

The Role of Tec Protein-tyrosine Kinase in T Cell Signaling*

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The Tec protein-tyrosine kinase family includes Btk, Itk/Tsk/Emt, Tec, Rlk/Txk, and Bmx which are involved in signals mediated by various cytokines or antigen receptors. Itk is expressed primarily in T cells and activated by TCR/CD3, CD28, and CD2. However, the defect in T cell signaling in *itk*-deficient mice is very modest. Thus, we looked for other Tec family kinases that could be expressed in lymphoid cells and involved in T cell signal transduction. Here, we demonstrate that Tec, expressed in T cells, is activated following TCR/CD3 or CD28 ligation and interacts with CD28 receptor in an activation-dependent manner. This interaction involves the Tec SH3 domain and the proline-rich motifs in CD28. We also show that Tec can phosphorylate p62^{dok}, one CD28-specific substrate, whereas Itk cannot. Overexpression of Tec but not Itk can enhance the interleukin-2 promoter activity mediated by TCR/CD3 or CD28 stimulation and introduction of a kinase-dead Tec but not Itk can suppress interleukin-2 expression, indicating that Tec is directly involved in T cell activation. Altogether, these data demonstrate that Tec kinase is an integral component of T cell signaling and that the two Tec family kinases, Tec and Itk, have distinct roles in T cell activation.

The Tec family is a recently emerging subgroup of non-receptor PTKs.¹ The Tec family is currently the second largest PTK subfamily encompassing five members: Tec, Btk, Itk/Tsk/Emt, Rlk/Txk, and Bmx (1). However, little is known about this family. They are all characterized by a pleckstrin homology domain (PH)¹ (except Rlk/Txk), a Tec homology domain (TH) containing a region homologous to the GAP1 family of Ras GTPases at the N terminus and one or two proline-rich motifs at its C terminus (2), an Src homology (SH) 3 domain, an SH2 domain, and an SH1/kinase domain. They are devoid of N-terminal myristoylation sites and C-terminal tyrosine residues corresponding to Tyr⁵²⁷ of c-Src involved in its regulation. PH domains bind to phosphorylated inositol lipids and are thought to anchor proteins possessing such domains to membrane (3–6). Itk SH3 domains bind to cellular ligands such as Sam-68, Wiskott-Aldrich syndrome protein, and human RNP-K (7, 8).

In the same way, Tec TH domains interact with SH3 domains of Grb-2, Src family PTKs (Fyn, Lyn, and Hck) (9, 10), Vav (11), or c-Kit (12). In the unactivated state, the interaction of Itk SH3 domain with the proline-rich motif (KPLPPTP) present in its TH domain would result in an intramolecular binding that could prevent the interaction of these domains with other cellular interaction motifs and probably inhibit their enzymatic activity. Regulation of Tec family depends on at least two events, phosphorylation by Src family kinases (9, 13) and recruitment to phospholipids produced by phosphatidylinositol 3-kinase on membrane (4, 14).

Many members of Tec family are abundantly expressed in hematopoietic tissues. Btk is expressed in B cells and myeloid cell lineages (15). Itk/Tsk/Emt is primarily expressed in T cells (16–18), natural killer cells, and mast cells (19, 20). Itk is activated by various T cell surface receptors such as TCR/CD3, CD28, and CD2 (21, 22). Interestingly, mice lacking Itk have both decreased numbers of mature thymocytes and reduced proliferative responses to allogeneic major histocompatibility complex stimulation or TCR cross-linking (23). These signaling defects of the antigen receptors can be overcome by stimulation with phorbol esters (PMA) and calcium ionophores (ionomycin), indicating that this protein kinase functions at an early stage in the signaling pathways. Tec is expressed in most hematopoietic cells (24) and B cells (25). It is involved in the intracellular signaling system of numerous cytokines such as IL-3, IL-6, stem cell factor, granulocyte colony stimulating factor, erythropoietin, and thrombopoietin (11, 12, 26–29). So far, Tec functions are hardly understood, and its substrates still remain to be identified. However, it was recently suggested that Tec can phosphorylate Jak-2 and the transient expression of Tec in BAF3 cells resulted in the marked elevation of the promoter activity of the *c-fos* proto-oncogene (30, 31).

The full activation of T cells requires both interaction of the TCR with antigen bound to major histocompatibility complex molecules and costimulatory signals. The major costimulatory molecule is the adhesion molecule CD28 that interacts with its ligands B7.1/CD80 and B7.2/CD86 on antigen presenting cells. These two signals induce T cell proliferation, cytokine production, and regulation of T cell apoptosis and survival. The signaling pathways for TCR/CD3 stimulation and CD28 costimulation still remain poorly understood. TCR/CD3 or CD28 signal transduction induces various biochemical events inclusive of calcium mobilization, tyrosine phosphorylation of downstream substrates, PTK activation, activation of PI 3-kinase, and activation of p21^{ras}. TCR/CD3 and CD28 must recruit PTKs to phosphorylate their substrates upon activation since they lack intrinsic kinase activities. For example, it has been shown that Lck, Fyn, ZAP-70, and Itk can be recruited and activated in TCR/CD3 pathway and that Lck, Fyn, and Itk can be recruited and activated in CD28 pathway. Although similar PTKs can be recruited, TCR/CD3 and CD28 induce phosphorylation of their specific substrates such as SLP-76 (p76) and LAT (p36/38) for TCR/CD3 and p62^{dok} and the catalytic subunit of phosphatidylinositol 3-kinase (32–35) for CD28 besides common substrates

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¹ The abbreviations used are: PTK, protein-tyrosine kinase; PH, pleckstrin homology; TH, Tec homology; IL, interleukin; HA, hemagglutinin; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; TCR, T cell receptor; GAM, goat anti-mouse; PR, proline-rich; WT, wild type; AU, arbitrary units; aa, amino acid(s); GST, glutathione S-transferase; PI, phosphatidylinositol.

such as phospholipase C γ and p95^{uv}. PTKs are critical for T cell signaling since inhibition of PTKs abrogated the substrate phosphorylation and IL-2 production. Nevertheless, in the *itk*-deficient mice, CD3-mediated proliferative response was severely compromised, whereas CD28-mediated proliferation was significantly enhanced when compared with cells from control animals (36). These data suggest that Itk negatively regulates the amplitude of CD28 costimulation. Intriguingly, instead of being involved in the amplification of IL-2 production which is the hallmark of CD28 costimulation, Itk could down-modulate these events. These observations prompted us to look for other Tec family kinases that could be expressed in lymphoid cells and involved in the amplification of IL-2 production.

Here, we demonstrate that Tec kinase is also expressed in T cells and activated on TCR/CD3 or CD28 stimulation. We also show that Tec inducibly binds to CD28 via SH3 domain proline-rich motif interaction and Tec can phosphorylate *in vivo* p62^{dok} protein, a Ras GAP-associated adaptor that can be phosphorylated in CD28 but not TCR/CD3 pathway. Furthermore, Tec but not Itk can induce IL-2 promoter activity. In addition, kinase-dead Tec can suppress IL-2 activation in TCR/CD3 and CD28 pathways. These data suggest that two Tec family kinases, namely Itk and Tec, are involved in T cell signal transduction and that Tec is a likely candidate for regulating T cell activation.

EXPERIMENTAL PROCEDURES

Cell Lines—Murine T hybridoma parental cells DC27.1, clone DWT6.11+— expressing wild-type huCD28 cDNA, mutant DYF173+200 where Tyr has been substituted by Phe at positions 173 and 200, and DEL 21 where 21 amino acids in the C terminus of CD28 have been truncated and contained no functional proline-rich motifs were previously described (37). Here we renamed clones DWT6.11+ and DYF173+200 for WT and Y173F+Y200F. Clones P181A+P183A, P193A, and P194A were derived from DC27.1 cells that were stably transfected with the plasmids PSR α CD28P181A+P183A, PSR α CD28P193A, and PSR α CD28P194A. Clone WTHAp62^{dok} was obtained from WT stably transfected with the plasmid pMSCV-HADok. All T murine hybridoma cells were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum, penicillin, streptomycin, β -mercaptoethanol, sodium pyruvate, and glutamate. COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamate. Besides human Jurkat T leukemia cells JH6.2 (38), murine fibroblast cell line 3T6, murine IL-3-dependent pro-B cell line BAF3, human monocytic cell line THP1, human myeloid cell line KG1a, human pre-B cell line Nalm6, and human myeloma cell line U266 were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamate except BAF3 medium which was further supplemented with IL-3.

