Guanine nucleotide exchange factor PIX leads to activation of the Rac 1
GTPase/glycogen phosphorylase pathway in interleukin (IL)-2-stimulated T cells

Llavero, Francisco; Urzelai, Bakarne; Osinalde, Nerea; Gálvez, Patricia; Lacerda, Hadriano
M; Parada, Luis A; Zugaza, José L

Published in:
Journal of Biological Chemistry

DOI:
10.1074/jbc.M114.608414

Publication date:
2015

Document version
Publisher's PDF, also known as Version of record

Citation for published version (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Download date: 08. dec.. 2018
Guanine Nucleotide Exchange Factor αPIX Leads to Activation of the Rac 1 GTPase/Glycogen Phosphorylase Pathway in Interleukin (IL)-2-stimulated T Cells

Francisco Llavero, Bakare Urzelai, Nerea Osinalde, Patricia Gálvez, Hadriano M. Lacerda, Luis A. Parada, and José L. Zugaza

From the †Department of Genetics, Physical Anthropology and Animal Physiology, University of the Basque Country, 48940 Leioa, Spain, the ‡Achucarro Basque Center for Neuroscience, Bizkaia Science and Technology Park, Building 205, 48170 Zamudio, Spain, the §Department of Biochemistry and Molecular Biology, University of Southern Denmark, 5230 Odense, Denmark, the ¶Pharmascience Division, Technological Park of Health Sciences, Avda. de la Ciencia, s/n 18100 Armilla, Granada, Spain, the ‡‡Department of Biomedical Sciences and Human Oncology, Unit of Cancer Epidemiology, Universidad degli Studi di Torino, 10124 Torino, Italy, the ‡§Instituto de Patología Experimental, Universidad Nacional de Salta, 4400 Salta, Argentina, and the §§Ikerbasque, Basque Foundation for Science, Maria Diaz de Haro 3, 48013 Bilbao, Spain

Background: Rac 1 GTPase mediates glycogen phosphorylase activation and controls IL-2-stimulated T cell proliferation. PKCθ activates αPIX by serine phosphorylation and now this Rho-GEF activates Rac 1.

Results: IL-2-stimulated T cells migration and proliferation require the involvement of the PKCθ/αPIX/Rac 1/PYGM pathway.

Significance: This new signaling cascade may be a viable therapeutic target to block the inflammatory response mediated by T cells.

Recently, we have reported that the active form of Rac 1 GTPase binds to the glycogen phosphorylase muscle isoform (PYGM) and modulates its enzymatic activity leading to T cell proliferation. In the lymphoid system, Rac 1 and in general other small GTPases of the Rho family participate in the signaling cascades that are activated after engagement of the T cell antigen receptor. However, little is known about the IL-2-dependent Rac 1 activator molecules. For the first time, a signaling pathway leading to the activation of Rac 1/PYGM in response to IL-2-stimulated T cell proliferation is described. More specifically, αPIX, a known guanine nucleotide exchange factor for the small GTPases of the Rho family, preferentially Rac 1 mediates PYGM activation in Kit 225 T cells stimulated with IL-2. Using directed mutagenesis, phosphorylation of αPIX Rac 1/Rho-GEF serines 225 and 488 is required for activation of the Rac 1/PYGM pathway. IL-2-stimulated serine phosphorylation was corroborated in Kit 225 T cells cultures. A parallel pharmacological and genetic approach identified PKCθ as the serine/threonine kinase responsible for αPIX serine phosphorylation. The phosphorylated state of αPIX was required to activate first Rac 1 and subsequently PYGM. These results demonstrate that the IL-2 receptor activation, among other early events, leads to activation of PKCθ. To activate Rac 1 and consequently PKCθ phosphorylates αPIX in T cells. The biological significance of this PKCθ/αPIX/Rac 1 GTPase/PYGM signaling pathway seems to be the control of different cellular responses such as migration and proliferation.

Co-stimulation of T cell receptor and CD28 T cell receptors lead to IL-2 expression and secretion. An autocrine effect is necessary for expressing the IL-2 receptor (IL-2R) α chain (1, 2) and ultimately to culminate in T cell clonal expansion (3, 4). The IL-2 effect on T cells is not only restricted to the induction of lymphocyte proliferation only but, in the inflammatory response it is also necessary for T lymphocytes differentiation into effector T lymphocytes as well as regulatory T lymphocytes (5).

