TCR Engagement Induces Proline-Rich Tyrosine Kinase-2 (Pyk2) Translocation to the T Cell-APC Interface Independently of Pyk2 Activity and in an Immunoreceptor Tyrosine-Based Activation Motif-Mediated Fashion

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TCR Engagement Induces Proline-Rich Tyrosine Kinase-2 (Pyk2) Translocation to the T Cell-APC Interface Independently of Pyk2 Activity and in an Immunoreceptor Tyrosine-Based Activation Motif-Mediated Fashion

David Sancho,* María C. Montoya,* Alicia Monjas,† Mónica Gordón-Alonso,* Takuya Katagiri,‡ Diana Gil,† Reyes Tejedor,* Balbino Alarcón,§ and Francisco Sánchez-Madrid**

The relocation of kinases in T lymphocytes during their cognate interaction with APCs is essential for lymphocyte activation. We found that the proline-rich tyrosine kinase-2 (Pyk2) is rapidly translocated to the T cell-APC contact area upon T cell-specific recognition of superantigen-pulsed APCs. Stimulation with anti-CD3-coated latex microspheres was sufficient for Pyk2 reorientation, and the coengagement of CD28 boosted Pyk2 redistribution. Nevertheless, Pyk2 translocation did not result in its recruitment to lipid rafts. Two results support that Pyk2 translocation was independent of its kinase activity. First, Lck activity was required for TCR-induced Pyk2 translocation, but not for TCR-induced Pyk2 activation. Second, a kinase-dead Pyk2 mutant was equally translocated upon TCR triggering. In addition, Lck activity alone was insufficient to induce Pyk2 reorientation and activation, requiring the presence of at least one intact immunoreceptor tyrosine-based activation motif (ITAM). Despite the dependence on functional Lck and on phosphorylated ITAM for Pyk2 translocation, the ITAM-binding tyrosine kinase ζ-associated protein 70 (ZAP-70) was not essential. All these data suggest that, by translocating to the vicinity of the immune synapse, Pyk2 could play an essential role in T cell activation and polarized secretion of cytokines. The Journal of Immunology, 2002, 169: 292–300.

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elocation of membrane and cytosolic proteins is essential for lymphocyte activation (1–4). Studies on the molecular reorganization that occurs during the specific interaction of T lymphocytes with APCs revealed that different molecules involved in T cell stimulation are compartmentalized, favoring the activation of these cells (4–8). Upon engagement with an APC in a productive contact, T cells undergo a rapid cytoskeletal polarization, which includes the formation of a tight collar of polymerized actin at the T cell-APC interface and the reorientation of the microtubule-organizing center (MTOC)† toward the bound APC (9, 10). Repositioning of the MTOC leads to the polarized secretion of cytokines at the T cell-APC interface (11–13).

The TCR is coupled to intracellular signaling pathways by two noncovalently associated signal-transducing complexes, namely CD3 (γ, δ, and ε) and TCR-associated ζimer. Signaling through these complexes requires the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAM) by protein tyrosine kinases (PTK) of the Src family, mainly Lck (14, 15). Phosphorylated ITAMs serve as docking sites for Src homology domain 2 (SH2)-containing signaling molecules, such as the PTKs of the Syk/ζ-associated protein 70 (ZAP-70) family. After its recruitment, ZAP-70 is activated by phosphorylation and contributes to the initiation of downstream signaling events. The importance of Lck and ZAP-70 in T cell activation has been demonstrated in kinase-negative Jurkat cells that show defects in Ca2+ mobilization, tyrosine phosphorylation, and IL-2 transcription (16, 17).

Besides the pivotal role of Lck, other molecules also participate in T cell activation. Proline-rich tyrosine kinase-2 (Pyk2) is a nonreceptor PTK from the focal adhesion kinase family (18), also known as related adhesion focal tyrosine kinase (19), cellular adhesion kinase β (20), and calcium-dependent tyrosine kinase (21). Pyk2 is predominantly expressed in hemopoietic cell lineages and in the CNS. The alternatively spliced isoform of Pyk2 (Pyk2H) (22, 23) is specifically expressed by T and B lymphocytes, monocytes, and NK cells. In T cells, Pyk2 is tyrosine phosphorylated and activated after TCR engagement (24–26). In addition, Pyk2 contributes to IL-2 production in activated T cells (27). Despite its important role in T cell activation, its subcellular location during TCR-mediated T cell activation is currently unknown. Therefore, to better understand the function of Pyk2 in Ag-specific response, it was of interest to unveil the subcellular location of Pyk2 in T

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cells engaged through the TCR and the mechanisms that regulate this distribution.