Plasmids—Expression plasmids pSR α CD28, p48Src, pF10, and BtkHA containing human wild-type CD28, Src, Fyn, and Btk tagged with HA epitope cDNAs were gifts from Drs. R. Sweet, S. Courtneige, C. Bebbington, and R. Guinamard. Expression plasmids pCDNALck, pCDNAItkflag, and pCDNAHAp62^{dok} possessing Lck, Itk tagged in 3' end, and p62^{dok} tagged with HA epitope were constructed by subcloning of pSMLck gifted from Dr. M. Marsh, p52.2.1, a gift from Dr. S. Desiderio, and pMSCV-HADok gifted from Dr. Y. Yamanashi to pCDNA3 vector, respectively. Plasmid pCDNAflagTec containing a wild-type full-length murine Tec cDNA tagged with the FLAG epitope in 5' end was described previously. This enzyme corresponds to the full-length enzyme, type IV or TecA (24). Plasmid pCDNAflagTecdk was the kinase-dead version of pCDNAflagTec with a point mutation at the ATP-binding site of Tec corresponding to amino acid 397 (Lys to Glu). pCDNAItkdkflag was the kinase-dead version of pCDNAItkflag with a point mutation at the ATP-binding site of Itk corresponding to amino acid 396 (Lys to Glu). Plasmid pCD2flagTec was constructed by in frame subcloning Tec *Eco*RI fragment of pCDNAflagTec downstream of rat CD2 corresponding to amino acids 1–219 in pCDNArCD2BE vector. Plasmid pCD2flagTecdk was a kinase-dead version of pCD2flagTec with a point mutation at the ATP-binding site of Tec corresponding to amino acid 397 (Lys to Glu). Plasmids PSR α CD28P181A+P183A, PSR α CD28P193A, and PSR α CD28P194A in which human CD28 was point-mutated from Pro to Ala at amino acid residues 181 and 183, 193, and 194, respectively, were generated using the Transformer Site-directed Mutagenesis kit (CLONTECH) according to the manufacturer's instructions. The GST fusion plasmids pGSTTECSH1, pG-

STTECSH2, and pGSTTECSH3 were constructed by respectively subcloning the polymerase chain reaction product of SH1, SH2, and SH3 regions of Tec into pGEX4T3 vector (Pharmacia Biotech Inc., Uppsala, Sweden). pGSTTECSH1 containing PH and TH region of Tec has been described previously (12). These constructs are further depicted in Fig. 3. pGSTITKSH3 containing Itk SH3 domain was a gift from Dr. R. Guinamard. The promoter assay plasmids pIL-2-Luc composed of IL-2 promoter fused with firefly luciferase reporter gene and p β -actin-RLuc composed of β -actin promoter fused with *Renilla* luciferase reporter gene were gifts from Drs. E. Verdin (39) and R. Castellano, respectively.

Antibodies—Anti-human CD28 monoclonal antibodies (mAbs) 248, CD28.2, and CD28.6 and anti-human CD3 mAb 289 have already been described (40). The anti-Itk, anti-Tec, anti-p62^{dok} rabbit polyclonal antisera were raised against murine Itk, Tec, and p62^{dok} proteins using synthetic peptides corresponding to amino acid residues 605–625 (DRP-PFSQLLSQLAEIAEAGL), amino acid residues 162–179 (EIKKRRPP-PPIPPEENT), and amino acid residues 424–437 (PQGLILPESGT-TRGS), respectively. Anti-Itk mAb Tuk-N1 has been previously described (41). Anti-FLAG and 4G10 mAbs were purchased from Eastman Kodak Co. and Upstate Biotechnology Inc., respectively. Anti-rat CD2 mAb (OX34) was a kind gift from Dr. D. A. Cantrell (London, UK) and 145 2C11 mAb is a hamster IgG specific for murine CD3 ϵ chain (ATCC). Anti-Lck, anti-Src, and anti-phosphotyrosine antisera were bought from Santa Cruz Biotechnology, and goat anti-mouse IgG (GAM) antiserum was from Jackson ImmunoResearch. CD28-bound beads was obtained by incubation of CD28.2 mAb with avidin-conjugated magnetic beads (Immunotech) bound to biotinylated GAM (Jackson ImmunoResearch) according to the manufacturer's instructions (Immunotech, France).

Cell Stimulation, Immunoprecipitation, and Immunoblot—Ten million cells per ml medium were stimulated at 37 °C for 2 min with 10 μ g of CD28.2 mAb, then cross-linked with 30 μ g of GAM antiserum for 5 min at 37 °C. For nonstimulated controls, 10 \times 10⁶ of cells per ml of medium were left at 37 °C for 2 min and then cross-linked with 30 μ g of GAM antiserum for 5 min at 37 °C. The cells were lysed with lysis buffer (1% Triton X-100, 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate) on ice for 15 min and centrifuged at 4 °C for 15 min at 13,000 rpm. Postnuclear supernatants were precipitated with antibody. The precipitates were washed and subjected to SDS-polyacrylamide gel electrophoresis, then electro-transferred to polyvinylidene difluoride membrane (Millipore), and the membrane was probed with antibody. Alternatively, 10 \times 10⁶ cells per ml of medium were stimulated at 4 °C for 15 min with 20 μ g of CD28 mAb bound to beads and prepared according to the above described protocol. The cells were lysed with lysis buffer, and CD28 molecules were precipitated using a magnet. For nonstimulated control, 10 \times 10⁶ cells per ml of medium were left unstimulated at 4 °C for 15 min, lysed, and centrifuged at 4 °C for 15 min at 13,000 rpm. Postnuclear lysates were precipitated also with CD28 mAb bound to beads. The precipitates were processed as described above.

GST Fusion Protein Production and Pulldown Precipitation—The bacterial hosts *Escherichia coli* DH5 α or BL21, transformed with GST fusion plasmids, were cultured according to the manufacturer's instructions (Pharmacia Biotech Inc., Uppsala, Sweden). GST fusion proteins were purified according to the manufacturer's instructions (Pharmacia Biotech Inc., Uppsala, Sweden). For pulldown precipitation, 5 \times 10⁶ hybridoma cells were stimulated, lysed, and centrifuged at 4 °C for 15 min at 13,000 rpm. Postnuclear lysates were incubated with 10 μ g of GST recombinant protein coupled with Sepharose at 4 °C overnight, and the precipitates were treated as described above.

Transient Transfection—COS-7 (6 \times 10⁶) cells were transiently transfected by the DEAE-dextran method as described (42).

In Vitro Kinase Assay—The immune complex containing Tec was precipitated from T murine hybridoma cells expressing endogenous Tec with anti-Tec polyclonal antiserum and then washed three times with lysis buffer and once with kinase buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 2 mM MnCl₂, 1 mM Na₃VO₄). The immune complex was then incubated in 30 μ l of kinase buffer containing 10 μ Ci of [γ -³²P]ATP at room temperature for 1 h. The immune complex was subjected to gel electrophoresis, and gels were dried and phosphorylated proteins were analyzed by autoradiography.

Luciferase Assay—Jurkat cells (10 \times 10⁶) were electroporated at 960 microfarads and 250 V using a Bio-Rad Gene Pulser with 15 μ g of pIL-2-Luc plasmid, 5 μ g of p β -actin-RLuc, and 15 μ g of the other plasmids expressing Tec and Itk or their mutants. The cells were incubated for 2 h and then left unstimulated or stimulated for 6 h with PMA (Sigma, 50 ng/ml), PMA plus 248 (1/400 dilution), or PMA plus 289

(10 μ g/ml). Following the centrifugation, the cells were washed and lysed. Proteins were quantified by Bradford reagent (Bio-Rad). Ten μ g of cell lysates were subjected to dual luciferase reporter assay (Promega) according to the manufacturer's instructions. The efficiency of transfection was corrected by the activity of firefly luciferase normalized by that of *Renilla* luciferase in the lysates.

RESULTS

Tec Expression in Myeloid and Lymphoid Cells Including T Cells—Four isoforms of Tec protein-tyrosine kinase type I to type IV (527, 608, 602, and 630 aa long) have been described, resulting from alternative splicing (26). They have estimated molecular masses of 62, 70, 68, and 72 kDa, respectively. The largest type IV which has an estimated molecular mass of 72 kDa is also known as TecA. For clarity we will hereafter refer to TecA/Tec type IV as Tec. By using specific anti-Tec antiserum, Tec with an apparent molecular mass of 72 kDa was found to be predominantly expressed in all cell lines examined here with the exception of fibroblast cell line (Fig. 1A). These cells include a monocytic cell line (THP1), B cell lines (Nalm6, BAF3, and U266), a myeloid cell line (KG1a), T cell lines (WT and JH6.2), and resting T cells. Moreover, another Tec isoform with 70 kDa can be found in myeloid cell line KG1a, pro-B cell line BAF3, and T hybridoma cell line WT (Fig. 1A, lanes 2, 5, and 7). The other band at 79 kDa as described previously by Kitanaka *et al.* (25) is probably nonspecific since it does not correspond to any predicted molecular weight of Tec protein, is expressed in the fibroblast cell line, and is not detected in COS-7 transfected with Tec plasmid (data not shown). Fig. 1B illustrates the anti-Tec antiserum specificity since anti-Tec antiserum can only recognize Tec but not Itk or Btk protein expressed in COS-7 cells. Hence, Tec is expressed in lymphoid cell lines, primary resting T cells and myeloid cell line.

