Protein-tyrosine Kinase Pyk2 Is Involved in Interleukin-2 Production by Jurkat T Cells via Its Tyrosine 402*

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We established Jurkat transfectants that overexpress Pyk2 or its mutants, K457A (lysine 457 was mutated to alanine), Pyk2-Y402F (tyrosine 402 to phenylalanine), and Pyk2-Y881F to investigate the role of Pyk2 in T cell activation. Pyk2 as well as kinase-inactive Pyk2-K457A, was phosphorylated at tyrosine residues 402, 580, and 881 upon T cell antigen receptor cross-linking, indicating that these residues are phosphorylated by other tyrosine kinases. However, no tyrosine phosphorylation of Pyk2-Y402F was detected while more than 60% of the tyrosine phosphorylation was observed in Pyk2-Y881F. Pyk2-Y402F inhibited the activation of endogenous Pyk2. The degree of activation of both c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase but not extracellular signal-regulated protein kinase after concurrent ligation of T cell antigen receptor and CD28 was reduced by more than 50% in the clones expressing Pyk2-Y402F. Consistent with this inhibition, IL-2 production was significantly diminished in the Pyk2-Y402F-expressing clones. Furthermore, we found that Pyk2, when overexpressed, associates with Zap70 and Vav. Taken together, these findings suggest that Pyk2 is involved in the activation of T cells through its tyrosine 402.

Engagement of T cell antigen receptor (TCR) by antigen or ligation of TCR by anti-CD3 Ab rapidly activates the Src family protein-tyrosine kinases (PTKs), Fyn and Lck, leading to the phosphorylation of the immunoreceptor tyrosine-based activation motifs in the CD3 complex (1). Phosphorylated immunoreceptor tyrosine-based activation motifs interact with the Src homology (SH) 2 domains of the Syk/Zap70 family PTKs, while not extracellular signal-regulated protein kinase after restimulation. Production of IL-2 by T cells requires activation of three distinct mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 MAP kinase (3–5). In T cells, JNK and p38 MAP kinase are synergistically activated by costimulation of the TCR/CD3 and CD28 receptors, while no synergy is observed in the ERK activation (4, 5). Although these MAP kinases are supposed to be regulated by different PTKs and small GTPases (6), the more precise mechanisms for the activation of these MAP kinases, especially of JNK and p38 MAP kinase, in T cell activation remain unsolved.

A new family of PTKs has been identified as the focal adhesion PTK family consisting of the non-receptor focal adhesion kinase (Fak) (7) and proline-rich tyrosine kinase 2 (Pyk2) (8), also known as cellular adhesion kinase β (CAK β) (9), related adhesion focal tyrosine kinase (RAFTK) (10), and calcium-dependent tyrosine kinase (CADTK) (11). Pyk2 is predominantly expressed in the cells derived from hematopoietic lineages and in the central nervous system, while Fak is ubiquitously expressed. The recently discovered alternatively spliced isoform of Pyk2 (Pyk2H) (12, 13) is specifically expressed in the T and B lymphocytes, monocytes, and natural killer cells. Pyk2 contains the canonical binding site (Tyr-881) for the SH2 domain of Grb2 and the interacting site (Tyr-402) for the SH2 domain of several Src family kinases in addition to the proline-rich region for binding of the SH3 domains of p130CAS and HEP1 (14, 15). Pyk2 COOH terminus binds to Hic-5 (16) and PAP (Pyk2 COOH terminus-associated protein) (17), and its NH2 terminus associates with Nirs (Pyk2 NH2-terminal domain-interacting receptors) (18). It has been shown that Pyk2 is one of the signaling mediators critical for the G protein-coupled receptors (19) and that, in many cells, Pyk2 is activated by signals that elevate the intracellular Ca2+ concentration (8, 11). Several reports have demonstrated that Pyk2 is requisite for the activation of ERK, JNK, and/or p38 MAP kinase in non-lymphoid cells (8, 11, 19–21). In primary T cells and T cell lines, Pyk2 is tyrosine-phosphorylated and activated upon ligation of TCR (22, 23) as well as integrins (24, 25). Recently, it was demonstrated that the tyrosine phosphorylation of Pyk2 but not Fak is induced by CD28 ligation in Jurkat T cells (26).
The Role of Pyk2 in T Cell Activation

In the current study, we investigated the role of Pyk2 in T cell activation by analyzing the Jurkat transfectants expressing Pyk2 mutants. In the clones expressing a mutant Pyk2-Y402F (tyrosine 402 to phenylalanine) but not expressing Pyk2-Y881F, both tyrosine phosphorylation and activation of Pyk2 that occur upon TCR stimulation were prevented. The degree of activation of both JNK and p38 MAP kinase after concurrent ligation of TCR and CD28 was also reduced by more than 50% in Pyk2H-Y402F-expressing cells. Consistent with these inhibition, IL-2 production was significantly diminished in the same clones. These results strongly suggest that Pyk2 is involved in the activation of T cells via its tyrosine 402.