In this study, we describe that Pyk2 is rapidly translocated to the area of cell-cell contact after TCR stimulation via superantigen-pulsed APCs or anti-CD3-coated latex beads. Pyk2 translocation takes place independently of its activation, although both activation and relocation of Pyk2 require the presence of functional ITAMs.

Materials and Methods
Cells, chimeric constructions, and cell transfections
Jurkat T cell clones J77cl20 (37) (28), JCAM 1.6 (29), and P116 (17) as well as the lymphoblastoid B cell lines Raji and LG2 were cultured in complete medium (RPMI 1640, 10% FCS; Life Technologies, Gaithersburg, MD). T cell clones stably expressing the chimeric proteins containing the extracellular and transmembrane domains of human CD8ε were purchased from the functional and nonfunctional ITAM of CD3ε (BG10, CD8ε-YY, and A10, CD8ε-FF cell lines, respectively) (30, 31), or to the second ITAM of CD3ε (E7b3D, CD3εb) (32), were obtained by transfection of the corresponding plasmids into 3T13 cells, a TCR-negative variant of the human Jurkat T cell line.

The Jurkat stable transfectants expressing the wild-type (wt) and the kinase-dead mutant of Pyk2H have been previously described (27), and were grown in complete medium supplemented with G418 (1 mg/ml; Cal-biochem, San Diego, CA). The CD4 T cell clone S3058B (53), which responds to staphylococcal enterotoxin B (SEB), was generated by limiting dilution from S3 T cells that were stimulated with irradiated LG2 cells and PBMCs in growing medium supplemented with 0.5 μg/ml SEB and 50 U/ml human rIL-2 every 2 wk.

To generate the Pyk2H-green fluorescent protein (GFP) construct, Pyk2H wt in pME185 (27) was subcloned as an EcoRI fragment into the pEGFP1 vector (Clontech Laboratories, Palo Alto, CA). The resultant chimeric construct was sequenced and the correct expression was confirmed by SDS-PAGE of lysates from transient transfected cells. Jurkat cells (107) were transiently transfected with Pyk2H-GFP by electroporation (280 V, 1200 μF; BioRad GenePulsor II electroporator (Hercules, CA).

Abs and reagents
The following mouse anti-human mAbs were used: T3b (anti-CD3), CD28.2 (anti-CD28) (34), D39 (anti-CD45, IgG1 isotype matched to CD28.2), and OKT8 (Ortho Diagnostic System, Rantian, NJ). The sheep anti-mouse Ab used for cross-linking was purchased from Sigma-Aldrich (St. Louis, MO); the BV8 biotin anti-human Vβ8 mAb was from BD Biosciences (San Diego, CA), and the anti-paxillin P13520 mAb was from Transduction Laboratories (Lexington, KY). The goat polyclonal Ab C-19 (anti-Pyk2) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and the mouse monoclonal 4G10 (anti-phosphotyrosine (PTyr)) was from Upstate Biotechnology (Lake Placid, NY). Recombinant chimeric ICAM-1-Fc was from R&D Systems (Minneapolis, MN). The total extracellular domain fused to the ICAM-1 Fc fragment, was obtained as previously described (35). Fibronectin (FN), poly-(l-lysine) (PLL), genitin, and SEB were purchased from Sigma-Aldrich. Staphylococcal enterotoxin E (SEE) was obtained from Toxin Technology (St. Petersburg, FL), and the pertussis toxin was from Calbiochem. The fluorescent cell tracker chloromethyl derivative of aminocoumarin (CMAC) and chloromethylbenzoyl aminomethylrhodamine (CM-TMR) were obtained from Molecular Probes (Eugene, OR). Human rIL-2 from M. Gately (Hoffmann-LaRoche, Nutley, NJ) was provided by the National Institutes of Health AIDS Research and Reference Reagent program, Division of AIDS, National Institute of Allergy and Infectious Diseases. [32P]ATP (400 Ci/mmol) was from ICN (Costa Mesa, CA), and protein A- and G-Sepharose were from Roche Molecular System (Alameda, CA). ECL reagents were from Amersham (Arlington Heights, IL), and all other reagents used were of the purest grade available.

