. J. Mayor for the pEBG vector, C. I. E. Smith for the Btk cDNA, S. Desiderio for the Itk cDNA, D. Weil for the Bmx cDNA, T. J. Parsons for the Fak cDNA, T. Yi for the Lyn cDNA, M. Okada for the Csk cDNA, T. Mustelin for the Syk cDNA, J. N. Ihle for the Jak2 cDNA, Y. Maru for the c-Abl cDNA, J. Schlessinger for the Pyk2 cDNA, D. Shalloway for pGEX-RBD, and the Kirin Brewery Co. (Tokyo, Japan) for cytokines.