MATERIALS AND METHODS

Reagents—Geneticin disulfate (G418) was obtained from Wako Chemicals (Osaka, Japan). [32P]ATP and the Renaissance enhanced chemiluminescence (ECL) detection system were purchased from NEN Life Science Products. Polyvinylidine fluoride (PVDF) membrane was obtained from Millipore (Bedford, MA), and protein G-Sepharose 4B and glutathione-Sepharose 4B were from Amersham Pharmacia Biotech. Poly(Glu-Tyr) 4:1, Biotin 97, piceatannol, and myelin basic protein (MBP) were obtained from Sigma. Glutathione S-transferase (GST)-c-Jun (amino acids 1–N-19), anti-Pyk2 (C-19), and anti-Fak (C-20) Abs and polyclonal or monoclonal Abs, anti-Lck, anti-Vav, anti-Zap70, anti-phospholipase C-γ, anti-c-Src, anti-Yes, and anti-Fyn were purchased from Santa Cruz Biotechnology. Moloney marine leukemia virus vector was purchased from the University of California, CA. Moloney marine leukemia virus p55gene reverse transcriptase RNase H minus was obtained from Toyobo Co. Ltd. (Tokyo, Japan). Human IL-2 ELISA kit was obtained from Endogen Inc. (Woburn, MA).

Antibodies—Anti-human CD3 (OKT3) monoclonal antibody (mAb) was obtained from American Type Tissue Culture Collection (ATCC, Bethesda, MD), and anti-human CD28 (CD28.2) was obtained from Immunotech (Marseille, France). Polyclonal anti-Pyk2 (N-19), anti-Pyk2 (C-19), and anti-Fak (C-20) Abs and polyclonal or monoclonal Abs, anti-Lck, anti-Vav, anti-Zap70, anti-phospholipase C-γ, anti-c-Src, anti-Yes, and anti-Fyn were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-labeled anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG Abs were obtained from Amersham Pharmacia Biotech, and anti-FLAG mAb (M2) was from Sigma. Anti-phosphotyrosine mAb (4G10) was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phosphotyrosine 402, 579, 580, or 881 of Pyk2 Abs were purchased from BIOSOURCE. Abs each detecting active form of ERK, JNK, or p38 MAP kinase were obtained from Promega Corp. (Madison, WI), and anti-ERK, anti-JNK, and anti-p38 MAP kinase Abs were obtained from Santa Cruz Biotechnology.

Establishment of Jurkat Transfectants Expressing Mutated Pyk2—Expression vector pME18s neo was kindly donated by Dr. K. Maruyama (Tokyo Medical and Dental University, Tokyo, Japan). Pyk2 and mutant Pyk2 were subcloned into pME18s neo downstream of FLAG tag under the control of SRa promoter. Hematopoietic cell-specific Pyk2 (Pyk2H) and point mutants of kinase-inactive PK (K457A, lysine 457 mutated to alanine), autophosphorylation site (Y402F, tyrosine 402 to phenylalanine), and Grb2 SH2-binding site (Y881F, tyrosine 881 to phenylalanine) mutants were constructed by PCR-based mutagenesis. These mutations were confirmed by DNA sequencing. Acute human T cell leukemia (Jurkat) cells, clone E6–1, were obtained from ATCC and maintained in RPMI 1640 containing 10% fetal calf serum and 5 μM 2-mercaptoethanol. To generate stable lines, 50 μg of the expression vector were linearized and transfected into Jurkat T cells (107 in 800 μl) by electroporation at 960 microfarads, 250 V using Gene Pulser (Bio-Rad), and the transfectants were selected by 1 mg/ml G418. Neomycin-resistant clones were selected by 1 mg/ml G418. Neomycin-resistant clones were screened for expression of FLAG-tagged Pyk2 by Western blotting and surface expression of CD3 and CD28 by flow cytometry. To assess CD3 and CD28 expression, cells were stained with OKT3 and CD28.2, respectively, followed by goat anti-mouse fluorescein isothiocyanate-conjugated IgG (Cappel, Aurora, OH). The clones expressing FLAG-tagged Pyk2 mutants with equivalent amounts of CD3 and CD28 to those of Jurkat or Jurkat cells transfected with pME18s vector alone as controls were selected for analyses as described below.