In vitro kinase assays and Western blot
Cell lines were stimulated or not with the appropriate primary Ab (anti-CD3 or anti-CD8) for 10 min at 4°C. Then, a sheep anti-mouse Ab was added as cross-linker, and cells were incubated for 5 min at 37°C. Thereafter, the cells were lysed in 300 μl of cold lysis buffer (50 mM Tris-HCl, pH 7.65, 5 mM EDTA, 150 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 2 mM sodium orthovanadate, 1% Triton X-100, 50 μg/ml aprotinin, 50 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM PMSF). Cell lysates were clarified by centrifugation at 14,000 rpm for 10 min, and supernatants were immunoprecipitated at 4°C overnight with protein G-agarose coupled to goat polyclonal Ab specific for Pyk2 (C-19) or mAb 3A5 for Lck (Upstate Biotechnology). Immunoprecipitates were washed three times with lysis buffer, and used for either in vitro kinase assays or Western blot. The in vitro kinase assay was performed as described (36). Briefly, immunoprecipitates were washed three times in lysis buffer and twice with kinase buffer (20 mM HEPES, 3 mM MnCl2, pH 7.35), pellets were dissolved in 40 μl kinase buffer, and reactions were initiated by adding 1 μCi [γ-32P]ATP. The reactions were conducted at 30°C for 15 min, and stopped by transferring to ice and adding 10 mM EDTA. Pellets were then washed in lysis buffer containing 10 mM EDTA, extracted for 5 min at 95°C in 2× SDS-PAGE sample buffer, and analyzed by SDS-PAGE. Autoradiograms were processed using an AGFA Studio ScanIIsi scanner, and bands were quantified using the Bio-Rad MultiAnalytser Software.

To perform the Western blot assay, immunoprecipitated proteins were transferred to Immobilon membranes after SDS-PAGE, using a Bio-Rad Transblot. Membranes were then blocked with 3% nonfat dry milk in PBS, pH 7.2, and incubated for 2 h at room temperature with the C-19 anti-Pyk2 at a 1/500 dilution, the 4G10 anti-PTyr at 1 μg/ml, and the 3A5 anti-Lck at a dilution in PBS containing 3% nonfat dry milk. Bound Abs were detected with HRP-conjugate secondary Abs, followed by visualization by ECL reagents.

Cell conjugate formation and Pyk2 translocation assays
To distinguish APCs from T lymphocytes, Raji and LG2 B lymphoblastoid cells were loaded with the blue fluorescent cell tracker CMAC. Briefly, cells were preincubated in HBSS containing 10 μM CMAC for 20 min at 37°C. T cells were added at a ratio of 5 × 105/ml in HBSS. The cells were incubated for 20 min, in the presence or absence of 5 μg/ml SEE (Raji) or 1 μg/ml SEB (LG2). J77 or S3 T cells (2 × 105 cells/slide) were slide with an equal number of Raji or LG2 cells, respectively, in a final volume of 80 μl/slide and placed in an Eppendorf tube. Then, cells were centrifuged at low speed, and incubated for 15 min at 37°C. Thereafter, conjugates were gently resuspended, plated onto PLL-coated slides in humidified incubation chambers, and allowed to settle for additional 15 min at 37°C. PLL-adhered cells were fixed and permeabilized for 5 min in 2% formaldehyde-dehydrate-0.5% Triton X-100 in PBS, and stained with the appropriate Abs, using highly cross-absorbed donkey anti-goat Abs labeled with 488 and rhodamine red X as secondary reagents (Molecular Probes). Quantitative analysis of cell conjugate formation was assessed by fluorescence microscopy. Conjugates were first identified by direct observation under differential interference contrast (DIC) and then confirmed by detecting the blue fluorescent CMAC-labeled APCs. The proportion of conjugates with Pyk2 redistributed next to the T cell-APC contact area was calculated by random choice of 500 to 600 different conjugates from, at least, three independent experiments. Results were expressed as the percentage of conjugates with Pyk2 redistributed to the contact area.

Ab-coated beads were made as previously described (37). In brief, 107 styrene beads, diameter 6.4 μm (Sigma-Aldrich), were incubated for 90 min at room temperature with a suboptimal (1 μg/ml) or optimal (10 μg/ml) concentration of anti-CD3, or 10 μg/ml anti-CD28 (CD28.2) under continuous rotation. Beads were then saturated with 1% BSA HBSS, washed with HBSS, and stored at 4°C until use. For conjugation assays, Jurkat T cells and Ab-coated beads were mixed at 1:1 ratio in medium and allowed to interact for 15 min at 37°C. Then, conjugates were gently resuspended and plated onto PLL-coated coverslips for 30 min, fixed, permeabilized, and stained with the anti-Pyk2, as stated above. When samples were visualized under the fluorescence microscope, the beads appeared round and refringent. Pyk2 was scored as reoriented when it was located in close proximity to the T cell plasma membrane between the nucleus and the cell contact area. Up to 200 conjugates were scored in each experiment.