Tec Is Activated Following TCR/CD3 or CD28 Ligation—Itk, a member of Tec family kinases, has been shown to be expressed in T cells and be activated following TCR/CD3 or CD28 ligation. Because the defect in *itk*-deficient mice is quite modest and Tec kinase is expressed in T cells, we wanted to know whether another Tec family kinase member, Tec kinase, could be involved in T cell signaling. To evaluate Tec activation, *in vitro* autokinase assay indicating the Tec kinase activity (Fig. 2A, upper panel) and Western blotting to quantify the Tec kinase amount in each lane (Fig. 2A, lower panel) were carried out. Relative activity is shown at the bottom of Fig. 2A. Ligation of CD3 or CD28 on the CD28-transfected hybridoma line (WT) elicited a 2.3- or 2-fold increase in Tec autokinase activity, respectively (Fig. 2A, lanes 2 and 3), in contrast to non-stimulated condition (Fig. 2A, lane 4). As in autokinase assay, tyrosine phosphorylation in different stimulations was also evaluated by immunoblot with anti-phosphotyrosine antibody (Fig. 2B). Relative activity is shown at the bottom of Fig. 2B. Ligation of CD3 or CD28 on the CD28-transfected hybridoma line (WT) also induced a 3- or 3.2-fold increase in tyrosine phosphorylation level. The increase in relative activity of Tec following ligation was relatively modest but reproducible because Tec family kinases have a much lower autokinase activity than Src family kinases, and there is no known substrate available for evaluating their transphosphorylation activity. For example, the relative activity of Itk is about 2–3-fold following CD28 or CD3 ligation (43, 44). Hence, these results suggest that Tec can be involved in TCR/CD3 or CD28 pathway in T cells.

Tec Can Bind to CD28 via SH3 Domain Proline-rich Domain Interaction Following CD28 Ligation—Tec expression in T cells and activation following CD28 ligation incited us to determine how Tec is involved in CD28 signaling. Indeed, another member of the Tec family Itk/Emt/Tsk is expressed in T cells and inducibly binds to CD28 (21). This interaction implicates Itk

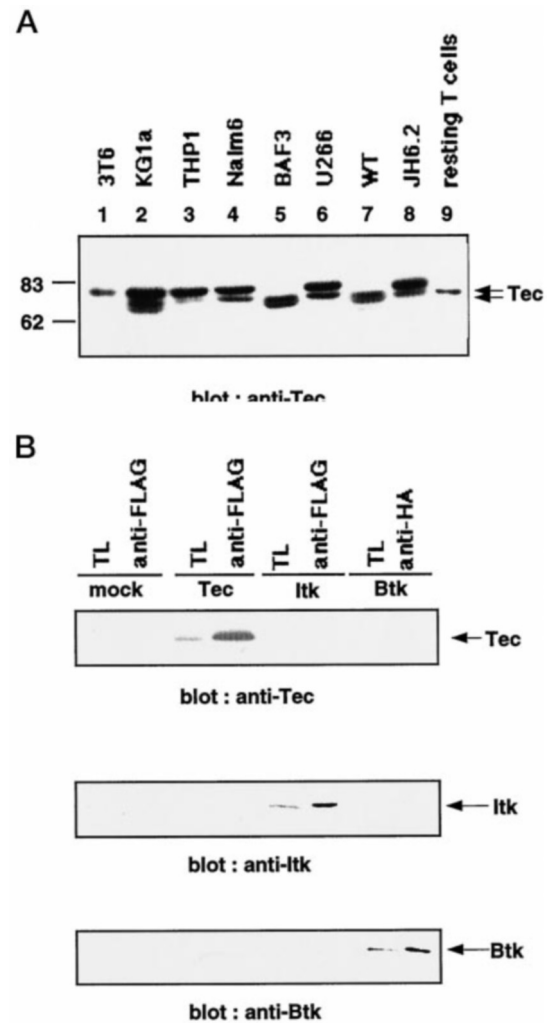


FIG. 1. Expression pattern of Tec protein in hematopoietic cells. A, 50 μ g of total lysates from different cells were analyzed by Western blotting with polyclonal rabbit antiserum anti-Tec. Total lysates were prepared from different cell lines 3T6 (lane 1), KG1a (lane 2), THP1 (lane 3), Nalm6 (lane 4), BAF3 (lane 5), U266 (lane 6), WT (lane 7), Jurkat clone JH6.2 (lane 8), and human primary resting T cells (lane 9). Data are representative of three independent experiments. B, the anti-Tec antiserum was specific for Tec rather than Itk or Btk proteins. COS-7 cells transfected with pCDNA3 vector (lanes 1 and 2), pCDNAflagTec (lanes 3 and 4), pCDNAItkflag (lanes 5 and 6), or pSR α BtkHA (lanes 7 and 8) were lysed. Total lysates (odd lanes), or immunoprecipitates with anti-epitope mAbs (even lanes) were subjected to electrophoresis. Then membrane was blotted with anti-Tec antiserum alone (upper panel) or, following stripping, reblotted with anti-Itk antiserum (middle panel) or anti-Btk antiserum (lower panel).

SH3 domain and the first proline-rich motif of CD28 (45). As depicted in Fig. 3, endogenous Tec inducibly bound to CD28 following cross-linking (Fig. 2, lane 3), although a weak association was found in unstimulated cells (Fig. 2, lane 2). Hence, Tec can bind to CD28 *in vivo* in an activation-dependent manner. In our hybridoma T cells, Itk associated with CD28 receptor following its cross-linking (data not shown), confirming previous works (45). Interestingly, the ratio of Itk and Tec that became recruited to CD28 receptor in comparison to the total kinase amount detected in total cell lysates was similar (data not shown). This may mean that both Tec and Itk play similarly important roles in CD28 pathway.

Tec possesses various domains that are likely candidates for protein-protein and protein-lipid interaction, PH, TH, SH3, and SH2 domains. So far, the only region of Tec involved in its functional interaction with defined proteins is its TH domain

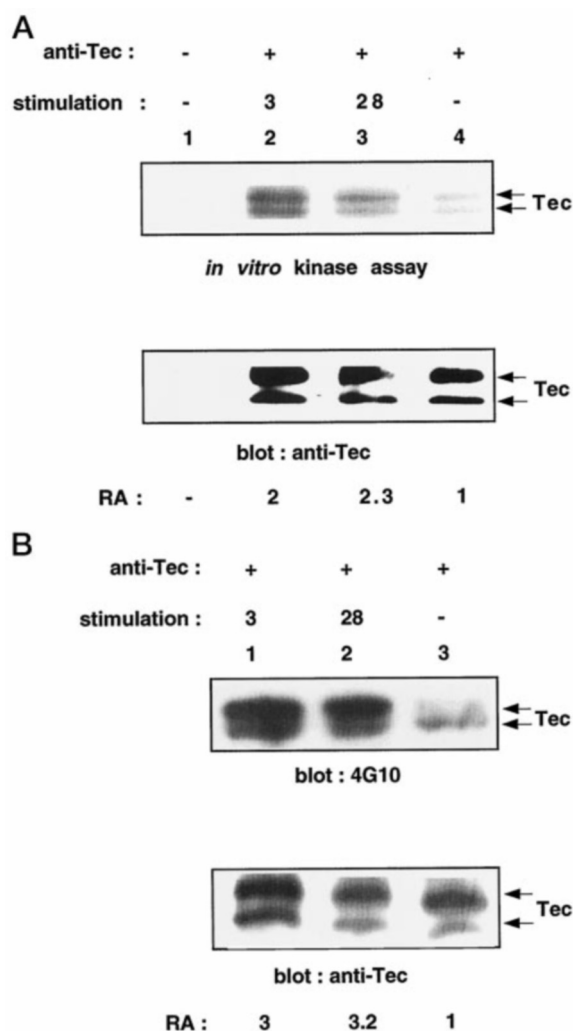


FIG. 2. Tec is activated following CD3 and CD28 cross-linking. A, hybridoma cells WT were left unstimulated (lanes 1 and 4) or stimulated with anti-CD3 mAb cross-linking (lane 2) and with anti-CD28 mAb cross-linking (lane 3) and then lysed. Total cell lysates were precipitated by normal rabbit serum (lane 1) or anti-Tec antibody (lanes 2–4). Immunoprecipitates were divided into two parts, one for *in vitro* kinase assay (upper panel) and the other for Western blotting (lower panel). All values detected from upper and lower panels were determined by densitometric analysis using the BioImage system (Millipore); relative activities (RA) were obtained by normalization of *in vitro* kinase activities of Tec to the protein amounts. B, hybridoma cells WT were left stimulated with anti-CD3 mAb cross-linking (lane 1), with anti-CD28 mAb cross-linking (lane 2), or unstimulated (lane 3). Following lysis, total cell lysates were precipitated by anti-Tec antibody. Immunoprecipitates were subjected to gel electrophoresis and then blotted with 4G10 (upper panel). Following stripping, the membrane was blotted with anti-Tec antiserum (lower panel). All values detected from upper and lower panels were determined by densitometric analysis using the BioImage system (Millipore); relative activities were obtained by normalization of Tec tyrosine phosphorylation to the protein amounts. Data are representative of two independent experiments.