Cell Activation and Preparation of Cell Lysates—Jurkat cells were serum-deprived for 4 h to become “quiescent” cells with low levels of phosphorytions. These cells were treated on ice for 15 min with saturating concentrations (5–10 μM/ml) of anti-CD3 (OKT3) mAb or/and anti-CD28 (CD28.2) mAb. This treatment did not result in any activation. Ten μg/ml goat anti-mouse IgG Ab were added to the cell suspensions to cross-link CD3/TCR and/or CD28 on the cells, and the cells were then immediately placed at 37 °C for the indicated time periods. Following the desired time point, the cells were lysed in a lysis buffer (1% Triton X-100 or Brij 97, 25 mM Hesper, pH 7.4, 150 mM NaCl, 5 μM EDTA, 1 μM Na3VO4, 10 μM β-glycerophosphate, 10 mM pyrophosphate, 100 units/ml aprotinin, 10 μg/ml leupeptin, 25 μM N-nitrophenyl p-guanidino-benzoate, 1 μM phenylmethylsulfonyl fluoride, 10 mM iodoacetamide) for 30 min at 4 °C. For piceatannol treatment, Jurkat cells expressing Pyk2H (JT-Pyk2H) 2 × 107 were cultured with 25 μg/ml piceatannol for 30 min at 37 °C, washed, and then stimulated with anti-CD3 mAb as described above.

Pyk2 in Vivo Kinase Assays—Equal amounts of lysates from various lines of Jurkat transfectants were subjected to immunoprecipitation with anti-FLAG mAb. Immunoprecipitation was performed as described previously (27). One-half of the immunoprecipitates were washed three times with lysis buffer and twice with kinase buffer (40 mM Hesper, pH 7.4, 0.1% Nonidet P-40, 10 mM MgCl2, 3 mM MnCl2, 30 μM sodium orthovanadate). One-half of the immunoprecipitates was analyzed by SDS-PAGE and immunoblotting with anti-Pyk2 antibody, whereas the other half was subjected to kinase assay and incubated with 50 μl of kinase buffer supplemented with 2 μM ATP including 5 μCi of [32P]ATP for 20 min at 30 °C. For exogenous substrate assay, 20 μg of poly(Glu-Tyr) (4:1) were added to the reaction mixture. The reaction was stopped by the addition of 50 μl of 2× SDS sample buffer, boiled for 5 min, and the products were resolved by 7.5% SDS-PAGE. The gel was dried, and the [32P]-labeled Pyk2 or poly(Glu-Tyr) was made visible by autoradiography. The degree of phosphorylation of Pyk2 or poly(Glu-Tyr) was determined by quantitation with a bioimaging analyzer (Fuji BAS2000), and the amounts of Pyk2 were quantitated by using NIH Image. The activities of Pyk2 were determined as a function of the amount of Pyk2 assayed (relative specific activities).

Glutathione S-Transferase Fusion Protein Binding Studies—The Fyn SH2 and two SH3 domains of Vav (amino acids 605–662 and 786–844) were produced in Escherichia coli as fusion proteins with GST using the pGEX-2T expression vector (Amersham Pharmacia Biotech). For the binding experiments, cell lysates from Jurkat transfectants (2 × 107) were first mixed with GST-glutathione-Sepharose 4B for 1 h to preabsorb nonspecific binding proteins to the complex and then mixed with GST-Fyn SH2- or Vav SH3-glutathione-Sepharose 4B complex for 3 h at 4 °C on a rotatory shaker. The beads were centrifuged and washed five times with lysis buffer and Triton X-100 lysis buffer. The bound Pyk2 was eluted by boiling in 1× SDS sample buffer and subjected to 7.5% SDS-PAGE and Western blot analysis.

Determination of ERK, JNK, and p38 MAP Kinase Activities—Activated state of MAP kinases (ERK, JNK, and p38 MAP kinase) were assessed by Western blotting using polyclonal antibodies that recognize the dually phosphorylated, active form of ERK, JNK, or p38 MAP kinase as described previously (27). To normalize the amount of loaded samples, the blots were reblotted with anti-ERK, anti-JNK, or anti-p38 MAP kinase Abs. For further confirmation, we used another method to evaluate activities of ERK and JNK. To measure ERK kinase activity, immune complex kinase assay using MBF as a substrate was performed as described (28). JNK activity was determined by solid phase kinase assay using GST-e-Jun(-1–79) as a substrate and was described (29). The reaction products were resolved by SDS-PAGE, and the incorporation of [32P]phosphate was quantitated by PhosphorImager analysis (Molecular Dynamics).