Time-lapse fluorescence confocal microscopy
Coverslips were coated with FN (20 μg/ml) for 20 h at 4°C, and then saturated with HBSS containing 1% BSA for 30 min at 37°C. Thereafter, coverslips were washed with HBSS and mounted in Attofluor open chambers (Molecular Probes) and placed on the microscope stage. Then, 6 × 105 J77 cells transiently transfected with Pyk2H-GFP in 500 μl HBSS medium containing 2% FCS were allowed to adhere to these chambers for 30 min at 37°C. Raji cells (2 × 105), previously loaded with 5 μM CM-TMR for 20 min in complete medium, were added to the chambers. Cells were loaded with the blue fluorescent CMAC-labeled APCs. Coverslips were washed with HBSS and mounted in Attofluor open chambers (Molecular Probes) and placed on the microscope stage. Then, 6 × 105 J77 cells transiently transfected with Pyk2H-GFP in 500 μl HBSS medium containing 2% FCS were allowed to adhere to these chambers for 30 min at 37°C. Raji cells (2 × 105), previously loaded with 5 μM CM-TMR for 20 min in complete medium, were added to the chambers. Cells were maintained at 37°C in a 5% CO2 atmosphere using an incubation system (La-con GBr Pe-con). A foil cover was placed on top of the chamber, to prevent liquid evaporation without affecting CO2 diffusion.
Confocal images were acquired using a Leica (Deerfield, IL) TCS-SP confocal laser scanning unit equipped with Ar and He/Ne laser beams and attached to a Leica DMIRBE inverted epi-fluorescence microscope. Serial fluorescence and DIC images were simultaneously obtained at the indicated intervals. Optical sectioning was necessary to capture all the green fluorescent signal. The most representative section of the green channel (GFP signal), its corresponding DIC image (which provides information about the morphology of the cell), and the image from the red channel showing the staining for Raji cells (CM-TMR signal) were overlaid in a single image. Experiments were conducted using either the /H11003 63 or /H11003 100 objectives. Images were processed and assembled into movie using the Leica Confocal Software.

Lipid raft isolation

Cells (3 × 10^7) were lysed on ice in 1 ml 0.5% Triton in TNE buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA) for 1 h at 2°C and mixed with 2 ml 68% sucrose (m/v) in TNE. After centrifugation for 16–20 h at 200,000 g in a Beckman SW40 rotor, fractions of 1 ml were collected from the top of the gradient, precipitated with the same volume of acetone, mixed with SDS reducing sample buffer, and analyzed by SDS-PAGE under reducing conditions.

Results

Pyk2 is translocated during specific TCR-mediated activation of T cells

Since Pyk2 is phosphorylated during TCR signaling and plays a crucial role in T cell activation (25–27), we decided to study the subcellular localization of Pyk2 during specific TCR stimulation. We used the human nontransformed T cell clone S3 that specifically recognizes the superantigen SEB presented by LG-2 B cells. Pyk2 formed a cluster in the cytoplasm of unstimulated T cells. This cluster translocated specifically in the presence of SEB to a submembrane location in the area of contact with SEB-preloaded APC (Fig. 1, a and b). The T cell line J77, a Vβ8+ Jurkat clone that specifically recognizes SEE presented by Raji B cells, showed the same superantigen-driven Pyk2 reorientation (Fig. 1, a and b).

The process of Pyk2 rearrangement was dynamically analyzed by live time-lapse fluorescence confocal microscopy. J77 T cells transiently transfected with Pyk2H-GFP were monitored during their interaction with Raji cells pretreated or not with SEE. A rapid (2- to 5-min) Pyk2 relocation in the T cell toward the contact area.
with the APC was observed when conjugates were formed in the presence of superantigen (Fig. 2).

**TCR engagement is sufficient for Pyk2 translocation in T cells**

To characterize whether TCR engagement was sufficient for Pyk2 translocation, an in vitro assay was conducted using J77 as effector cells, and protein-coated, cell-sized latex microspheres as APCs. This system has been used in previous studies to show cytoskeletal rearrangements during Ag-specific cell conjugate formation (37, 38). Anti-CD3-coated latex beads efficiently induced the relocation of Pyk2 to the area of contact between the T cell and the microsphere (Fig. 3a). In contrast, ICAM-1-coated beads did not have a significant effect on Pyk2 reorientation (Fig. 3a), despite the fact that ICAM-1 activates Pyk2 (39). Because CD28 acts as a co-stimulatory molecule in Pyk2 activation (27, 40), we evaluated its effect on Pyk2 reorientation. CD28 engagement, although unable to translocate Pyk2 by itself, cooperated with suboptimal doses of anti-CD3 to induce the change in subcellular redistribution of Pyk2 (Fig. 3, b and c).