that binds to protein kinase, adaptor molecules, and to the receptor tyrosine kinase c-Kit (9–12). To elucidate which region of Tec kinase is involved in CD28 binding, recombinant proteins corresponding to these different regions were constructed as GST fusion proteins, expressed and used to precipitate CD28 from the murine T cell hybridoma transfected with huCD28 cDNA. As depicted in Fig. 4, GSTTECSH3 can precipitate CD28 from nonstimulated (Fig. 4, lane 5) or stimulated cells by anti-CD28 antibody (Fig. 4, lane 6) to a similar degree. In contrast, the GSTTECSH2 domain (Fig. 4, lanes 7 and 8) did not bind CD28 even after CD28 cross-linking which was shown

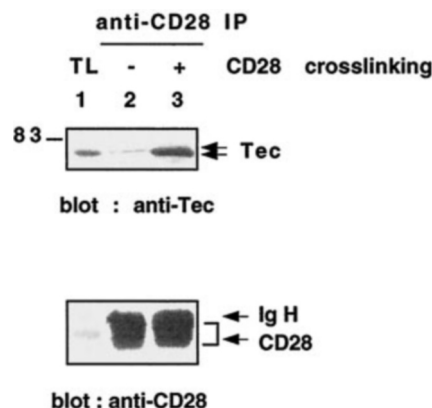
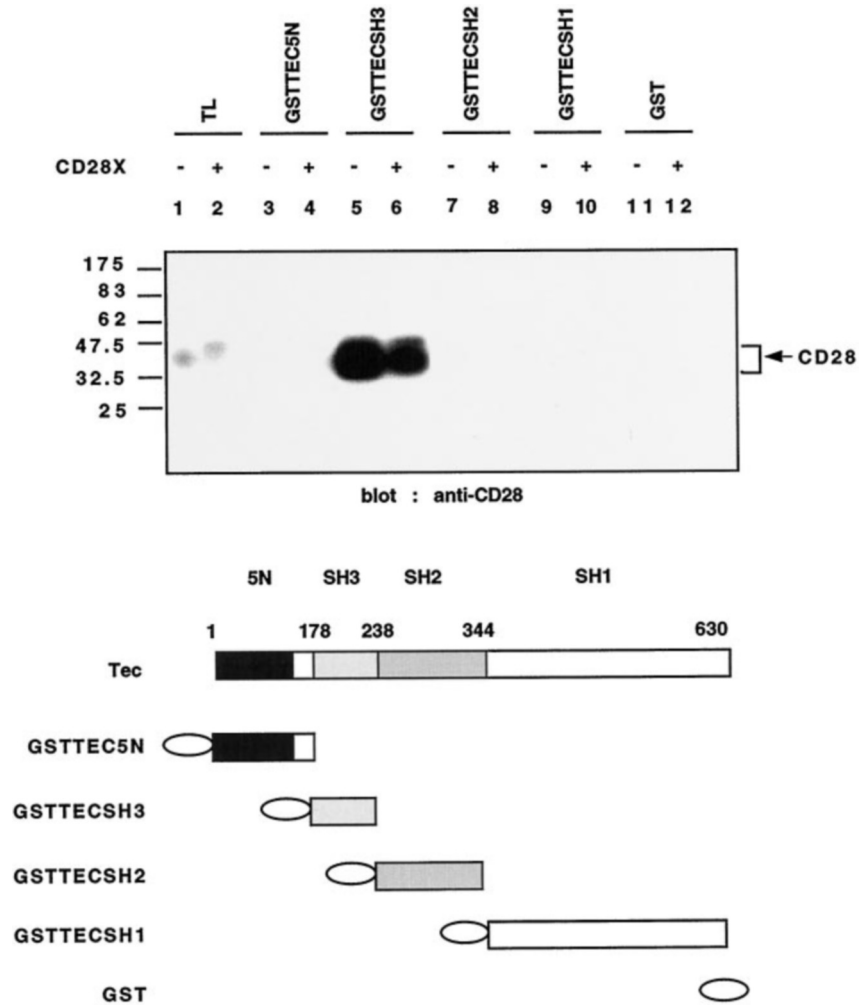


FIG. 3. CD28 receptor can bind Tec kinase in T cells *in vivo*. Hybridoma cells WT were left unstimulated (–) or stimulated with anti-CD28 mAb and goat anti-mouse antiserum (+), before lysis in lysis buffer and precipitation. IP, immunoprecipitation. Total cell lysate (TL, lane 1) and precipitates from unstimulated cells (lane 2) or from stimulated cells (lane 3) were subjected to gel electrophoresis and immunoblotted with anti-Tec antiserum (upper panel), or following stripping membrane was reblotted with anti-CD28 antibody (lower panel). Immunoglobulin heavy chain (Ig H) was indicated. Data are representative of two independent experiments.

to induce the phosphorylation of its intra-cytoplasmic domain and permit its association with p85 and Grb-2 SH2 domains. The GSTTECSH3 containing the PH and TH domains was poorly effective in precipitating CD28 (Fig. 4, lanes 3 and 4). Thus, Tec SH3 domain seems to be the only domain responsible for CD28 interaction.

So far, some regions have been identified within CD28 intra-cytoplasmic domain involved in the binding of adaptor molecules and tyrosine kinases. The ¹⁷³Tyr-Met-Asn-Met motif is involved in both the binding of p85 and Grb-2 SH2 domains (46, 47). Two putative proline-rich motifs, N-terminal ¹⁷⁸Pro-Arg-Arg-Pro-Gly-Pro and C-terminal ¹⁹⁰Pro-Tyr-Ala-Pro-Pro-Arg, called PR1 and PR2, respectively (Fig. 5A), are also likely candidates for SH3 domain binding. PR1 was recently identified as the major binding site for Itk SH3 domain, whereas the binding partner of the latter is unknown. We next investigated whether the proline-rich motifs of CD28 are involved in Tec SH3 domain interaction. We mutated prolines 181 and 183 in PR1 and prolines 193 and 194 in PR2 within CD28 (Fig. 5A). By using transfection of mutant plasmids in murine T cell hybridoma, stable transfectants with similar surface expression of CD28 molecules were selected and analyzed by flow cytometry (Fig. 5B). We used as a control the recombinant GST SH3 domain of Itk to precipitate different CD28 molecules. As already reported, Itk SH3 domain binds to wild-type CD28 in an activation-dependent manner (45). These two PR motifs are functionally essential for Itk SH3 binding since CD28 mutated or deleted within PR1 and PR2 abolished almost or completely this binding, respectively. CD28 mutant Y173F+Y200F was still precipitated by Itk SH3 with enhanced binding following CD28 ligation (Fig. 5C). This also confirms correct expression of CD28 molecules in these stable clones. As indicated in Fig. 5D, Tec SH3 domain binds to wild-type CD28 molecules, but in contrast to Itk no enhanced binding was detected upon receptor ligation. The two PR motifs are functionally essential for Tec SH3 binding since deletion mutant without PR motifs abrogated completely this binding. Similarly to Itk SH3, mutations on tyrosines 173 and 200 did not impede Tec SH3 precipitation. This further demonstrates that its binding is independent of p85 and Grb-2 since these two adaptor molecules are recruited upon CD28 ligation (46, 47). In agreement with results of *in vitro* binding, the *in vivo* binding between endogenous Tec and PR motif-mutated CD28 molecules was impaired following

FIG. 4. SH3 domain of Tec is the only domain responsible for the interaction of Tec with CD28 receptor *in vitro*. GST fusion proteins containing different domains of Tec were used to precipitate wild-type human CD28 receptor from unstimulated cells (–, *odd lanes*) or cells stimulated by CD28 antibody cross-linking (+, *even lanes*). Following gel electrophoresis, proteins were transferred to membranes and blotted with anti-CD28 antibody shown in *upper panel*. CD28X represented CD28 cross-linking. GST fusion proteins containing different domains of Tec are indicated at *bottom* of this figure, the *numbers* correspond to the amino acid residues as follows: GST 5N aa 1–178, SH3 aa 179–238, SH2 aa 239–344, and SH1 aa 345–630. Data are representative of two independent experiments. *TL*, total cell lysate.