IL-2 Assay—Jurkat transfectants (2 × 106) were stimulated with monoclonal antibodies to human CD3 (OKT3) and CD28 (CD28.2) cross-linked by goat anti-mouse IgG and cultured for 24 h. IL-2 secreted in the culture supernatant was measured using ELISA (Endogen) according to the manufacturer’s instructions. For RT-PCR assay, 105 cells were stimulated with anti-CD3 mAb plus anti-CD28 mAb and cultured for 7 h at 37 °C and total cellular RNA was prepared by acid guаниdinum-phenol-chloroform method. These samples were treated with DNase 1, and the 10 μg of RNA were then reverse transcribed using random hexanucleotides (Roche Molecular Biochemicals) and AMV reverse transcriptase (Promega, Madison, WI) to produce cDNA. Dilutions of cDNAs were then performed to allow for a semiquantitative estimate of IL-2 mRNA levels by PCR using IL-2- or glyceraldehyde-3-phosphate dehydrogenase-specific primers (Stratagene, La Jolla, CA). PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

RESULTS

Characterization of Jurkat Clones Expressing Mutated Pyk2—It has been reported that Pyk2 is tyrosine-phosphorylated upon stimulation of the T cells through TCR (24, 25). To investigate the role of Pyk2H, a dominant Pyk2 species in T cells, in the T cell activation, we established Jurkat transfect-
Pyk2H-Y402F Prevents Activation of Endogenous Pyk2 upon TCR Stimulation—To investigate further the role of tyrosine PTKs other than Pyk2 phosphorylate Pyk2. Similar levels as Pyk2H (Fig. 1, panel A), we investigated the tyrosine phosphorylation at Tyr-402, Tyr-881, and Tyr-580 to kinase-negative mutant, Pyk2H-K457A (PKM) underwent tyrosine phosphorylation was observed in Pyk2H-Y402F, while upon ligation of TCR (25), we investigated the binding of GST-Fyn ligation of TCR induced tyrosine phosphorylation of Pyk2 at residues 402 (panel B), 881 (panel C), and 580 (panel D). As expected, substitution of tyrosine residues for phenylalanine abolished the phosphorylation of the corresponding sites (Pyk2-Y402F (panel B) and Pyk2-Y881F (panel C)). It is noted that a kinase-negative mutant, Pyk2H-K457A (PKM) underwent tyrosine phosphorylation at Tyr-402, Tyr-881, and Tyr-580 to similar levels as Pyk2H (Fig. 1, panel A), lane 3). This suggests that PTK(s) other than Pyk2 phosphorylate Pyk2.

Pyk2H-Y402F Prevents Activation of Endogenous Pyk2 upon TCR Stimulation—To investigate further the role of tyrosine 402 and tyrosine 881 in the tyrosine phosphorylation of Pyk2 in the activated T cells, we examined the tyrosine phosphorylation of Pyk2H-Y402F and Pyk2H-Y881F in these Jurkat transfec-tants after stimulation with OKT3. As shown in Fig. 2A, no tyrosine phosphorylation was observed in Pyk2H-Y402F, while that of Pyk2H-Y881F was 60–70% of Pyk2H, indicating that tyrosine 402 is indispensable for the phosphorylation of Pyk2 upon T cell activation.

As Fyn associates with Pyk2 through its SH2 domain upon ligation of TCR (25), we investigated the binding of GST-Fyn SH2 to the mutated Pyk2 by pull-down assay. We observed the association of Fyn SH2 with Pyk2H or Pyk2H-Y881F but not with Pyk2H-Y402F (Fig. 2B). These associations were detected only when the Jurkat transfec-tants were stimulated through TCR, indicating that phosphorylated tyrosine 402 provides a binding site for Fyn. In addition, we found that the amount of Fyn SH2 associated with Pyk2 was significantly increased upon concurrent ligation of TCR and CD28 as compared with that upon ligation of TCR alone (Fig. 2B).

Furthermore, as demonstrated in Fig. 2 (C and D), Pyk2H-Y402F provided a dominant inhibitory effect on both tyrosine phosphorylation and activation of endogenous Pyk2. Thus, elevation of the level of both tyrosine phosphorylation (Fig. 2C) and the activity (Fig. 2D) of endogenous Pyk2 were signifi-cantly prevented in the TCR-stimulated JT-Pyk2-Y402F cells as compared with those of JT-pME18s. PKM(K457A) also in-hibited the activation of Pyk2 (Fig. 2D).