**Pyk2 is not recruited to lipid rafts after its reorientation**

Upon engagement, the TCR has been shown to be partly localized in lipid rafts (8). This effect is increased if CD28 is coengaged (7). Since Pyk2 translocates to the vicinity of the plasma membrane in the T cell-APC cognate interaction (Figs. 1 and 2) and CD28 engagement reinforces the effect (Fig. 3), the possible association of Pyk2 with membrane rafts was studied. After stimulation with anti-CD3/anti-CD28-coated beads, the localization of GM1, Lck, and Pyk2 in lipid rafts was analyzed by sucrose density centriugation of cell lysates obtained after 0, 5, or 15 min of stimulation. As shown in Fig. 4, GM1 and Lck were present in the detergent-insoluble, lipid raft fractions (3–5), and the engagement of TCR and CD28 induced a Lck-phosphorylated form that was preferentially located at the insoluble fractions (3–5). However, Pyk2 remained in the soluble fractions. This suggests that Pyk2 does not associate with lipid rafts after its translocation.

**Pyk2 relocation and activation are independent processes**

To assess the possible relation between the activation of Pyk2 and its translocation, we first investigated the effect of some pharmacological inhibitors on the Pyk2 relocation induced by the interaction of J77 cells with either SEE-pulsed Raji cells or anti-CD3-coated latex beads. The tyrosine kinase inhibitor genistein and the Src family inhibitor PP2 significantly blocked Pyk2 translocation in both systems, indicating the important role of these kinases in this process (Table I). In contrast, piceatannol, an inhibitor of Syk/ZAP-70, did not show a significant effect, despite the crucial role of ZAP-70 in early TCR-mediated signaling. In contrast, the PKC physiological inhibitors bis-indoleyl-maleymide-II had a mild, but significant effect on the Pyk2 translocation induced by anti-CD3-coated beads, while it did not affect the relocation triggered by SEE-primed APC.

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**FIGURE 3.** Role of T cell stimulation via CD3 and CD28 in Pyk2 translocation. a, ICAM-1- or anti-CD3-coated (10 μg/ml) latex polystyrene beads were allowed to form conjugates with J77 T cells, adhered to PLL, and stained for Pyk2, as indicated in Materials and Methods. Matched fluorescence and DIC images are shown. b, Conjugates of J77 cells with latex beads coated with anti-CD28 (10 μg/ml), or isotype-matched anti-CD45 (IgG1 control, 10 μg/ml), and with (anti-CD3 subopt.) or without (−) suboptimal doses of anti-CD3 (1 μg/ml) were adhered to PLL and stained for Pyk2. Asterisks point out the latex bead interacting with the T cell. c, The stimulatory effect of anti-CD28 was studied with latex beads coated with different doses of anti-CD3 in the presence or absence of CD28 (10 μg/ml). Data correspond to the arithmetic mean ± SD of Pyk2 translocation frequency in >600 conjugates from three independent experiments. *, p < 0.05, compared with the translocation frequency in the presence or absence of anti-CD28 at each anti-CD3 dose (Student’s t test).

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**FIGURE 4.** Pyk2 is not located in rafts after TCR-mediated Pyk2 translocation. J77 cells were incubated for the indicated times with polystyrene beads precoated with a mixture of anti-CD3 and anti-CD28 Abs (10 μg/ml each). After lysis in 0.5% Triton X-100, detergent-soluble and insoluble membrane fractions were separated by centrifugation on sucrose density gradients. An aliquot of each fraction was run by SDS-PAGE, and the distribution of Pyk2 and Lck was analyzed by immunoblotting with appropriate Abs. The distribution of the ganglioside GM1 was analyzed by dot-blot analysis. One experiment of three is shown.
Finally, wortmannin and Ly-294002 did not exert a significant effect at doses that effectively inhibited Akt phosphorylation (Table I, and not shown). This result suggests that PI3-kinase activity is not essential for this phenomenon, although Jurkat is not an adequate model to study PI3-kinase-dependent events (41). The involvement of PTK in Pyk2 activation and reorientation was further explored using the mutant Jurkat cell lines JCaM1.6, which lacks functional Lck molecules (29), and P116, a ZAP-70-deficient mutant (17). Although basal activation of Pyk2 is reduced in JCaM1.6 cells (Fig. 5a) (24), we were interested in comparing Pyk2 activation and translocation in lck- or ZAP-70-deficient T cells after TCR triggering. a, Upper panels, J77 cells, the Lck-deficient JCaM1.6 cell line, and the ZAP-70-deficient P116 cell line were treated (+) or not (−) with anti-CD3 (10 μg/ml) for 5 min at 4°C and then cross-linked with sheep anti-mouse (20 μg/ml). After 5 min of incubation at 37°C, cells were lysed and immunoprecipitated with anti-Pyk2, and in vitro kinase assay was performed. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and exposed to detect radioactive signal. The same membrane was blotted with anti-Pyk2 to quantify the levels of the protein by Western blot. Lower panel, The results of the in vitro kinase assays performed as indicated above are expressed as arithmetic mean ± SD of OD relative to the level of Pyk2 protein detected by Western blot in the same membranes. The results represent three independent experiments. *, p < 0.05; **, p < 0.01, with respect to the translocation frequency in conjugates formed with J77 cells without pretreatment (Student’s t test).

