

# Tyrosine Phosphorylation of Pyk2 Is Selectively Regulated by Fyn During TCR Signaling

By Dapeng Qian,\* Sima Lev,<sup>†</sup> Nicolai S.C. van Oers,\* Ivan Dikic,<sup>§</sup> Joseph Schlessinger,<sup>§</sup> and Arthur Weiss\*

From the \*Howard Hughes Medical Institute, Departments of Medicine and of Microbiology and Immunology, University of California, San Francisco, California 94143; <sup>†</sup>SUGEN, Redwood City, California 94063; and <sup>§</sup>the Department of Pharmacology, New York University Medical Center, New York 10016

## Summary

The Src family protein tyrosine kinases (PTKs), Lck and Fyn, are coexpressed in T cells and perform crucial functions involved in the initiation of T cell antigen receptor (TCR) signal transduction. However, the mechanisms by which Lck and Fyn regulate TCR signaling are still not completely understood. One important question is whether Lck and Fyn have specific targets or only provide functional redundancy during TCR signaling. We have previously shown that Lck plays a major role in the tyrosine phosphorylation of the TCR- $\zeta$  chain and the ZAP-70 PTK. In an effort to identify the targets that are specifically regulated by Fyn, we have studied the tyrosine phosphorylation of Pyk2, a recently discovered new member of the focal adhesion kinase family PTK. We demonstrated that Pyk2 was rapidly tyrosine phosphorylated following TCR stimulation. TCR-induced tyrosine phosphorylation of Pyk2 was selectively dependent on Fyn but not Lck. Moreover, in heterologous COS-7 cells, coexpression of Pyk2 with Fyn but not Lck resulted in substantial increases in Pyk2 tyrosine phosphorylation. The selective regulation of Pyk2 tyrosine phosphorylation by Fyn in vivo correlated with the preferential phosphorylation of Pyk2 by Fyn in vitro. Our results demonstrate that Pyk2 is a specific target regulated by Fyn during TCR signaling.

Engagement of the TCR evokes a series of signal transduction events critical for the functional activation of T cells (reviewed in reference 1). Signal transduction through the TCR is also important for T cell development (1). The earliest detectable signaling event after TCR stimulation is the activation of protein tyrosine kinases (PTKs)<sup>1</sup>, resulting in the tyrosine phosphorylation of cellular proteins (1). Lck and Fyn, two cytoplasmic PTKs of the Src family, have been implicated as the initiating PTKs for TCR signaling. Lck is critical for TCR signaling. Mutant T cell lines lacking functional Lck or T cells from *lck*<sup>-/-</sup> mice respond to TCR stimulation with very limited tyrosine phosphorylation of cellular proteins, greatly decreased calcium mobilization, and reduced proliferation (2–4). Lck also plays a critical role in T cell development, as *lck*<sup>-/-</sup> mice have a pronounced reduction in thymocyte numbers and a block in thymocyte development at the early CD4<sup>+</sup>CD8<sup>+</sup> stage (2). The residual progression of thymocytes from CD4<sup>-</sup>CD8<sup>-</sup> to CD4<sup>+</sup>CD8<sup>+</sup> stage in *lck*<sup>-/-</sup> mice depends upon the redundant function of Fyn. Combined disruption of both

Lck and Fyn (*lck*<sup>-/-</sup>/*fyn*<sup>-/-</sup>) completely arrests thymocyte development at the CD4<sup>-</sup>CD8<sup>-</sup> stage (5, 6). Fyn is also important for TCR signaling. Mature CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes from *fyn*<sup>-/-</sup> mice are severely impaired in TCR signaling as measured by calcium mobilization, protein tyrosine phosphorylation, IL-2 production, and proliferation (7, 8). Peripheral T cells from *fyn*<sup>-/-</sup> mice are also impaired in TCR signaling, albeit to a lesser degree (7, 8).

The mechanisms by which Lck or Fyn regulates proximal TCR signaling are still not completely understood. One important issue is whether Lck and Fyn have specific targets or only provide functional redundancy during TCR signaling. We have studied the TCR signaling pathway in T cells from *lck*<sup>-/-</sup> or *fyn*<sup>-/-</sup> mice in an attempt to identify targets for Lck and Fyn. We have shown previously that Lck is the primary PTK that regulates the tyrosine phosphorylation of the TCR subunits and of ZAP-70 (9), a Syk family PTK critical for TCR signaling (reviewed in reference 10). The identity of the downstream target(s) for Fyn has not been identified. In the present study, we have focused our efforts in identifying target(s) whose phosphorylation is specifically regulated by Fyn. Previous studies have shown that Fyn interacts with a number of proteins in T cells (11–16), including several proteins migrating from 110 to

<sup>1</sup>Abbreviations used in this paper: FAK, focal adhesion kinase; PTK, protein tyrosine kinase; ZAP-70,  $\zeta$ -associated protein 70.