Binding of IL-2 to its high affinity receptor (IL-2R) drives the activation of a signaling network giving rise to many cellular responses, among them the three major signaling cascades best characterized are the Janus kinase (Jak)/STAT and MAPK pathways, which modulate gene expression and PI3K-mediated cell survival (4). To accomplish these cellular responses, IL-2-dependent T cells possibly require not only activation of these pathways but also a complex cooperation with other signaling networks mediated by tyrosine kinases such as lck, BTK (6), PLCγ (7, 8), and serine/threonine kinases such as protein kinase C family members (9, 10), and some GTPases of the Rho family. In fact, it has been reported that RhoA cooperates with ERK-dependent signaling pathways to transcribe c-fos in response to IL-2 (11). Moreover, Rac 1 has also been found to participate in IL-2-induced actin cytoskeleton rearrangement in a murine T cell line (12). More recently, our group reported that Rac 1 binds and activates the glycogen phosphorylase muscle isoform (PYGM) and thus established a novel metabolic pathway that participates actively in IL-2-stimulated cell proliferation in human T cells (13).

Signals emanating from a large variety of membrane receptors: growth factor receptors (14, 15), G protein-coupled receptors (16, 17), and tyrosine kinases-linked receptors such as TCR

1 Supported by Dept. of Education, Basque Government Grant BFI-2010-184.
3 The abbreviations used are: PYGM, glycogen phosphorylase muscle isoform; RBD, Rho/Rac-binding domain; GEF, guanine nucleotide exchange factor; BCR, B cell receptor; PMA, phorbol 12-myristate 13-acetate; EGFP, enhanced green fluorescent protein.
PKC\(\theta\) Regulates \text{\textalpha} PIX/Rac 1/PYGM Pathway

(5, 18), BCR (19, 20), and IL-2-R (13), actively regulate Rho GTPase effects. Like other small GTPases, Rho GTPases function as molecular switches that cycle between the inactive GDP-bound and the active GTP-bound state. In the active state, GTPases interact with downstream effectors to promote a variety of biological responses, such as control of the appropriate actin cytoskeleton reorganization in response to extracellular signals, and their significant implications in additional biological processes, where gene expression regulation, cell polarity, and cell migration have also been reported (21–23).

The transition between the inactive to the active state is regulated by guanine nucleotide exchange factors (GEFs) (21–23). A key factor in the functioning of small GTPases lies in their selection and regulation of these GEFs. It is well established that upon IL-2/IL-2R ligation, Ras GEF, Son of Seven (Sos), associates to Grb2 and it is recruited through the adapter protein Shc, to the tyrosine-phosphorylated IL-2R \(\beta\) chain. In this configuration, Sos activates Ras and consequently the MAPK pathway (24, 25). Therefore, Sos exchange activity is indirectly regulated by tyrosine phosphorylation. However, the exchange activity of some GEFs of the Dbl family that activates Rac 1 GTPase are directly regulated by phosphorylation. In fact, in the immune system, Vav (Rac 1-specific GEF) must be tyrosine phosphorylated at residue 174 to turn on its GTPase activity (26, 27). Nevertheless, Tiam-1 and STEF, both members of the Tiam GEF family where the former is mainly expressed in the brain and in the immune system and the latter in the brain, are two additional GEFs with higher specificity for Rac 1 (28, 29) that are activated by threonine (30) and serine/threonine phosphorylation (31), respectively. Like Tiam-1, \text{\textalpha} PIX (also known as ARHGEF6 or Cool-2) (32–34), a Rac-GEF primarily expressed in neurons and hematopoietic cells (34), has its exchange activity predicted to be regulated by serine/threonine kinases phosphorylation by phosphoproteomic analysis (35–38). In the last few years, GTPases of the Rac subfamily gained increasing relevance in T cell biology (39, 40). In contrast to its well-established Sos-mediated Ras activation mechanism in IL-2-stimulated T cells, the identity of the Rac GEF responsible for Rac activation in IL-2-stimulated cells has not been determined.