Table I. Effect of pharmacological inhibitors in Pyk2 translocation during TCR engagement

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* J77 T cells were pretreated with the pharmacological inhibitors PP2 (20 μM), piceatannol (100 μM), genistein (20 μM), H-89 (20 μM), bis-indolyl-maleymide-II (BIM-II, 0.5 μM), and pertussis toxin (Ptx, 1 μg/ml) 30 min at 37°C. The standard dose of genistein, PP2, piceatannol, and BIM-II effectively inhibited CD3-mediated induction of CD69 in this system. Wortmannin (WMN, 2 μM) and Ly-294002 (20 μM) were pretreated 90 min at 37°C, and the parallel inhibition of basal and CD3-induced Akt phosphorylation was tested (not shown). Thereafter, conjugates of J77 T cells with Raji APC in the absence (−) or presence of SEE (4 μg/ml), or with anti-CD3-coated latex beads were formed. Pyk2 translocation frequency is expressed as arithmetic mean ± SD of more than 600 conjugates in three independent experiments.

*, p < 0.05; **, p < 0.01, with respect to the translocation frequency in conjugates formed with J77 cells without pretreatment (Student’s t test).
Pyk2 activation in each cell line in response to CD3 stimulation. The induction of Pyk2 activity by a cross-linked anti-CD3 mAb was not apparently affected by the defect in Lck or the absence of ZAP-70 (Fig. 5a). In contrast to the mild effect on Pyk2 activity, relocation of Pyk2 induced by anti-CD3-coated latex beads was completely inhibited in the absence of Lck (Fig. 5, b and c). This effect was totally reverted when Lck activity was reestablished by transfection of JCaM1.6 cells with Lck-GFP (Fig. 5c). In contrast, the absence of ZAP-70 only slightly reduced Pyk2 reorientation (Fig. 5, b and c). These results suggest that while Pyk2 can be activated by Lck- and ZAP-70-independent pathways, Pyk2 translocation is mainly a Lck-dependent phenomenon independent of ZAP-70.

The above results suggested that Pyk2 activation is not sufficient for its translocation. To further prove it, we analyzed Jurkat cells stably transfected with either Pyk2H wt or the kinase-dead mutant Pyk2H-K457A (27). We found that the overexpression of the K457A kinase-dead mutant did not interfere with the translocation of Pyk2 induced by SEE stimulation (Fig. 6, a and b), indicating that Pyk2 activation is not necessary for its redistribution.

Pyk2 activation and reorientation are dependent on the presence of functional ITAMs

ITAM phosphorylation is essential for signaling through the TCR (14, 42). To ascertain the role of ITAM phosphorylation in Pyk2 activation and relocation, we used stable clones of a TCR-negative Jurkat T cell variant (31.13) expressing chimeras of CD8α coupled either to the second ITAM of CD3ζ (CD8b), or the cytoplasmic tail of CD3ε containing or not a functional ITAM (CD8ε-YY and CD8ε-FF, respectively) (Fig. 7a). All these clones expressed comparable amounts of CD8 and associate to Lck through their cytoplasmic tails (43). Although association of Lck to phosphorylated CD3ε ITAM tail from the chimeric receptors is more efficient than to the unphosphorylated tail present in CD8ε-FF chimera (30), cross-linking of CD8 receptors resulted in an equivalent phosphorylation of Lck (Fig. 7b). However, only the stable clones expressing functional ITAMs induced Pyk2 activity (Fig. 7c). Likewise, Pyk2 translocation was induced by anti-CD8-coated latex beads only in those clones bearing functional ITAM (Fig. 7, d and e). These results suggest that Lck activation alone is not sufficient and at least one functional ITAM is necessary to mediate Pyk2 activation and translocation. Since this process is mainly independent of ZAP-70, another phosphorylated ITAM-associated protein would be responsible for the ITAM-mediated effect on Pyk2 reorientation.