130 kD. These proteins are potential targets for Fyn, though their identities have not been fully elucidated. A recently discovered cytoplasmic PTK Pyk2 has a molecular mass of 112 kD (17–19). Pyk2 is a member of the focal adhesion kinase (FAK) family and has been shown to play important roles in signal transduction of neuronal cells (17, 20, 21). Because Pyk2 is also expressed in T cells (18, 19), we examined whether it might be a target for Fyn during TCR signaling. Our data demonstrate that Pyk2 is a novel Fyn-dependent tyrosine-phosphorylated substrate during TCR signaling.

## Materials and Methods

**Mice.** Wild-type mice (C57BL/6 strain) were purchased from The Jackson Laboratory (Bar Harbor, ME). *lck*<sup>-/-</sup> mice (2) and *fyn*<sup>-/-</sup> mice (7) were obtained from Drs. T. Mak (Amgen and the Ontario Cancer Institute, Toronto, Ontario, Canada) and R. Perlmutter (University of Washington, Seattle, WA), respectively.

**Cells.** Preparation of thymocytes and lymph node T cells were carried out as described (9). Jurkat, the human leukemic T cell line, was described previously (3). COS-7 cells (derived from monkey kidney), which do not express Lck, Fyn (T), ZAP-70, and Pyk2 endogenously, have been described (22). TAG Jurkat cells, obtained from Dr. G. Crabtree (Stanford University, Palo Alto, CA), are Jurkat T cells stably transfected with SV40 TAG.

**Antibodies.** Anti-CD3 mAb 145-2C11 was obtained from the American Type Culture Collection ([ATCC] Rockville, MD). C305 is an anti-TCR mAb. The anti-ZAP-70 mAb 2F3 was described (22). The rabbit anti-ZAP-70 antiserum no. 1598 and the anti-ZAP-70 mAb 1E7.2 were produced against a KLH-peptide immunogen with a peptide corresponding to the human ZAP-70 amino acid sequence 282–307. Antibodies against Pyk2 were raised in rabbits immunized against a synthetic peptide corresponding to 15 amino-terminal residues (D1) (17) or with GST fusion protein containing amino acids 362–647 (antibody nos. 1 and 3) (17) or 679–830 (antibody no. 600) (21) of Pyk2. The rabbit anti-Fyn antibody and the anti-Lck mAb 1F6 were obtained from Drs. A. Veillette (McGill University, Montreal, Canada) and J. Bolen (DNAX Research Institute, Palo Alto, CA), respectively. The rabbit anti-Lck antibody was generated using a TrpE fusion protein containing the amino-terminal unique region of Lck as an immunogen. The anti-Fyn mAb was obtained from Transduction Laboratories, Lexington, KY. 12CA5 (Boehringer Mannheim, Indianapolis, IN) is a murine mAb to the hemagglutinin (HA) epitope. OKT8, obtained from ATCC, is a murine mAb to the CD8- $\alpha$  chain. Anti-phosphotyrosine mAbs 4G10 and RC20 were obtained from Upstate Biotechnology, Lake Placid, NY and Transduction Laboratories, respectively.

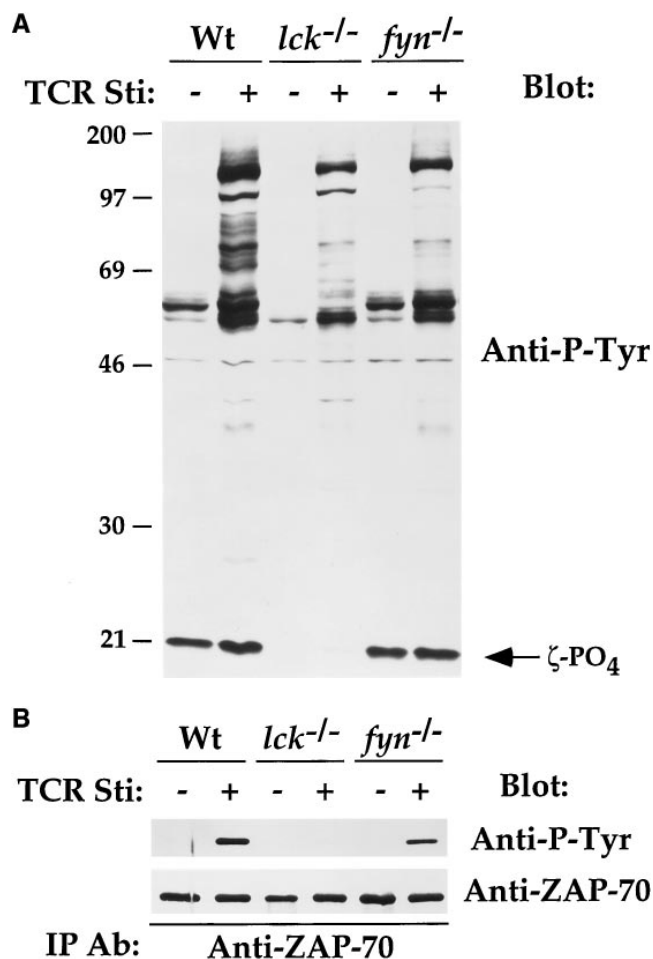
**Plasmids.** Lck and Fyn (T) were expressed with the pSM vector and pSV7d vector, respectively, as described (22). pSM was derived from pSV7d. Pyk2 and PKM, a kinase-inactive Pyk2 mutant, were expressed with the pRK5 vector (17, 20). ZAP-70 (K369A) was expressed with the pBJ1 vector (23). HA-Pyk2 was created by fusing the HA-epitope tag (YPYDVPDYAS) to the carboxy-terminal end of Pyk2 and subcloned into pRK5 vector.