Here we show that subsequent to IL-2 stimulation \text{\textalpha} PIX-Rho-GEF mediates PYGM activation in Kit 225 T cells; an IL-2-dependent human T cell line. Serines 225 and 488 of \text{\textalpha} PIX are critical to active Rac 1 and mediate PYGM activation in IL-2-stimulated cells. By combining pharmacological and genetic approaches, we identified PKC\(\theta\) as the serine/threonine kinase that controls the phosphorylation of these serines and consequently the Rac 1/PYGM axis.

These results reveal that Rac 1/PYGM pathway activation stimulated by IL-2 is achieved through \text{\textalpha} PIX. Furthermore, our results identify PKC\(\theta\) as the intermediary between the activated IL-2/IL-2R complex and \text{\textalpha} PIX. This novel intracellular signaling pathway actively participates in the regulation of the IL-2-stimulated T cell migration and proliferation.

**EXPERIMENTAL PROCEDURES**

Reagents—PKA inhibitor H-89 dihydrochloride, PKC inhibitors Gö6976 and Rottlerin, and PI 3-kinase inhibitor LY29004 hydrochloride were obtained from Sigma. Mouse monoclonal anti-HA antibody was obtained from Covance, mouse monoclonal anti-phosphoserine clone PSR-45 and rabbit monoclonal anti-glutathione \(S\)-transferase (GST) antibodies were from Sigma, mouse monoclonal anti-Rac 1 clone 23A8 antibody was obtained from Millipore, rabbit monoclonal anti-PKC\(\alpha\) was obtained from Cell Signaling, and enhanced chemiluminescence (ECL) reagent was obtained from GE Healthcare. Phorbol 12-myristate 13-acetate (PMA) was obtained from Sigma. MISSION\(\superscript{\text{\textregistered}}\) esiRNA human PRKCQ (gene synonym PKC\(\theta\), reference EH093601), MISSION esiRNA human ARHGEF6 (gene synonym \text{\textalpha} PIX, reference EH133681), and MISSION esiRNA targeting EGFP (reference EHUEGFP) were from Sigma. IL-2 cytokine was provided by the “AIDS Research and Reference Reagent Program,” Division of AIDS (NIAD, National Institutes of Health).

**Cell Culture and DNA/esiRNA Transfections**—Kit 225 T cells were cultured as described by Hori et al. (41) in the presence of 16 units/ml of recombinant human IL-2. For transient transfections, cells were cultured in complete RPMI 1640 medium in the absence of IL-2 for 24 h. Thereafter, cells were washed, resuspended in 200 \(\mu\)l of serum-free medium, and placed in an electroporation cuvette (0.4 mm, Sigma) containing 10–20 \(\mu\)g of different plasmids, or 15 ng of esiRNAs. Electroporation was carried out in a Gene Pulser Xcell Electroporator (Bio-Rad) at 260 V and 950 microfarads (13). The cuvette content was collected into 1 ml of complete RPMI 1640 medium and cultured in the absence of IL-2 for an additional 24 h.

**Agonists and Inhibitors**—Kit 225 T cells were maintained in the absence of IL-2 for 48 h and subsequently stimulated with 500 units/ml of IL-2 at 37 \(^\circ\)C (13). In some experiments, Kit 225 T cells were pretreated with 10 \(\mu\)M H-89 (PKA inhibitor) or 20 \(\mu\)M LY29004 (PI3K inhibitor) or 100 nm Gö6976 and different concentrations of Rottlerin (PKC inhibitors) for 1 h prior to IL-2 or PMA stimulation (13, 42).

**Plasmid Construct and Site-directed Mutagenesis**—The \text{\textalpha} PIX comprising amino acids 204–532 (\text{\textalpha} PIX\(^{204–532}\)) was generated by PCR amplification using pMT2-HA-\text{\textalpha} PIX wt as template (forward oligonucleotide, 5`-CGG GAT CCC GAT CGG TCT CTC-3`) and reverse oligonucleotide 5`-CGG GAT CCT GTC TCA TTC C-3`, and reverse oligonucleotide 5`-CGG GAT CCT GTG CAG TCA TTC C-3`, each harboring BamHI restriction sites (underlined). The BamHI \text{\textalpha} PIX\(^{204–532}\) fragment was subcloned into pGEX-4T3 (GE Healthcare) to generate the GST-\text{\textalpha} PIX\(^{204–532}\) fusion protein. pMT2-2HA-\text{\textalpha} PIX\(^{S488A}\), pMT2-2HA-\text{\textalpha} PIX\(^{S488A}/\text{HA}\) and pMT2-2HA-\text{\textalpha} PIX\(^{S488A}/\text{HA}\) single and double mutated constructs were generated according to manufacturer’s instructions (QuikChange Lightning Site-directed Mutagenesis Kit, Stratagene). Oligonucleotides used for S225A mutation were: 5`-GAG AGA CCT CTC GCC CCA AAA GCC GTC-3` (forward) and 5`-GAC GGC TTT TGG GGC GAG AGG TCT CTC-3` (reverse) and for S488A mutation were: 5`-AGT CCT CGG ATG GCT GGT ATT ATC TAT-3` (forward) and 5`-ATA GAT AAA GCC AGC CAT CCG AGG ACT-3` (reverse).