Discussion

PTK play a critical role in the TCR-mediated activation of T cells. The nonreceptor tyrosine kinase Pyk2 is phosphorylated during TCR signaling and is involved in T cell activation (22, 25–27). Although the subcellular distribution of kinases is crucial for the regulation of lymphocyte function (1, 5, 36), the localization of Pyk2 during T cell-APC cognate interaction had not been studied to date. In this study, we demonstrate that Pyk2 is translocated toward the vicinity of the immune synapse after TCR triggering. This reorientation is mostly independent of Pyk2 activation and ZAP-70, but requires both functional Lck and the presence of phosphorylated ITAMs.

Our initial results in T cells showed an apparent parallelism between activation and translocation of Pyk2. In this regard, we found that CD28, which has been proved to cooperate with CD3 in Pyk2 activation (27, 40), also induced the relocation of this kinase. However, our dynamic studies revealed that Pyk2 reorientation occurred between 2 and 5 min after the initial interaction of the T cell and the APC, while it has been described that Pyk2 activation occurs as early as 1 min upon TCR triggering (25, 26). These data suggested that activation of Pyk2 is previous to its translocation. Supporting a possible dissociation of Pyk2 activation and relocation, we have observed that some Pyk2-activating stimuli, such as ICAM-1 (39), were not able to induce any change in the subcellular localization of Pyk2. By using two independent experimental approaches, we demonstrated that Pyk2 activation and translocation are independent events. First, we found that only specific inhibitors of Src kinases, among a wide spectrum of compounds that affect Pyk2 activation after TCR engagement, diminished Pyk2 reorientation. Second, JCaM-1 cells, which lack functional Lck, showed Pyk2 activation, but not relocation after TCR triggering.

The analysis of chimeric constructs of CD8 and TCR ITAMs revealed that Lck activation alone, which occurred after receptor engagement in the cell line expressing nonfunctional ITAMs, is not sufficient for Pyk2 activation and reorientation. Instead, the presence of functional ITAMs was necessary for the receptor-mediated Pyk2 activation and translocation. Although ZAP-70 is the main mediator of signaling of T cells through its association to

![FIGURE 6. Pyk2 kinase-dead mutant does not affect Pyk2 translocation.](http://www.jimmunol.org/)

- a. Cell conjugates were formed between SEE-pulsed Raji APCs and the Jurkat stable transfectants expressing Pyk2H wt, and its kinase-dead mutant form K457A. After adhesion to PLL, cells were stained for Pyk2, as stated in Materials and Methods. DIC pictures merged with blue fluorescence images from CMAC-loaded APCs are shown. b. Conjugates between Raji cells and the Jurkat stable transfectants Pyk2H wt and K457A were formed in the presence (solid bars) or absence (open bars) of SEE, adhered to PLL, and stained for Pyk2. Results represent the arithmetic mean ± SD of Pyk2 translocation frequency corresponding to >600 conjugates from three independent experiments. *, p < 0.05, compared with the random reorientation in the absence of SEE (Student’s t test).
FIGURE 7. Pyk2 activation and translocation are dependent on the presence of functional ITAMs. a, Schematic representation of the CD8-ITAM chimeric constructs used in the generation of CD8e-FF, CD8e-YY, and CD8b stable cell lines. b, Upper panels, Parental cells (31.13) or their derived stable cell lines expressing CD8e-FF, CD8e-YY, and CD8b chimeras were stimulated with anti-CD8 (10 µg/ml) 10 min at 4°C and then cross-linked with sheep anti-mouse for 3 min at 37°C. Cells were lysed and immunoprecipitated with anti-Lck Ab. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and Western blot was performed with anti-PYr (4G10). The membrane was then stripped and reblotted for Lck detection. Lower panels, Lck phosphorylation levels are expressed as the arithmetic mean ± SE of the OD relative to the levels of Lck protein detected by Western blot. Results represent three independent experiments. *, p < 0.05, compared with basal activation in the absence of anti-CD8. c, Upper panels, Cells with the same treatment as in b were lysed and immunoprecipitated with anti-Pyk2, and then the in vitro kinase and subsequent processes were performed as in Fig. 5a. Lower panels, Statistical analysis of Pyk2 activation was performed as indicated in Fig. 5a. The results represent three independent experiments. *, p < 0.05, compared with basal activation in the absence of anti-CD3. d, Anti-CD8-coated latex polystyrene beads were allowed to form conjugates with 31.13, CD8e-FF, CD8e-YY, and CD8b cells. After adhesion to PLL, conjugates were stained for Pyk2, as indicated in Materials and Methods. Matched fluorescence and DIC images are shown. e, Quantification of Pyk2 translocation in conjugates between 31.13, CD8e-FF, CD8e-YY, or CD8b cells, and anti-CD8-coated latex beads. Results represent the arithmetic mean ± SD of Pyk2 translocation frequency in 500 to 600 conjugates counted from three independent experiments. *, p < 0.01, compared with the random basal level of translocation in the 31.13 CD8-negative cells.