**Stimulation, Solubilization, Immunoprecipitation, and Immunoblot Analysis.** Stimulation of mouse thymocytes and T cells with 10  $\mu$ g/ml purified anti-CD3 mAb 145-2C11 was carried out as described (9). Stimulation of Jurkat with the anti-TCR mAb C305 or 1  $\mu$ M ionomycin was performed as described (3). Cells were

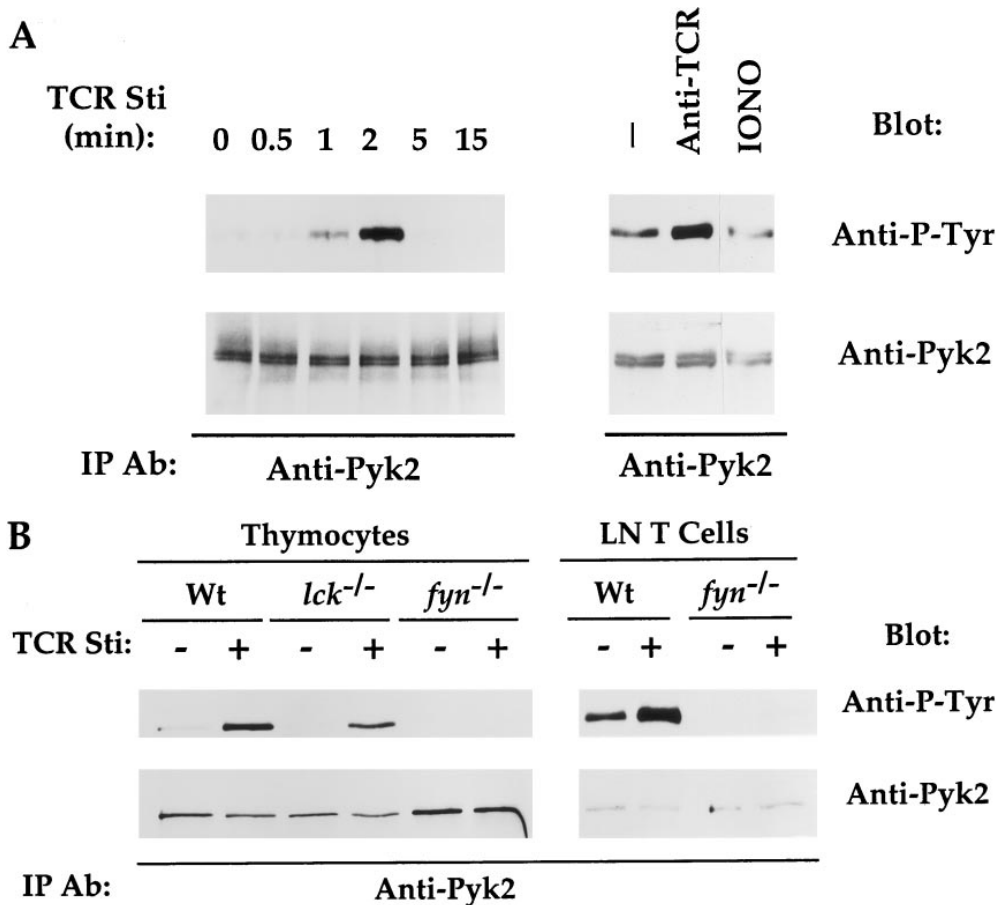
lysed in a buffer containing 1% NP-40, 10 mM Tris, pH 7.6, 150 mM NaCl, 2 mM EDTA, protease and phosphatase inhibitors, as described (3). Immunoprecipitation, immunoblotting, stripping, and reprobing blots were performed as described (23).