Concurrent Ligation of TCR and CD28 Enhances Tyrosine Phosphorylation and Activation of Pyk2—As the concurrent ligation of TCR and CD28 increased the association of Fyn with Pyk2 (Fig. 2B), we investigated the tyrosine phosphorylation and activation of Pyk2 in the co-stimulated Jurkat cells in comparison with those in cells stimulated by TCR alone (Fig. 3A). The tyrosine phosphorylation of both Pyk2 and Pyk2H upon concurrent ligation is enhanced up to 2-fold of that upon ligation of TCR alone (Fig. 3A). Consistent with this result, the activity of Pyk2 and Pyk2H was further elevated by co-stimulation as compared with TCR stimulation alone, as detected by autophosphorylation (Fig. 3B) and by phosphorylation of the endogenous Fyn at Tyr-394 as determined by Western blotting using a phospho-specific antibody.
exogenous substrate, poly(Glu-Tyr) (4:1) (Fig. 3C). Kinase activity of Pyk2H-K457A was negligible, and that of Pyk2H-Y402 was little activated upon TCR stimulation (Fig. 3C). We could not detect either the tyrosine phosphorylation or the activation of Pyk2 upon ligation of CD28 alone (data not shown). These results suggest that Pyk2 is involved in the full activation of T cells leading to cytokine production.

Pyk2 Is Involved in the Activation of JNK and p38 MAP Kinase Accompanying Co-stimulation of TCR and CD28—The MAP kinase superfamily comprises three distinct kinases: ERK, JNK/stress-activated protein kinase, and p38 MAP kinase (3). Activation of these three kinases is required for the full activation of T cells (4, 5). It has been reported that Pyk2 could induce activation of these kinases in non-lymphoid cells (8, 11, 19–21). Thus, we next investigated the activation of MAP kinases in the Jurkat transfectants accompanying TCR stimulation. Consistent with a previous report (6), in our system, JNK was just weakly activated by TCR stimulation, but its activity was markedly activated by co-stimulation with TCR and CD28 (data not shown). JNK activity was compared in Jurkat transfectants stimulated by concurrent ligation of TCR and CD28. The activity was assessed by both immunoblotting using the anti-active form of JNK (Fig. 4, A and C) and solid phase kinase assay using GST-c-Jun-(1–79) as a substrate (Fig. 4, B and D). In both systems, the JNK activity after stimulation was enhanced by 30–80% over control cells in JT-Pyk2, JT-Pyk2H, and JT-Y881F, while reduced in JT-K457A and JT-Y402F by 30% and 60%, respectively (Fig. 4, A and B). These inhibitory effects of Pyk2H-K457A and Pyk2H-Y402F were confirmed by using three to four independent clones that expressed the mutated Pyk2H (Fig. 4, C and D). In the respective clones, about 30% (JT-K457A) and 60% (JT-Y402F) of the JNK activity were reduced compared with that of JT-pME18s clones. These data suggest that Pyk2 is involved in the activation of

FIG. 3. Concurrent ligation of TCR and CD28 promotes tyrosine phosphorylation and activation of Pyk2. A, JT-Pyk2 and JT-Pyk2H (10^7 cells) were stimulated as in Fig. 2D, and the lysates were immunoprecipitated with anti-FLAG mAb. The precipitates were subjected to immunoblotting with anti-phosphotyrosine mAb (4G10). Arrows indicate the position of Pyk2 and Pyk2H. B, the same immunoprecipitates as in A were subjected to in vitro kinase assay for autophosphorylation. Arrows indicate the position of Pyk2 and Pyk2H. C, the same immunoprecipitates as in A were subjected to assay for kinase activity against exogenous substrate, poly(Glu-Tyr). The numbers indicate the relative activities (R.A.) of Pyk2.