The crucial role of functional ITAMs in Pyk2 translocation resembles the requirements for MTOC reorientation. We have been unable to detect a direct association of Pyk2 to CD3 ITAMs or even colocalization with CD3 at membrane rafts. However, we have previously found the colocalization of Pyk2 and MTOC in NK cells, and it has been proposed also in T cells. All these data suggest that MTOC and Pyk2 translocation in T cells can be associated. However, in this study, we rule out a significant role for ZAP-70 in Pyk2 translocation, whereas a partial inhibition of MTOC reorientation by a dominant-negative ZAP-70 SH2 (N+C) construct was suggested. This partial inhibitory effect of the SH2 (N+C) ZAP-70 could be due to the occupancy of the phosphorylated ITAMs, in which it could therefore block the binding of any SH2 domain-containing effector molecules capable of binding to the tyrosine-phosphorylated TCR ITAMs. All these data suggest that Lck-dependent translocation of Pyk2 and the MTOC is mainly mediated by signaling protein(s) that binds to phosphorylated TCR ITAMs, but that does not belong to the Syk family of kinases. In addition, since the Ca²⁺ response after TCR stimulation in the ZAP-70-deficient P116 cell line is very poor, our data also suggest the existence of a significant Ca²⁺-independent pathway that regulates Pyk2 and MTOC reorientation.

The use of a Pyk2H-K457A kinase-dead mutant demonstrated that the enzyme activity of Pyk2 is not required for its reorientation in response to superantigen stimulation. The lack of effect of the phosphorylated ITAMs, the specific inhibitor piceatannol did not show a significant effect in TCR-mediated Pyk2 relocation. Accordingly, our analysis of the ZAP-70-deficient Jurkat cell line P116 revealed that the translocation of Pyk2 was only slightly affected. In this regard, a recent report shows that superantigen-induced T cell-APC conjugation mediated by LFA-1 requires signaling through Lck, but not ZAP-70 (44). In this study, it is suggested that Lck would mediate its effect through the direct activation of phospholipase C, independently of ZAP-70. Our results also suggest the existence of a ZAP-70-independent pathway that could regulate Pyk2 translocation.

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kinase-dead mutant and the likely existence of alternative signaling pathways in T cell activation would also explain the apparent lack of a significant phenotype in T cells from the Pyk2-deficient mice (46). However, we have previously found that a high over-expression of Pyk2 inhibits the relocation of Pyk2 and MTOC during the recognition of a specific target by NK cells (36). It is feasible that the biological effect of Pyk2 that is independent of its kinase activity could be mediated by its function as an adapter molecule. In this regard, it has been reported that this kinase-negative mutant undergoes tyrosine phosphorylation to similar levels as Pyk2H wt, suggesting that PTKs(s) other than Pyk2 itself phosphorylates Pyk2 (27). Therefore, possible SH2 and SH3 docking sites would be conserved in this mutant. Pyk2 contains the interacting site (Tyr103) for the SH2 domain of the PTK Fyn, and the canonical binding site (Tyr881) for the SH2 domain of the adapter protein Grb2, in addition to a proline-rich region for binding the SH3 domains of proteins such as Pap or pleckstrin homology and SH3 domain-containing rho GTPase-activating protein (27, 47, 48). Therefore, Pyk2 associates to an array of signaling proteins that could be mediating MTOC complex translocation. In this regard, Fyn association to Pyk2 (25–27) is important to modulate TCR-mediated production of IL-2 (27). Interestingly, it has been recently reported that Pyk2, in concert with Fyn, phosphorylates and inactivates pleckstrin homology and SH3 domain-containing rho GTPase-activating protein, thus activating Cdc42 (48), an important mediator of MTOC translocation (49). Another possible role of MTOC-associated Pyk2 could be the regulation of vesicular sequestration, since an essential component in controlling late sequestration of cytokines has been proposed for MTOC translocation (11, 13). In this regard, the interaction of Pyk2 with Pap (47), which acts as an Arf-GTPase-activating protein, has been described, thus regulating vesicular transport, which could be important for cytokine sequestration. In contrast, the lack of association of Pyk2 to rafts could indicate that Pyk2 is not directly related with the early signaling events induced through the TCR, but is involved in MTOC-dependent processes, either in those early processes that are beginning to be unveiled (50), or in other late well-established processes (11, 13). Whether Pyk2 plays an essential role as an adapter molecule for MTOC translocation or in the control of MTOC-mediated vesicular sequestration is interesting issues that deserve further investigation.

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