**Transfections.** COS-7 cells were transiently transfected with 2  $\mu$ g of each construct using DEAE-dextran (22). The total amount of DNA for each transfection was equalized with vector DNA. Cells were harvested 72 h after transfection and lysed with the lysis buffer as described above. Transient transfection of TAG Jurkat cells with 40  $\mu$ g pRK5.HA-Pyk2 expression construct and stimulation of transfected cells were carried out as described (23).

**Kinase Assays.** For in vitro kinase reactions using poly(Glu:Tyr) (4:1) as an exogenous substrate, immunoprecipitates were washed three times with the RIPA buffer (1% NP-40, 1% deoxy-



**Figure 1.** (A) Lck and Fyn regulate TCR stimulation-induced tyrosine phosphorylation of shared and distinct substrates. Thymocytes isolated from wild-type (*Wt*), *lck*<sup>-/-</sup>, or *fyn*<sup>-/-</sup> mice were either left unstimulated (-) or stimulated (+) with the anti-CD3 mAb 145-2C11 for 3 min. Cells were lysed, and tyrosine phosphorylation of whole cell lysates was analyzed by immunoblotting with the anti-phosphotyrosine (anti-P-Tyr) mAb 4G10. The migration of tyrosine-phosphorylated TCR- $\zeta$  chain is indicated. Molecular mass markers are also indicated. (B) Tyrosine phosphorylation of ZAP-70 requires Lck but not Fyn. Thymocytes isolated from wild-type (*Wt*), *lck*<sup>-/-</sup>, or *fyn*<sup>-/-</sup> mice were either left unstimulated or stimulated with the anti-CD3 mAb for 3 min. Cells were lysed, ZAP-70 was immunoprecipitated (IP) with an anti-ZAP-70 mAb (1E7.2), and analyzed by anti-P-Tyr immunoblotting using mAb 4G10. The same blot was stripped and reprobed with anti-ZAP-70 (mAb 1E7.2).



**Figure 2.** Fyn, but not Lck, is required for TCR-stimulated Pyk2 tyrosine phosphorylation. (A) Pyk2 phosphorylation in Jurkat T cells. Left, Jurkat cells were stimulated with anti-TCR (mAb C305) for the indicated period of times. Right, Jurkat cells were stimulated with anti-TCR or ionomycin (*IONO*) for 2 min. Cells were lysed, Pyk2 was immunoprecipitated with anti-Pyk2 (antibody no. 3), and analyzed by immunoblotting using anti-P-Tyr mAb RC20. The level of Pyk2 in each sample was verified either by immunoblotting an aliquot of anti-Pyk2 immunoprecipitates with anti-Pyk2 (antibody no. 3) (*left*) or by stripping and reprobing the same blot with anti-Pyk2 (*right*). (B) Thymocytes isolated from wild-type (*Wt*), *lck*<sup>-/-</sup>, or *fyn*<sup>-/-</sup> mice were either left unstimulated or stimulated with the anti-CD3 mAb for 3 min. Cells were lysed, Pyk2 was immunoprecipitated with anti-Pyk2 (antibody no. 600), and analyzed by immunoblotting using anti-P-Tyr mAb 4G10. The same blot was stripped and reprobed with anti-Pyk2 (antibody no. 3).