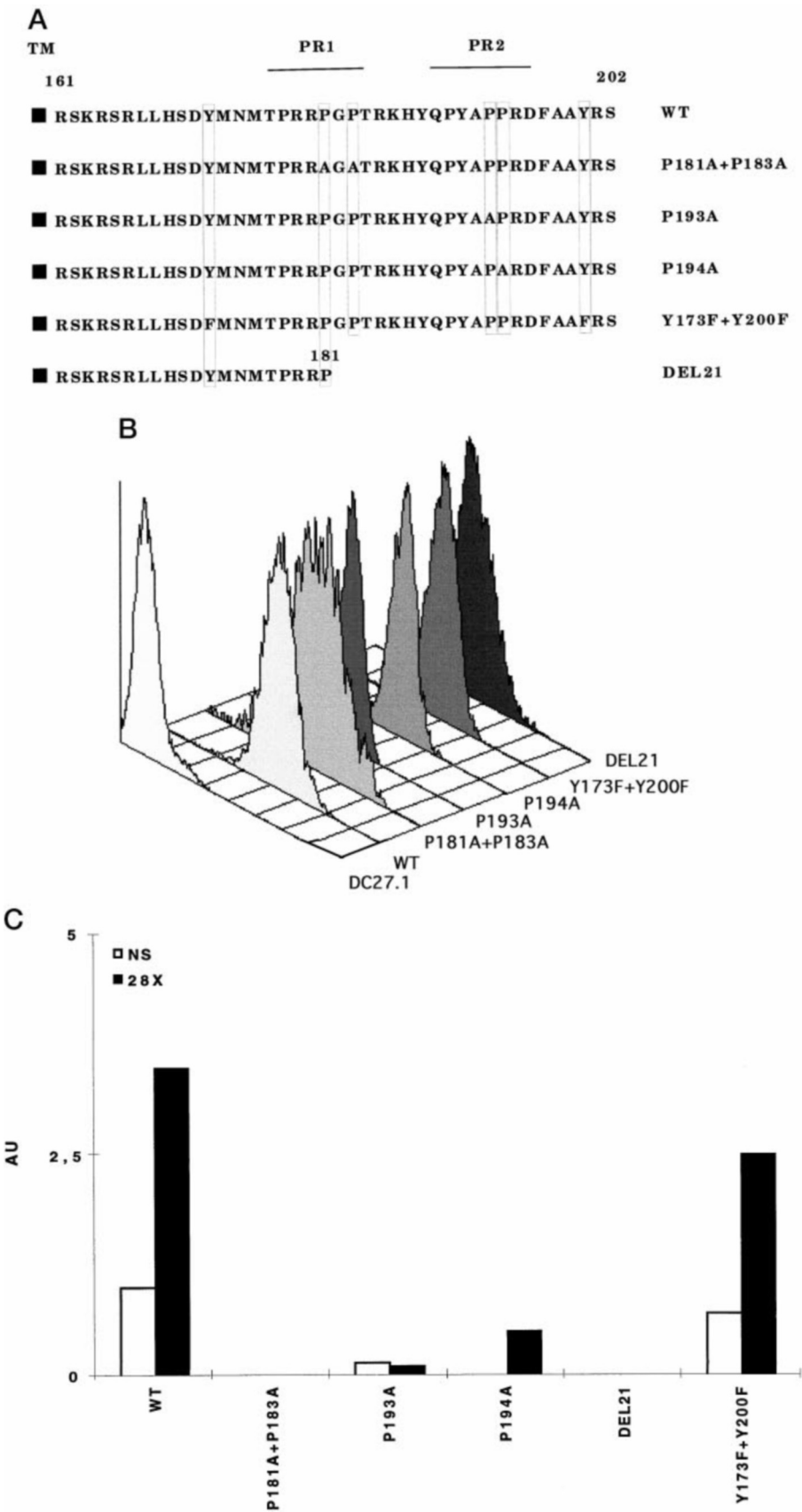
CD28 activation in comparison to that between endogenous Tec and wild-type CD28 molecule (Fig. 5E). Hence, we demonstrate that PR1 and PR2 within CD28 are functionally important for Itk and Tec SH3 domains although they are not typical proline-rich motifs (48, 49). Altogether, our data indicate that Tec uses its SH3 domain to bind to PR motifs of CD28.

p62^{dok} but Not CD28 Is an *In Vivo* Substrate for Tec—TCR activation results in tyrosine phosphorylation of substrates such as Cbl (p120), SLP76 (p76), and p36/38 (LAT) (50–52). CD28 activation also leads to tyrosine phosphorylation of substrates such as p95^{av}, an adaptor molecule p62, Cbl, the catalytic subunit of PI 3-kinase (p110), and CD28 itself (32–35, 46). To understand the role of Tec kinase in TCR and CD28 pathways, we tried to find its substrate(s). Molecule(s) of 62 kDa associated to Ras GAP was demonstrated to be phosphorylated upon CD28 but not CD3 stimulation (35). One molecule associated to Ras GAP has been recently cloned and called p62^{dok} (53, 54). To understand further whether or not p62^{dok} is actually a substrate for CD28 but not CD3 signaling, CD28-transfected murine T cell hybridoma WT was transfected with p62^{dok} cDNA tagged with the hemagglutinin (HA) epitope, and then stable transfected clones (WTHA62^{dok}) with high expression of p62^{dok} were selected by Western blotting. As shown in Fig. 6A, *upper panel*, lane 3, CD28 ligation induced the tyrosine phosphorylation of p62^{dok}. In contrast, CD3 stimulation did not induce detectable tyrosine phosphorylation of p62^{dok} (Fig. 6A, *lower panel*, lane 2). Fig. 6B showed the phosphorylation pattern of clones WTHA62^{dok} or WT stimulated with GAM, CD28 plus GAM, or CD3 plus GAM. The proteins p120, p95, p76, and

p36/38 were phosphorylated on CD3 cross-linking, whereas p95 and p62 were phosphorylated on CD28 cross-linking. These experiments confirm that p62^{dok} is a specific substrate of CD28 but not CD3 stimulation. We next investigated whether Tec can phosphorylate p62^{dok}. This protein p62^{dok} is a substrate for various kinases of the Src family (Src and Lck) but also Abl (55–57). HAp62^{dok} plasmid was cotransfected with Src, Fyn, Lck, Tec, or Itk expression vector in heterologous COS-7 cells. The relative phosphorylation level of p62^{dok} by PTKs was indicated in arbitrary units (AU). As shown in Fig. 7A, Src family kinases Src, Fyn, and Lck led to the tyrosine phosphorylation of p62^{dok} (lanes 3–5 in *upper panels*). Interestingly, Tec induced robust tyrosine phosphorylation of p62^{dok} (Fig. 7A, *upper panel*, lane 6). Under the same experimental conditions, Itk was unable to elicit a significant p62^{dok} tyrosine phosphorylation (Fig. 7A, *upper panel*, lane 7) although similar quantities of Tec and Itk proteins were detected (Fig. 7A, lanes 6 and 7 in *lower panels*). Fig. 7B shows phosphorylation level of Itk and Tec in heterologous COS-7 cells. Tec could be more activated than Itk in these cells. Hence, one of the CD28 tyrosine-phosphorylated substrates, namely p62^{dok}, can be phosphorylated by Tec.

So far, CD28 has been shown to be phosphorylated on tyrosine residues by Src family kinases such as Lck and Fyn and by Itk but not ZAP-70 (58, 59). Tec recruitment to CD28 receptor may be important for CD28 tyrosine phosphorylation. COS-7 cells were cotransfected with CD28 together with Lck or Tec. For the latter, we used Tec and its CD2 membrane-targeted versions either wild-type (rCD2flagTec) or kinase-dead mutant (rCD2flagTeckd) where the lysine of the ATP-binding site was

FIG. 5. Two proline-rich domains of CD28 receptor are important for Tec binding. *A*, protein sequences of CD28 molecules mutated in intra-cytoplasmic domain. These mutations are underlined. Stable clones with similar surface expression of CD28 molecules were selected and analyzed for interaction. *B*, flow cytometric analysis of T hybridoma cells. Parental murine T hybridoma cells DC27.1 without human CD28 transfection or different human CD28 stable clones were stained by anti-human CD28 mAb CD28.2. *C*, interaction between GSTITKSH3 and various CD28 molecules expressed in T hybridoma cells. Following stimulation with CD28 cross-linking (28×) or not (NS), lysates obtained from stable transfected murine T cell hybridoma clones expressing wild-type CD28 and its mutants were incubated with GSTITKSH3 protein coupled to Sepharose beads, washed three times with lysis buffer, and processed as described under "Experimental Procedures." The membrane was probed with anti-CD28 antibody. Following stripping, the same membrane was reprobed with anti-Sam 68 antibody. AU displayed on the y axis correspond to the ratio of the signal obtained by immunoblotting with anti-CD28 antibody to that obtained with anti-Sam 68 antibody. Data are representative of three independent experiments. *D*, interaction between GSTTECSH3 and various CD28 molecules expressed in T hybridoma cells. Similarly to *C*, GSTTECSH3 protein was used instead of GSTITKSH3 protein. *E*, interaction between endogenous Tec and various CD28 molecules expressed in T hybridoma clones. Different hybridoma clones were treated as in Fig. 3. CD28 immunoprecipitates from total lysates obtained from CD28-stimulated (+) or unstimulated (–) samples were subjected to gel electrophoresis and blotted with anti-Tec antiserum (*upper panel*). Following stripping, the membrane was reblotted with anti-CD28 mAb (*lower panel*).



replaced by glutamic acid. The tyrosine phosphorylation of CD28 was analyzed by immunoblot using anti-phosphotyrosine mAb. CD28 was phosphorylated when coexpressed together with Lck tyrosine kinase (Fig. 8, *upper panel*, lane 3). This

confirms previous observations (58). Conversely, we were unable to demonstrate a significant phosphorylation of CD28 using either coexpression of Tec or its membrane targeted forms (Fig. 8, *upper panel*, lanes 4–6). These experiments sug-