FIG. 4. Pyk2 is involved in the activation of JNK and p38 MAP kinase. A, upper, JT-pME18s, JT-Pyk2, JT-Pyk2H, JT-K457A, JT-Y881F, and JT-Y402F (2 × 10^7 cells) were stimulated with anti-CD3 mAb plus anti-CD28 mAb for 5 min. The total cell lysates equivalent to 10^6 cells were immunoblotted with anti-active form of JNK Ab. Arrows indicate the position of JNK (54 and 46 kDa). Lower, the blot was stripped and reprobed with anti-JNK Ab. B, JT-pME18s, JT-Pyk2, JT-Pyk2H, JT-K457A, JT-Y881F, and JT-Y402F (10^7 cells) were stimulated with anti-CD3 mAb plus anti-CD28 mAb for 10 min. The lysates were incubated with GST-c-Jun-(1–79) prebound to glutathione-Sepharose 4B for 12 h, and the precipitates were subjected to in vitro kinase assay using [γ-^32P]ATP. An arrow indicates the position of JNK (46 kDa). D, the JNK activities of the same lysates as in C were measured as in B. E (upper), JT-pME18s, JT-Pyk2, JT-Pyk2H, JT-K457A, JT-Y881F, and JT-Y402F (2 × 10^7 cells) were stimulated with anti-CD3 mAb plus anti-CD28 mAb for 5 min. The total cell lysates equivalent to 10^6 cells were subjected to immunoblot with anti-active form of JNK Ab. An arrow indicates the position of JNK (46 kDa). D, the JNK activities of the same lysates as in C were measured as in B. E (upper), JT-pME18s, JT-Pyk2, JT-Pyk2H, JT-K457A, JT-Y881F, and JT-Y402F (2 × 10^7 cells) were stimulated with anti-CD3 mAb plus anti-CD28 mAb for 5 min. The total cell lysates equivalent to 10^6 cells were immunoblotted with anti-active form of p38 MAP kinase Ab. An arrow indicates the position of p38 MAP kinase (38 kDa). Lower, the blot was stripped and reprobed with anti-p38 MAP kinase Ab.
JNK in Jurkat T cells induced by concurrent ligation of TCR and CD28.

The activation of p38 MAP kinase, another stress-activated MAP kinase, was assessed by immunoblotting using the anti-active form of p38 MAP kinase (Fig. 4E). Similar with the JNK activation described above, p38 MAP kinase activation was significantly inhibited in JT-Y402F and JT-457A.

Pyk2 Is Not Involved in the Activation of ERK upon Stimulation of T Cells through TCR—The ERK consists of two isoforms, ERK1 (41 kDa) and ERK2 (44 kDa), both of which are activated in the TCR-stimulated T cells (29). We applied two methods for the detection of activated ERKs; one is to use the antibody specific to the active form of the ERKs in immunoblotting (Fig. 5A), and the other is the immune complex kinase assay using MBP as a substrate (Fig. 5B). As shown in Fig. 5 (A and B), no significant differences in the level of activities of the ERKs were observed in any of the Jurkat transfectants, indicating that Pyk2 is not involved in the activation of ERKs in TCR-stimulated T cells.

Pyk2 Is Involved in Interleukin-2 Production by Jurkat Cells—Interleukin-2 production by Jurkat transfectants stimulated by concurrent ligation of TCR and CD28 was evaluated by ELISA (Fig. 6A). The amount of IL-2 in the culture supernatant of JT-Y402F was significantly reduced (p < 0.05), as shown in Fig. 6A. Some extent of the increase in the amount of IL-2 secreted by JT-Pyk2, JT-Pyk2H, or JT-Pyk2H-Y881F and a weak decrease in that by JT-K457A were reproducibly observed. The level of IL-2 mRNA in the Jurkat transfectants was analyzed by RT-PCR (Fig. 6B). The IL-2 mRNA was detected in cells co-stimulated with anti-CD3 and anti-CD28 Abs, but not detected in those without stimulation or with anti-CD3 Ab alone (data not shown). As demonstrated in Fig. 6 (B and C), consistent with the secreted amount of IL-2, the level of IL-2 mRNA was significantly low in Pyk2-Y402F. Thus, the amount of IL-2 mRNA was reduced by 50–60% in JT-Pyk2-Y402F, while slight decrease was observed in JT-Pyk2-K457A. These results indicate that Pyk2 is involved in the signal transduction pathway leading to IL-2 production.

Association of Pyk2 with Zap70 and Vav—The central role of Zap70 in T cell activation has been demonstrated (1). Vav is also required for T cell activation (30) and functions as a guanine nucleotide exchange factor for Rac1 (31). We found that these molecules are in complex with Pyk2H in JT-Pyk2H, as...
shown in Fig. 7. When overexpressed Pyk2H was immunoprecipitated by anti-FLAG, Zap70 was detected in the immunoprecipitates (Fig. 7A). The same immunoprecipitates were subjected to autophosphorylation reaction in the presence of \([^{32}P]ATP\), and was dissociated by boiling in SDS. Zap70 was recovered by anti-Zap70 Ab from the supernatants (Fig. 7B). These results revealed that Zap70 was associated with Pyk2H in JT-Pyk2H cells. Although Lck and SLP76 are abundant in Jurkat cells, they were not detected in the same precipitates (data not shown). This association was independent of TCR stimulation (Fig. 7A), and tyrosine phosphorylation at 402 or 881 of Pyk2 was not necessary (Fig. 7C). This observation was further confirmed by immunoprecipitation of Zap70, followed by immunoblot with anti-Pyk2 Ab (Fig. 7E). Pyk2H was not detected in the similarly treated immunoprecipitates of Lck (data not shown).