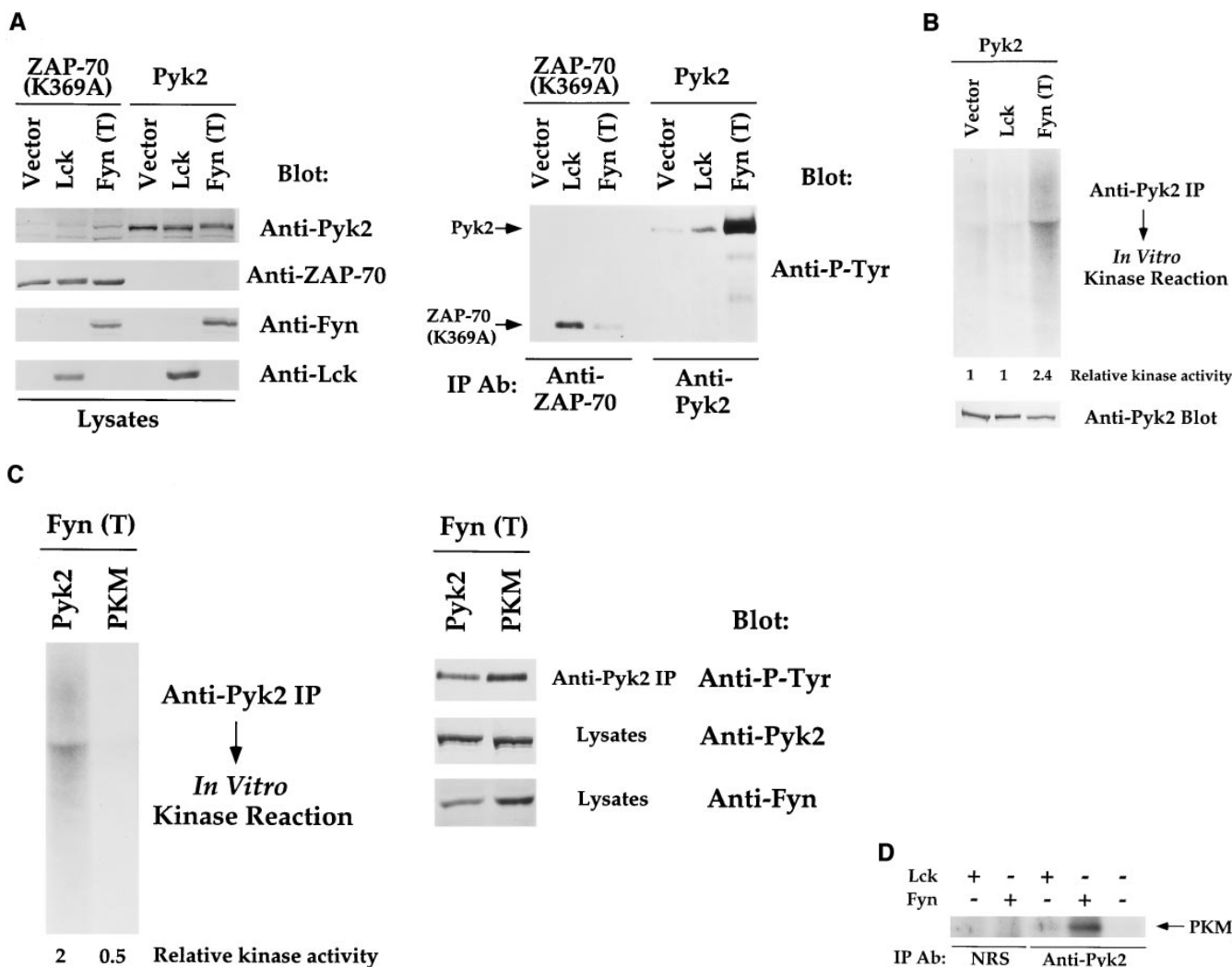
lcholate, 0.1% SDS, 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, and protease and phosphatase inhibitors), once with the RIPA buffer without EDTA, once with the kinase buffer I (50 mM Tris, pH 7.6, 5 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>), and incubated with 30 μl kinase buffer I containing 20 μg poly(Glu/Tyr) (4:1) (Sigma) and 10 μCi γ-[<sup>32</sup>P]ATP (6,000 Ci mmol<sup>-1</sup>; Amersham) for 12.5 min at room temperature. The reactions were stopped with SDS sample buffer and resolved on SDS-PAGE (10% gel). Autoradiography and quantitation of the kinase activity were performed as described (17). For in vitro kinase reactions using purified Lck or Fyn (Upstate Biotechnology, Lake Placid, NY), immunoprecipitates were washed three times with the RIPA buffer, once with the RIPA buffer without EDTA, once with the kinase buffer II (20 mM Hepes, pH 7.4, 100 mM NaCl, 5 mM MnCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>), and incubated with 30 μl kinase buffer II containing 3 U purified kinase (1 U is defined as 1 pmol phosphate transferred to a cdc2 [6–20] peptide min<sup>-1</sup> mg protein<sup>-1</sup>), 1 μM cold ATP, and 10 μCi γ-[<sup>32</sup>P]ATP for 10 min at room temperature. The reactions were stopped with SDS sample buffer and resolved on SDS-PAGE.