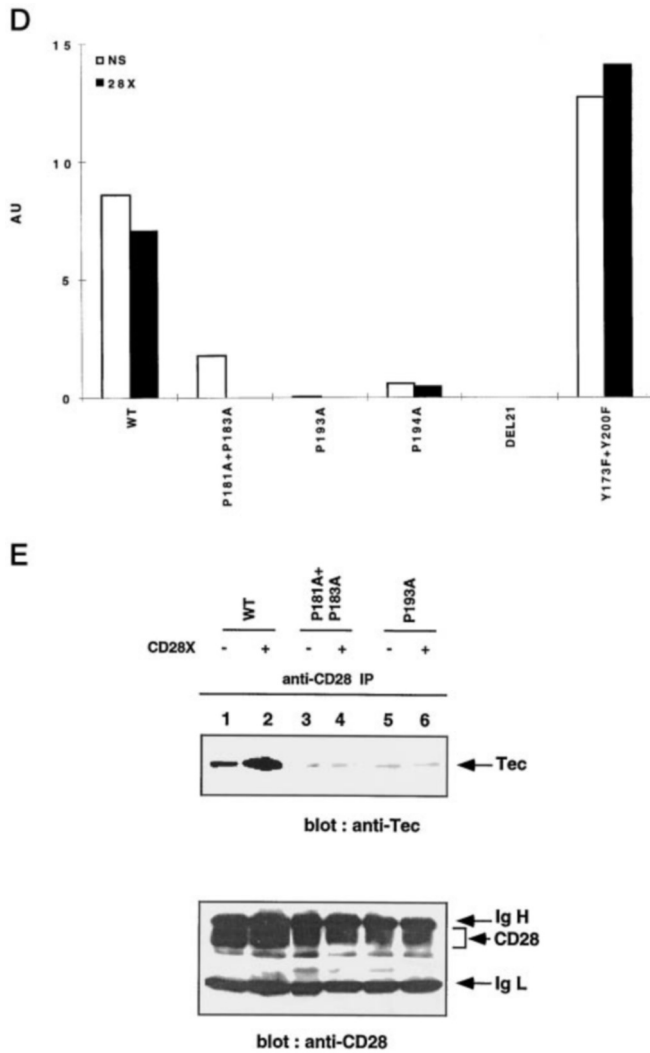


FIG. 5—continued

gest that CD28 is unlikely to be a major substrate for Tec in this experimental system. To sum up, these data show that p62^{dok} but not CD28 is a substrate for Tec kinase.

Tec, but Not Itk, Is Involved in the IL-2 Promoter Activity in T Cells—To address the question of the role of Tec in T cell activation, we analyzed its role in IL-2 transcription. We compared the ability of wild-type Itk and Tec to induce the IL-2 promoter activity upon transient transfection of Jurkat cells with plasmid vectors expressing the kinases and pIL-2 coupled to the luciferase gene (pIL-2-Luc). We used, as control kinase, kinase-dead mutants of Itk and Tec. These mutants have both been generated by substitution of glutamic acid for a critical lysine in the ATP-binding site at amino acid positions 396 and 397 for Itk and Tec, respectively. As already reported, activation of IL-2 promoter could be induced by pharmacological drugs such as PMA which mimics protein kinase C activation, PMA plus CD3 stimulation, and PMA plus CD28 costimulation in Jurkat cells. As shown in Fig. 9A, stimulation by PMA plus anti-CD3 antibody or PMA plus anti-CD28 antibody elicited IL-2 promoter-driven luciferase expression. The latter is stronger than the former for IL-2 promoter (mock). Overexpression of wild-type Tec (Tecwt), but not kinase-dead mutants of Tec and Itk or wild-type Itk (Tekkd, Itkkd, or Itkwt), can further increase significantly IL-2 promoter activity upon PMA, PMA plus anti-CD3 mAb, and PMA plus anti-CD28 mAb stimulations. Moreover, overexpression of kinase-dead mutant of Tec (Tekkd) can inhibit IL-2 promoter activity in CD3 or CD28 plus

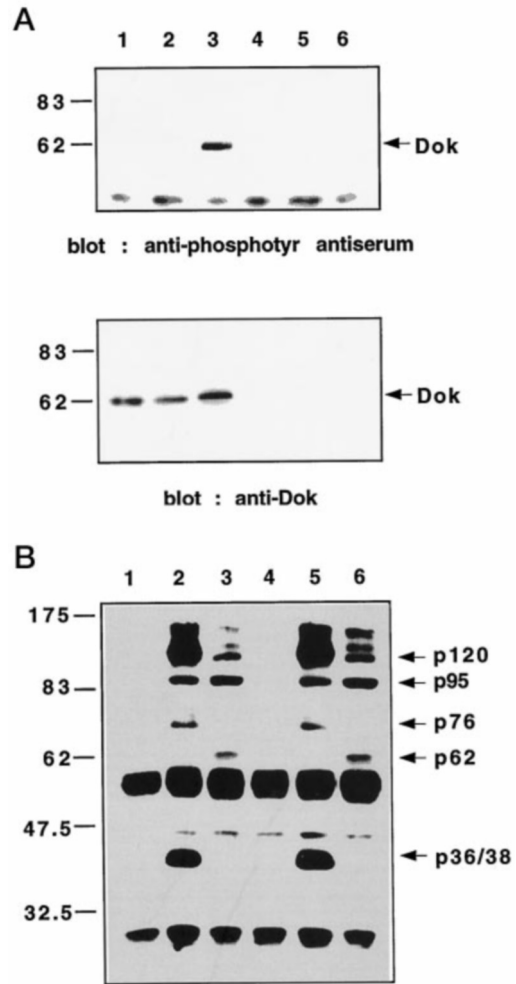


FIG. 6. p62^{dok} is phosphorylated following CD28 cross-linking but not CD3 cross-linking. A, murine T cell hybridoma transfected with expression vector containing HAP62^{dok} cDNA and puromycin resistance gene and selected for puromycin resistance, WTHADok (lanes 1–3) or the parental T cell hybridoma WT (lanes 4–6) were stimulated with GAM (lanes 1 and 4), anti-CD3 mAb plus GAM (lanes 2 and 5), or anti-CD28 mAb plus GAM (lanes 3 and 6). Lysates were incubated with anti-HA mAbs, and immunoprecipitates were divided in two halves, one processed and blotted with rabbit anti-phosphotyrosine antiserum (upper panel) and the other blotted with rabbit anti-p62^{dok} antiserum (lower panel). B, 50 µg of total lysates from these cells were subjected to gel electrophoresis as in A, probed with 4G10 mAb. p120, p95, p76, p62, and p36/38 were indicated. Data are representative of three independent experiments.

PMA stimulation, whereas kinase-dead mutant of Itk (Itkkd) was devoid of any significant effect. This inhibition by overexpressed kinase-inactive Tec suggests that Tec is located downstream of TCR/CD3 and CD28 receptors. More intriguingly, we measured endogenous IL-2 production in media collected in the above experiments by using enzyme-linked immunosorbent assay. In accordance, we got similar profiles (data not shown). That is to say, our isolated IL-2 promoter can completely reflect endogenous IL-2 promoter, and Tec has the similar effect on minimal and endogenous full-length IL-2 promoters. Expression levels of Itk, Tec, or their mutants are indicated in Fig. 9B. Cytomegalovirus promoter-driven expression of these expression vectors containing cDNA of Itk, Tec, or their mutants was poor in Jurkat cells, whereas PMA can strongly induce their expression. Apparently, PMA stimulation had no significant effect on Tec kinase activity in COS cells (data not shown). Although a similar quantity of wild-type Tec was overexpressed, CD3 or CD28 stimulation in synergy with PMA in-