Vav was also co-precipitated with Pyk2H in JT-Pyk2H as demonstrated in Fig. 7 (D and E). Like Zap70, this association was also independent of the TCR-stimulation (Fig. 7D) and of tyrosine phosphorylation at 402 and 881 of Pyk2 (data not shown). We prepared GST fusion proteins consisting of Vav SH3 N (605–662) or Vav SH3 C (786–844) and examined their association with Pyk2H by pull-down assay. As demonstrated in Fig. 7F, both SH3 domains of Vav interacted with Pyk2H.

### DISCUSSION

Multiple families of protein-tyrosine kinases participate in the TCR-mediated activation of T cells through interaction and cross-talking with each other (1). Co-stimulation of TCR and auxiliary molecules (co-receptors) such as CD28 induces activation of JNK and p38 MAP kinase, leading to full activation of T cells. However, little is known about the mechanisms for the activation of these MAP kinases. In this study, we have demonstrated that Pyk2 is involved in both the activation of JNK and p38 MAP kinase and the IL-2 production by Jurkat T cells. We further suggest that the interaction of Pyk2 with Fyn plays crucial roles in the T cell activation.

MAP kinases play critical roles in transmitting signals generated by PTKs to the nucleus (3). In T lymphocytes, JNK and p38 MAP kinase are synergistically activated by the co-stimulation of the TCR and CD28, while TCR engagement alone can fully activate ERK (6). The importance of simultaneous activation of these MAP kinases for IL-2 production by T cells has been genetically evidenced (4, 5). Furthermore, the enzymatic activities of ERK and JNK are reduced after costimulation with anti-CD3 and anti-CD28 Abs in murine anergic T cells (32). Therefore, it is crucial to clarify the signaling components from TCR and CD28 to MAP kinases that integrate the multiple signals and determine the T cell fate to be activated or to be anergic. In the current study, we demonstrated that a mutant Pyk2H-Y402, when overexpressed, significantly inhibits the activation of endogenous Pyk2 and prevents activation of JNK and p38 MAP kinase but not ERK. Pyk2H-Y402 seems to prevent activation of resident Pyk2H by interfering its interaction with Zap70 and other signaling molecules. Inhibitory effects of Pyk2H-K457A (PKM) were less than that of Pyk2H-Y402. We assume that PKM might be able to function to some extent since its tyrosine residues were fully phosphorylated in the TCR-stimulated Jurkat cells in this study. Our results indicate that Pyk2 is involved in the activation of JNK and p38 MAP kinase and that tyrosine 402 of Pyk2, which provides a binding site for Fyn, plays critical roles in the activation of these MAP kinases. Recent findings indicate that Rac1 is involved in the activation of JNK in T cells (33). Pyk2 has been reported to activate JNK in a Rac1-dependent manner in non-lymphoid cells (20). Thus, our results suggest that the Pyk2-Fyn complex may also activate JNK through activation of Rac1.
in T cells. Although some kinds of stimulation induce Pyk2-dependent activation of ERK kinases in non-lymphoid cells (8), Pyk2 did not affect the activation of ERK kinases induced in TCR-stimulated Jurkat cells.

Overexpression of Pyk2H-Y402F significantly prevented IL-2 production by Jurkat. Some extent of the increase in the IL-2 production was observed in JT-Pyk2, JT-Pyk2H, or JT-Pyk2H-Y881F and a weak decrease in that in JT-K457A. This profile of the effects exhibited by each Pyk2 mutants apparently correlated with that shown on activation of JNK and p38 MAP kinase. These results suggest that Pyk2 is involved in the IL-2 production through the activation of JNK and p38 MAP kinase. Pyk2H-Y402F inhibited at most 60–70% of these responses. This may be attributed to two possibilities. One is that Pyk2-dependent pathway is the major one leading to activation of JNK but exogenous Pyk2H-Y402F cannot prevent activation of Pyk2 completely, as demonstrated in Fig. 2 (C and D). Another possible explanation is that Pyk2-independent signaling pathway(s) also exert downstream of TCR and compensate Pyk2-mediated pathway or by itself give rise to some extent of activation of JNK and p38 MAP kinase leading to IL-2 production. At present, we cannot exclude the latter possibility.