## Results and Discussion

**Lck and Fyn Regulate the Tyrosine Phosphorylation of Shared and Distinct Substrates.** To determine whether Lck and Fyn have specific targets or only provide functional redundancy during TCR signaling, we first examined the induction of tyrosine-phosphorylated proteins after TCR stimu-

lation in thymocytes isolated from wild-type, *lck*<sup>-/-</sup>, or *fyn*<sup>-/-</sup> mice (Fig. 1 A). TCR stimulation-induced tyrosine phosphorylation of cellular proteins was generally decreased in thymocytes isolated from either *lck*<sup>-/-</sup> or *fyn*<sup>-/-</sup> mice compared with those from wild-type mice. More importantly, reductions in tyrosine phosphorylation of different proteins were evident. These results suggest that Lck and Fyn regulate the tyrosine phosphorylation of shared and distinct substrates. We have already shown (9) and confirm here that Lck is the primary Src family PTK regulating the tyrosine phosphorylation of the TCR-ζ chain and ZAP-70, whereas Fyn has only a minor redundant role in regulating these events (Fig. 1, A and B).

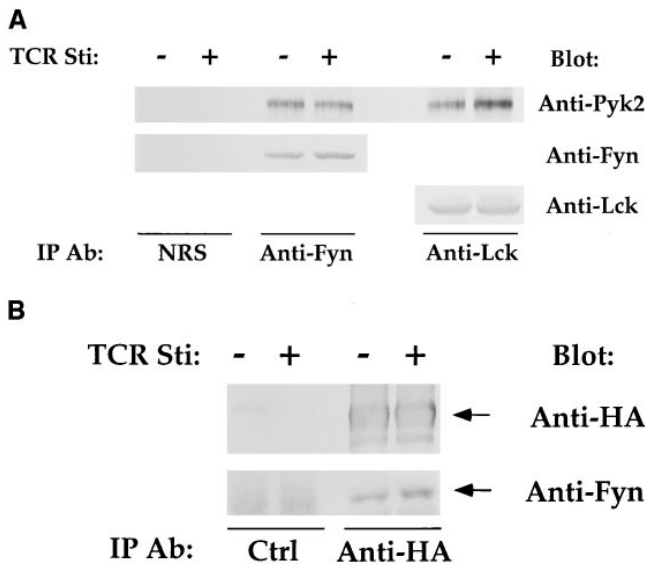
**Pyk2 Is a Novel Tyrosine-phosphorylated Substrate after TCR Stimulation.** To determine whether Pyk2 is a target for Fyn during TCR signaling, we first tested whether Pyk2 becomes tyrosine phosphorylated upon TCR stimulation. We found that Pyk2 was weakly tyrosine phosphorylated at basal state in T cells. Stimulation of the TCR resulted in a rapid increase in Pyk2 tyrosine phosphorylation in human Jurkat T cells (Fig. 2 A, *left*) or in normal mouse thymocytes and lymph node T cells (Fig. 2 B). Enhanced tyrosine phosphorylation of Pyk2 is relatively specific to TCR stimulation, as activation via Fas or the costimulator receptor CD28 did not lead to increased Pyk2 tyrosine phosphorylation (data not shown). Furthermore, in contrast with other



**Figure 3.** Pyk2 activation is selectively induced by Fyn, but not Lck. (A) Tyrosine phosphorylation of Pyk2. Pyk2 or the kinase-inactive ZAP-70 mutant, ZAP-70 (K369A), was transiently cotransfected with vector, Lck, or Fyn (T) into COS-7 cells. Tyrosine phosphorylation of Pyk2 or ZAP-70 (K369A) was determined by immunoblotting using anti-P-Tyr mAb 4G10 (right), after immunoprecipitation with anti-Pyk2 (antibody no. 1) or anti-ZAP-70 (antibody no. 1598). The expression of Pyk2, ZAP-70 (K369A), Fyn (T), or Lck in each transfected sample was determined by immunoblotting whole cell lysates with anti-Pyk2 (antibody no. 1), anti-ZAP-70 (mAb 2F3), a rabbit anti-Fyn antibody, or anti-Lck (mAb 1F6) (left). (B) Phosphorylation of an exogenous substrate by Pyk2. Pyk2 was transiently transfected into COS-7 cells with vector, Lck, or Fyn (T). Cells were lysed, and Pyk2 was immunoprecipitated with anti-Pyk2 (antibody no. 600). The immunoprecipitates were extensively washed with RIPA buffer, and subjected to kinase reactions in vitro in the presence of  $\gamma$ - $^{32}\text{P}$ ATP and the exogenous substrate poly(Glu/Tyr) (4:1). The phosphorylated products were resolved by SDS-PAGE, analyzed by autoradiography (top), and quantitated by a phosphorimager and Image Quant software (Molecular Dynamics). The kinase activities relative to vector cotransfected cells are indicated (middle). The expression of Pyk2 in each sample was determined by immunoblotting whole cell lysates with anti-Pyk2 (antibody no. 1) (bottom). (C) The kinase activity of Pyk2 is required for the ability of Pyk2 to phosphorylate an exogenous substrate but not Pyk2 tyrosine phosphorylation induced by Fyn (T). Pyk2 or the kinase inactive mutant Pyk2 with a point mutation at the ATP-binding site (PKM) was cotransfected with Fyn (T) into COS-7 cells. Cells were lysed, and Pyk2 or PKM was immunoprecipitated with anti-Pyk2 (antibody no. 600). Phosphorylation of poly(Glu/Tyr) (4:1) by the immunoprecipitates in in vitro kinase reactions was determined (left). The relative kinase activities to Pyk2 and vector cotransfected cells (data not shown) are also indicated. Tyrosine phosphorylation of Pyk2 and PKM was analyzed by immunoblotting using anti-P-Tyr mAb 4G10 (right). The expression of Pyk2, PKM, or Fyn (T) in each transfected sample was determined by immunoblotting whole cell lysates with the anti-Pyk2 (antibody no. 1) or an anti-Fyn mAb. (D) Phosphorylation of PKM by purified Lck or Fyn. COS-7 cells transfected with PKM were lysed, and lysates were immunoprecipitated with either normal rabbit serum (NRS) or an affinity-purified anti-Pyk2 (antibody D1). The immunoprecipitates were extensively washed with RIPA buffer and used in in vitro kinase reactions in the absence (-) or presence (+) of purified Lck or Fyn (3 U/reaction).