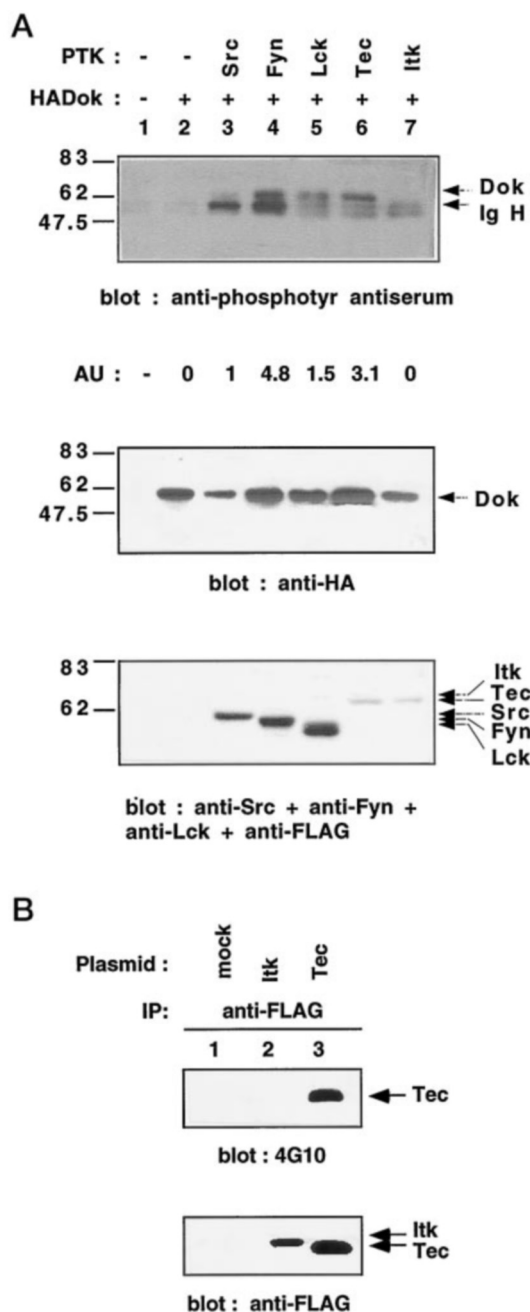


FIG. 7. p62^{dok} is a common substrate for Src, Fyn, Lck, and Tec rather than Itk in COS-7 cells. A, COS-7 cells transiently transfected with pCDNA3 empty vector (lane 1), pCDNAHA62^{dok} (lane 2), pCDNAHA62^{dok} plus p48Src (lane 3), pCDNAHA62^{dok} plus pF10 (lane 4), pCDNAHA62^{dok} plus pCDNALck (lane 5), pCDNAHA62^{dok} plus pCDNAflagTec (lane 6), and pCDNAHA62^{dok} plus pCDNAItkflag (lane 7) were harvested 2 days after transfection by trypsin treatment. Trypsinized cells were lysed, and anti-HA antibody immunoprecipitates were subjected to gel electrophoresis and then transferred to membranes. The membrane was probed with rabbit anti-phosphotyrosine (anti-phosphotyrosine) antiserum (upper panel), and following stripping, it was reprobed with rabbit anti-p62^{dok} antiserum (middle panel). Data obtained from upper and bottom panels by scanning using the BioImage system were converted into relative phosphorylation levels in AU by normalization of p62^{dok} tyrosine phosphorylation to the p62^{dok} protein amounts and are indicated at the bottom of the upper panel. Total lysates from the same cells subjected to gel electrophoresis were then blotted with anti-Src, anti-Fyn, anti-Lck, and anti-FLAG antisera. B, tyrosine phosphorylation of Tec and Itk in Cos-7 cells. PCDNA3 (mock, lane 1), pCDNAItkflag (Itk, lane 2), or pCDNAflagTec (lane 3) was transfected into COS-7 cells and treated as in A. Anti-FLAG immunoprecipitates (IP) were transferred to membrane, followed by immunoblotting with 4G10 (upper panel) or anti-FLAG (upper panel). Data are representative of three independent experiments.

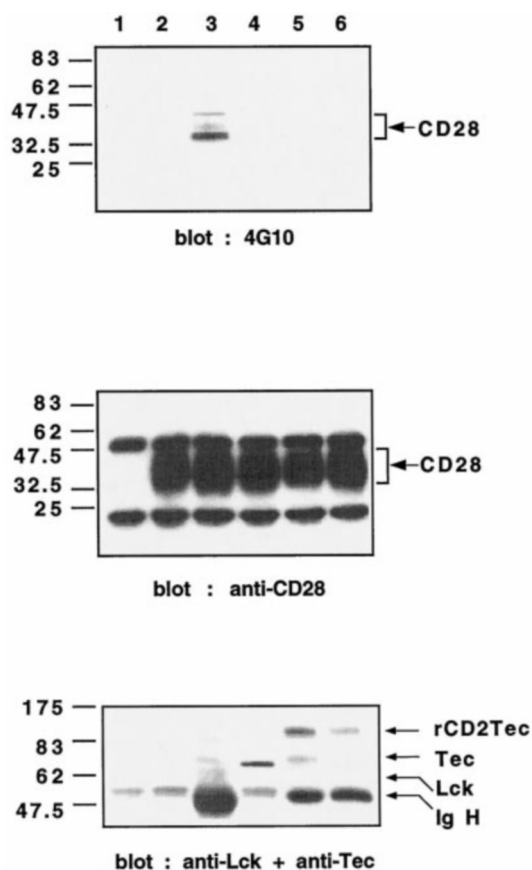


FIG. 8. CD28 can be phosphorylated by Lck but not Tec *in vivo*. COS-7 cells transiently transfected with different expression vectors, pCDNA3 empty vector (lane 1), pSRαCD28 (lane 2), pSRαCD28 plus pCDNALck (lane 3), pSRαCD28 plus pCDNAflagTec (lane 4), pSRαCD28 plus prCD2flagTec (lane 5), and pSRαCD28 plus prCD2flagTeckd (lane 6), were harvested with trypsin treatment 2 days after transfection. Trypsinized cells were lysed, and total cell lysates were divided in two parts. One was precipitated with anti-CD28.2 mAb. Immunoprecipitates were subjected to gel electrophoresis before transfer to membranes and immunoblotted with anti-phosphotyrosine antibody 4G10 (upper panel). Following stripping, the membrane was reprobed with anti-CD28 antibody CD28.6 (middle panel). The rest respectively precipitated with anti-FLAG mAb (lower panel, lanes 1, 2, and 4), anti-Lck (lower panel, lane 3), or anti-rat CD2 mAb OX34 (lower panel, lanes 5–6) was processed as described under "Experimental Procedures." The membrane was probed with anti-Lck and anti-Tec antibodies. Expression of Tec or Lck was depicted as indicated in the lower panel. Data are representative of two independent experiments.

creased IL-2 promoter activity to a greater extent than PMA alone (Tecwt). These results suggest that CD3- or CD28-mediated activation of Tec results in increase of IL-2 promoter in these transient transfections.

Altogether, these experiments demonstrate that Tec can specifically link signals from receptors TCR/CD3 and CD28 to IL-2 promoter (Fig. 10) and that the two Tec family members Itk and Tec differ dramatically in their ability to modulate IL-2 promoter following TCR/CD3 or CD28 stimulation.

DISCUSSION

We have demonstrated that Tec protein-tyrosine kinase participates in TCR/CD3 and CD28 pathways. CD3 or CD28 ligation induces Tec activation. Moreover, CD28 engagement can lead to the recruitment of Tec via its binding to proline-rich motifs (PR1 and PR2) found in the CD28 intra-cytoplasmic domain and phosphorylation of downstream substrates such as p62^{dok}, a substrate for Tec. Finally, Tec activation results in the activation of the IL-2 promoter regulated by TCR/CD3 and CD28 receptors.

FIG. 9. Tec but not Itk is implicated in IL-2 promoter activity following CD3 or CD28 ligation besides PMA stimulation in Jurkat cells. A, 10×10^6 Jurkat cells were electroporated with pCDNA3 (mock), pCDNAItkflag (Itkwt), pCDNAItkddflag (Itkdd), pCDNAflagTec (Tecwt), or pCDNAflagTeckd (Teckd) with pIL-2-Luc and p β -actin-RLuc. Following electroporation, cells were divided into four parts, one left unstimulated (NS), the other three stimulated with PMA, PMA plus 289, an anti-CD3 mAb (PMA+CD3), and PMA plus 248, an anti-CD28 mAb (PMA+CD28), respectively, for 6 h. After centrifugation, cell pellets were lysed. Postnuclear supernatants were analyzed according to the protocol described under "Experimental Procedures." IL-2 promoter activity was indicated in AU representing relative luminescence units of firefly luciferase normalized by that of *Renilla* luciferase. B, Western blot with anti-FLAG mAb indicates the expression level of transfected Itk, Tec, and their mutants. Data are representative of two independent experiments.

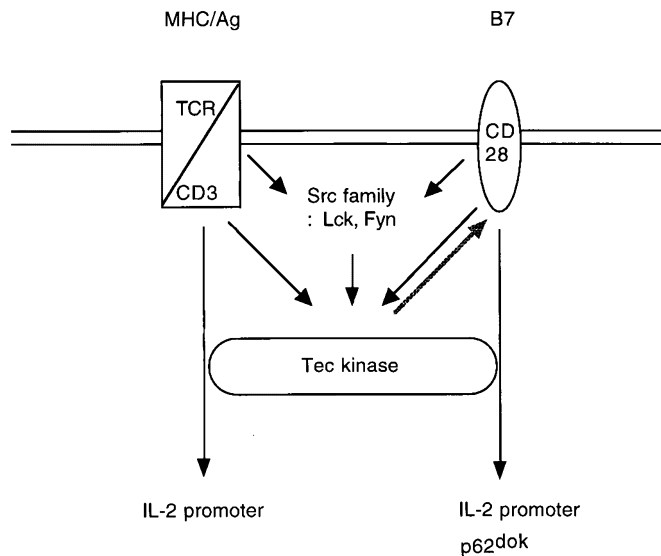
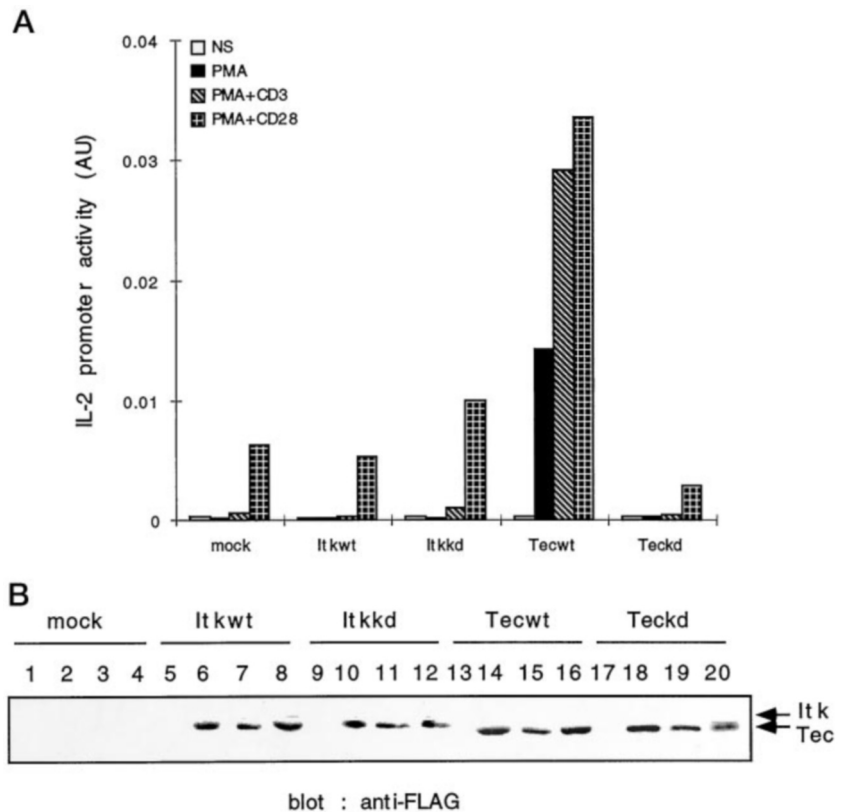