Tyrosine phosphorylation of Pyk2 is dependent on the cytoskeletal structure and partly on the intracellular Ca²⁺ (25, 26). Recently, it was shown that piceatannol, which specifically inhibits the activities of Zap70 and Syk, prevented tyrosine phosphorylation of Pyk2, suggesting the involvement of these kinases in the activation of Pyk2 (26). In rat tumor mast cell line RBL-2H3, Pyk2 functions downstream of Syk in FceRI-mediated signaling which is inhibited by piceatannol (34, 35). In our study, the equivalent degree of tyrosine phosphorylation occurred in the kinase inactive form of Pyk2H, PKM (Pyk2H-K457A), suggesting that tyrosine kinase other than Pyk2 phosphorylates Pyk2. In fact, piceatannol prevented the phosphorylation of tyrosine 402 of Pyk2H (data not shown). Furthermore, the constitutive association of Zap70 with Pyk2 was observed in Jurkat transfectants overexpressing Pyk2H. These results support the notion that Pyk2 functions downstream of Zap70 in T cell activation.

We demonstrated that tyrosine residues at 402, 580, and 881 of Pyk2 were markedly phosphorylated upon TCR engagement. Tyrosine 580 is located within a region responsible for phosphorylation dependent regulation of protein kinase activity, and its phosphorylation is assumed to be necessary for maximal Pyk2 kinase activity. Fyn and Fyn SH2 bind to Pyk2 (25) and tyrosine-phosphorylation of Pyk2 is diminished in Fyn-deficient T cells upon engagement of TCR (36). We showed that tyrosine 402 was requisite for the phosphorylation of other tyrosines on Pyk2 upon TCR stimulation. Furthermore, we demonstrated that phosphorylated tyrosine 402 of Pyk2 provides a binding site for Fyn SH2 in the T cell activation. Thus, we assume that Pyk2 is first phosphorylated at tyrosine 402 by Zap70 and then Fyn is recruited to Pyk2 to phosphorylate other tyrosine residues of Pyk2 and Pyk2-associated signaling molecules.

It was recently demonstrated that CD28 ligation induces the tyrosine phosphorylation of Pyk2 but not Fak in Jurkat T cells (26). In the current study, although tyrosine phosphorylation of Pyk2 was not detected upon CD28 ligation alone, enhanced tyrosine phosphorylation and activation of Pyk2 were observed in Jurkat cells stimulated by the concurrent ligation of TCR and CD28. In addition, we found the promoted binding of Fyn SH2 to Tyr-402 of Pyk2 upon the co-stimulation. These results further support the notion that Pyk2 is involved in the signaling pathway generated by the co-stimulation of TCR and CD28. T cells from mice deficient for Vav exhibit a blockage in the cell cycle progression and fail to produce IL-2 in response to anti-CD3 cross-linking (30). Vav is involved in the Rac-1-dependent activation of JNK (37) and in the TCR-mediated Ca²⁺ flux and reorganization of the actin cytoskeleton (38). In Jurkat transfectants overexpressing Pyk2H, Pyk2 is constitutively associated with Vav. Although 1–2% of the total amount of Vav in the cellular lysates bound to Pyk2 (data not shown), this observation indicates that Vav, in addition to Zap70, can bind to Pyk2. All of the Pyk2 mutants used here could bind similar amount of Vav and had little effect on the phosphorylation of Vav after TCR stimulation (data not shown). Therefore, it is possible that Pyk2 might exert an adapter-like function for Vav. The role of this association in T cell activation is now under examination in our laboratory.

In conclusion, Pyk2 is involved in the T cell activation mediated through TCR and CD28. Tyrosine 402 of Pyk2 is critical for its function, probably through formation of a Pyk2-Fyn complex leading to the activation of Rac1-JNK pathway followed by the production of IL-2. Recent findings suggest that Pyk2 may function downstream of LFA-1 (39) and of CD2 (40). As LFA-1 and CD2 are critical for the formation of immunological synapse (41), Pyk2 might function in forming or sustaining it for full activation of the T cells. Furthermore, Pyk2 is involved in the signaling mediated through IL-2 receptor on the T cells (42). Given these data and the fact that hematopoietic-specific Pyk2 isoform, Pyk2H, is dominantly expressed in T cells (16), we propose that Pyk2 is one of the crucial regulatory molecules for T cell functions.  |
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Protein-tyrosine Kinase Pyk2 Is Involved in Interleukin-2 Production by Jurkat T Cells via Its Tyrosine 402

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