systems, such as the nicotinic acetylcholine receptor expressed in neuronal cells, which induce Pyk2 tyrosine phosphorylation by stimulation of extracellular calcium influx (17, 20, 24), TCR-induced tyrosine phosphorylation of Pyk2 is  $\text{Ca}^{2+}$  independent. Treatment of Jurkat T cells (Fig. 2 A, right) or mouse thymocytes (data not shown) with the cal-

cium ionophore, ionomycin, did not stimulate Pyk2 tyrosine phosphorylation. Moreover, EGTA had no effect on TCR-stimulated Pyk2 tyrosine phosphorylation in these cells (data not shown). Thus, Pyk2 tyrosine phosphorylation is likely to be an event proximal to  $\text{Ca}^{2+}$  mobilization during TCR signaling. Together, these results demonstrate that Pyk2 is a



**Figure 4.** Physical association of Fyn with Pyk2 in T cells. (A) Thymocytes isolated from C57BL/6 mice were either left unstimulated or stimulated with the anti-CD3 mAb for 3 min. Cells were lysed, lysates were immunoprecipitated with NRS, a rabbit anti-Fyn antibody, or a rabbit anti-Lck antibody, and analyzed by immunoblotting with an affinity-purified anti-Pyk2 (antibody D1), an anti-Fyn mAb, and anti-Lck (mAb 1F6). (B) HA-tagged Pyk2 was transiently transfected into TAg Jurkat T cells. 40 h later, transfected cells were either left unstimulated or stimulated with anti-TCR (mAb C305) for 2 min. Cells were lysed, lysates were immunoprecipitated with either a control (*Ctrl*) antibody (OKT8) or anti-HA (mAb 12CA5), and analyzed by immunoblotting with an anti-HA mAb (12CA5) and an anti-Fyn mAb. The positions of HA-Pyk2 and Fyn are indicated by arrows.

newly identified physiological target of activated PTK(s) after TCR stimulation.

**Tyrosine Phosphorylation of Pyk2 during TCR Signaling Is Fyn Dependent.** We compared the status of Pyk2 tyrosine phosphorylation in thymocytes and T cells isolated from *lck*<sup>-/-</sup> or *fyn*<sup>-/-</sup> mice with those from wild-type mice (Fig. 2 B). Because *lck*<sup>-/-</sup> mice have few peripheral T cells, we focused our analysis on thymocytes. Pyk2 tyrosine phosphorylation was readily detected after TCR stimulation in thymocytes isolated from *lck*<sup>-/-</sup> mice. In contrast, thymocytes and lymph node T cells from *fyn*<sup>-/-</sup> mice exhibited little, if any, detectable basal and TCR-stimulated Pyk2 tyrosine phosphorylation. This experiment provides strong genetic evidence that Fyn, but not Lck, is required for tyrosine phosphorylation of Pyk2 during TCR signaling.