FIG. 10. Proposed model for Tec function in TCR/CD3 and CD28 pathway. Following TCR/CD3 or CD28 ligation, Tec kinase can be activated. Src protein kinases such as Lck and/or Fyn activated by these receptors could modulate Tec activation. Then Tec kinase can phosphorylate its substrates such as CD28 pathway-specific p62^{dok} protein and lead to IL-2 expression. In addition, Tec can associate with CD28 receptor following CD28 receptor ligation through SH3 and proline-rich motif interaction. Arrowhead in black shows activation or phosphorylation and arrowhead in gray shows association.

PTKs are important for T cell functions (32, 34). PTK inhibitors abolish TCR/CD3- and CD28-mediated protein phosphorylation and IL-2 production, a hallmark of activated T cells. The Src family kinases Lck and Fyn are recruited and/or activated in TCR/CD3 and CD28 pathways (60, 61). Besides, they have been shown to phosphorylate components such as CD3 or CD28 in these two pathways (58). Tec family member Itk/Etk/Tsk has been shown to be implicated in TCR/CD3 or CD28

pathway and can phosphorylate CD28 *in vitro*. Our data show that Tec kinase is expressed in T cells (Fig. 1) and activated in TCR as well as CD28 pathways (Fig. 2).

The activation of Tec is still elusive. TCR/CD3 or CD28 ligation induces Tec activation in T cells in addition to the activation of Src family kinases including Lck and/or Fyn (60, 62–64). One mechanism by which TCR/CD3 or CD28 can modulate Tec activity could be the activation of Lck and Fyn. Studies performed with Btk, Itk, and Tec have demonstrated that they are substrates of Src-related kinases that can act as positive regulators (10, 13, 43, 65–67). For instance, coexpression of Src family kinases induced the increased phosphorylation and activity of Btk at two sites Tyr⁵⁵¹ and Tyr²²³. This latter located in the SH3 domain is conserved among the different members of the Tec family and could be involved in enzyme regulation (68). An alternative possibility is the modulation of Tec by PI 3-kinase via targeting of its PH domain to membrane. PI 3-kinase has been shown to be activated following TCR/CD3 or CD28 ligation (69). This membrane targeting could render Tec accessible to its partners such as regulators or substrates (4, 6, 70).

Tec kinase can interact with CD28 in an activation-dependent manner *in vivo*. Tec SH3 domain seems to be responsible for direct binding to CD28, although we cannot exclude the indirect binding between the other regions than SH3 domain of Tec and CD28 (Fig. 4). The CD28 regions involved in Tec SH3 binding are the two proline-rich motifs (PR1 and PR2) (Fig. 5). This binding does not rely on inducible recruitment of p85 or Grb-2 to CD28 since mutants devoid of binding to p85 and Grb-2 retain their interaction with Tec. Endogenous Tec increases its binding to CD28 following CD28 ligation although there is basal binding of Tec to CD28 in unstimulated condition (Fig. 3). However, Tec SH3 domain has similar affinity for unstimulated or stimulated CD28 (Fig. 5B). Explanation may be that endogenous Tec may be more accessible for CD28 following ligation since activated Tec becomes "opened" via con-

formational change or induced indirect binding between other adaptor molecules, CD28 and Tec. Another interesting question is why two PR motifs of CD28 are essential for the binding of Tec containing one SH3 domain. This can be explained by cooperative binding between two Tec kinases to CD28, the dimerization of Tec, or only an artifact since GST proteins form a dimer. The protein sequence homology between Itk and Tec is about 50%. Three sites (Trp²⁰⁸, Ser²²³, and Ser²²⁴) located in murine Itk SH3-binding pocket have been suggested to be involved in binding to its own PR motif in TH domain (7). With the exception of Ser²²⁴, the first two sites are conserved between Itk and Tec. This may explain why Itk SH3 domain is partially different from Tec SH3 domain.

Substrates for Tec kinases are almost unknown. Tec has been shown to phosphorylate Jak kinase in hematopoietic cells and vice versa (31). Following TCR and CD28 ligations, PTKs are responsible for the phosphorylation of their specific substrates since TCR/CD3 and CD28 have no intrinsic kinase activity. p62^{dok} containing some functional domains (PH, PTB, and tyrosine residues) is a downstream substrate of kinases such as Lck, Src, or Abl. Stimulation of CD2 and CD28 but not CD3 can induce tyrosine phosphorylation of the p62^{ras} GAP-associated protein (35, 71). We first indicated that p62^{dok} is an *in vivo* substrate for Tec kinase but not Itk in COS-7 cells. This is probably because different Tec family members have different substrates or because distinct regulatory steps are involved in the regulation of Tec and Itk. For instance, Itk is less activated than Tec in COS-7 cells (Fig. 7B). One interesting question is why p62^{dok} is phosphorylated only in CD28 pathway but not in TCR/CD3 pathway, although TCR/CD3 and CD28 pathways appear capable of activating similar kinases (Lck, Fyn, Itk, and Tec). A possible explanation is that p62^{dok} is merely accessible to kinases including Tec upon CD28 activation. Which one(s) is responsible for p62^{dok} phosphorylation remains to be studied since kinases including Tec, Itk, Lck, and Fyn are activated and/or recruited in CD28 pathway. Studies on Tec-mediated phosphorylation of p62^{dok} could be important for determining the role of Tec in CD28 pathway. CD28 has been shown to be an *in vitro* substrate for Itk. However, neither Tec nor its dominant positive membrane-targeted forms of Tec kinase can phosphorylate CD28, whereas Lck can. These results suggest that CD28 is a regulator but not a potential substrate for Tec. On the contrary, CD28 could be a regulator and substrate of Itk (59).

IL-2 production in T cells is induced following activation of the TCR/CD3 together with costimulatory signals that are required for full activation. IL-2 production can be mimicked by anti-TCR (or anti-CD3) mAb and anti-CD28 mAb and be potentiated by pharmacological agents such as PMA in Jurkat cells. As shown in Fig. 9A, Tec overexpression but not Itk leads to the induction of IL-2 promoter activity in combination with PMA alone as well as PMA plus CD3 or CD28 triggering. This effect is dependent on Tec kinase activity since mutations that prevent ATP binding abrogated all Tec functions. Expectedly, overexpression of kinase-inactive Tec with CD3 or CD28 stimulation plus PMA leads to inhibition of IL-2 promoter, suggesting that Tec is a linker between receptors TCR/CD3 and CD28 and IL-2 promoter in a kinase-dependent manner. The mechanisms by which Tec but not Itk can regulate IL-2 promoter in T cell signaling need to be investigated, and this study is under way. IL-2 promoter can be induced via various transcriptional factors such as AP-1, NFAT, and NFkB activated by TCR/CD3 or CD28 signals. One candidate regulated by Tec may be the AP-1 site in IL-2 promoter since Ohya *et al.* (30) recently suggested a role for Tec in the regulation of *c-fos* transcription via ternary complex factors which is also involved in the acti-

vation of Jun-B promoter in hematopoietic cells. Another candidate may also be the NFAT site in IL-2 promoter since Btk regulates Ca²⁺ entry which could induce NFATp dephosphorylation and nuclear translocation via calcineurin activation (72).

Taken together, we first indicated that Tec kinase is involved in T cell signaling and that Tec can interact with CD28 (Fig. 10). More interestingly, Tec can phosphorylate its substrate p62^{dok} specifically implicated in CD28 pathway and regulate IL-2 promoter whereas Itk cannot. These results suggest that although two Tec family kinases Tec and Itk are involved in TCR and CD28 pathways, they have different functions.

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