**Selective Regulation of Pyk2 Tyrosine Phosphorylation and Activation by Fyn in COS-7 Cells.** Because it is possible that the differences in Pyk2 phosphorylation observed in *lck*<sup>-/-</sup> and *fyn*<sup>-/-</sup> thymocytes might reflect heterogeneity in the cellular subsets in these mice, the regulation of tyrosine phosphorylation of Pyk2 by Lck and Fyn was further studied in a heterologous cell system, COS-7 cells. Cotransfection of Pyk2 with the lymphocyte-specific form of Fyn, Fyn (T), resulted in a marked increase in Pyk2 tyrosine phosphorylation (Fig. 3 A, right). Hence, overexpression of Fyn (T) in the absence of other lymphoid-spe-

cific components in COS-7 cells is sufficient to induce Pyk2 tyrosine phosphorylation. Cotransfection with Lck only led to a slight increase in Pyk2 tyrosine phosphorylation. However, Lck induced substantial tyrosine phosphorylation of a kinase-inactive ZAP-70 mutant, ZAP-70 (K369A), even when expressed at a lower level (Fig. 3 A). Cotransfection of Pyk2 with Fyn (T), but not Lck, also enhanced the kinase activity of Pyk2 towards the exogenous substrate, poly (Glu/Tyr) (4:1) (Fig. 3 B). Mutation of the ATP-binding site in the kinase domain abolished the kinase activity of Pyk2 (Fig. 3 C, left), but had no effect on the overall tyrosine phosphorylation of Pyk2 induced by cotransfection with Fyn (T) (Fig. 3 C, right). Thus, the kinase activity of Pyk2 is not required for its tyrosine phosphorylation induced by Fyn (T) in these cotransfection experiments. Taken together, these results further demonstrate that Pyk2 tyrosine phosphorylation or activation is selectively regulated by Fyn, but not Lck.

**Pyk2 Is a Preferred Substrate for Fyn In Vitro.** The apparent differential regulation of Pyk2 tyrosine phosphorylation or activation by Lck and Fyn suggests that Pyk2 may be a preferred substrate for Fyn but not Lck. To determine whether Fyn or Lck could phosphorylate Pyk2, we immunoprecipitated the kinase-inactive Pyk2 mutant, PKM (17), that had been transfected into COS-7 cells and subjected it to kinase reactions in vitro with either purified Fyn or Lck (Fig. 3 D). When the same specific activities of Fyn and Lck were used in each reaction, PKM was strongly phosphorylated by Fyn. In contrast, little or no phosphorylation of PKM was induced by Lck. Thus, Pyk2 is a preferred substrate for Fyn in vitro.

**Association of Pyk2 with Fyn and Lck in T Cells.** Next, we examined whether Fyn or Lck may interact with Pyk2 in T cells as determined by coimmunoprecipitation. Anti-Fyn antibodies coimmunoprecipitated comparable levels of Pyk2 from thymocytes of C57BL/6 mice before and after TCR stimulation (Fig. 4 A). However, anti-Pyk2 antibodies did not coimmunoprecipitate Fyn from mouse thymocytes (data not shown). This likely results from the disruption of association by antibody binding. To circumvent this problem, we introduced a HA tag into the carboxyl terminus of Pyk2 and then transfected the HA-tagged Pyk2 into TAg Jurkat T cells. Similar levels of Fyn were coimmunoprecipitated by anti-HA antibodies from HA-Pyk2-transfected TAg Jurkat cells before and after TCR stimulation (Fig. 4 B). These data indicate that Fyn constitutively associates with Pyk2 in T cells. Interestingly, anti-Lck antibodies also coimmunoprecipitated Pyk2 from mouse thymocytes before and after TCR stimulation (Fig. 4 A). This result is consistent with an association between Fyn and Lck that was reported previously (25). It is possible that Pyk2 is present in the same multimolecular complex containing both Fyn and Lck. However, because Pyk2 is a preferred substrate for Fyn, it is selectively phosphorylated or activated by Fyn, but not Lck.

In summary, our results demonstrate that Pyk2 is selective target regulated by Fyn during TCR signaling. This selectivity is due, at least in part, to the fact that Pyk2 is a

preferred substrate for Fyn in vitro. Also, these findings provide evidence that Lck and Fyn have functional specificities during TCR signaling. Activation of distinct targets by Lck and Fyn may allow for the differential control of downstream signaling pathways by the TCR. Alternatively, full activation of a critical downstream signaling component may require synergism between two distinct upstream activating pathways regulated by TCR→Lck→ZAP-70

and TCR→Fyn→Pyk2. The defective TCR signaling responses in *lck*<sup>-/-</sup> and *fyn*<sup>-/-</sup> mice may result, in part, from the failure of ZAP-70 and Pyk2 to be activated, respectively. Further identification of other targets for Lck and Fyn, as well as the targets for ZAP-70 and Pyk2, should help clarify how these cytoplasmic PTKs coordinately mediate TCR signaling.

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Address correspondence to Dr. A. Weiss, the Howard Hughes Medical Institute, Departments of Medicine and of Microbiology and Immunology, University of California, 3rd and Parnassus Avenues, Box 0724, San Francisco, CA 94143.

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