**Activity Assay for Glycogen Phosphorylase**—The glycogen phosphorylase activity assay was performed as previously described (43, 44) with some modifications. Briefly, cells were washed twice with cold PBS and resuspended in 500 \(\mu\)l of TES.
buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 225 mM sucrose, 2.5 mM DTT, 0.1 mM PMSE, 1 µg/ml of leupeptin, 1 µg/ml of aprotinin). Samples were sonicated and centrifuged at 12,300 × g for 10 min at 4 °C. Total protein (100 µg) was used to measure PYGM activity in assay buffer (50 mM KH₂PO₄, pH 7.5, 10 mM MgCl₂, 5 mM EDTA, pH 8, 0.5 mM NADP⁺, 1.5 units/ml of glucose-6-phosphate dehydrogenase, 1 unit/ml of phosphoglucomutase, 0.1 mg/ml of glycogen (all from Sigma)). Assay buffer containing 300 µl of TES without NADP⁺, glycogen, phosphoglucomutase, and glucose-6-phosphate dehydrogenase was added to 100 µg of total protein as a blank control. To carry out the metabolic activity assay the mixture was incubated at 37 °C for 20 min. By placing samples on ice the reaction was stopped. Sample absorbances were detected at 340 nm in a spectrophotometer (Ultrospex 3100 pro, Amershams Biosciences). The amount of NADPH formed was determined using a standard curve of known NADPH concentrations (Sigma).

Rac 1 Activation Assay—Rac 1 pulldown assay was performed using a GST fusion protein containing the Rac 1 binding domain of PAK1 (GST-RBD-PAK1). Transfected and untransfected cells kept in the absence of IL-2 for 48 h were stimulated with IL-2 for 10 min and lysed as described in Ref. 45. Cell lysates were centrifuged at 12,300 × g for 10 min at 4 °C and incubated for 1 h at 4 °C with 50 µg of GST-RBD-PAK1 fusion protein coupled to glutathione-Sepharose beads. Precipitated proteins were eluted from beads using 2× loading buffer (12 mM Tris, pH 6.8, 5% glycerol, 0.4% SDS, 140 mM 2-mercaptoethanol, 0.02% bromphenol blue), separated by SDS-PAGE, and analyzed by immunoblots with specific monoclonal antibodies. Immunoreactive bands were visualized using anti-phosphoserine antibody and ECL.

Immunoprecipitation Assay—Transfected Kit 225 T cells with cDNA encoding for pMT2-HA-αPIX or empty vector (pMT2-HA) or esiRNAs to knock down PKCθ as indicated were treated or not with 500 units/ml of IL-2 for 10 min. Cells were washed three times in ice-cold PBS and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Igepal, 0.25% Na-deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM Na₃VO₄, 1 mM NaF, 1 µg/ml of aprotinin, 1 µg/ml of leupeptin). Ectopic HA-αPIX and endogenous αPIX were immunoprecipitated for 2 h at 4 °C using anti-HA or anti-αPIX antibodies. Immune complexes were recovered using Gamma Bind Plus-Sepharose beads (GE Healthcare, Pittsburgh, PA), washed, and eluted from beads and resolved electrophoretically by SDS-PAGE and analyzed by Western blot with anti-phosphoserine, anti-HA, or anti-αPIX antibodies. Immunoreactive bands were visualized using ECL.

In Vitro Kinase Assay—Kit 225 T cells transfected with PKCθ (wild type), PKCθΔ¹⁴⁸⁶ (dominant-negative), and/or the constitutively active forms of PKCa (PKCaΔ²⁵⁶), PKCe (PKCeΔ¹³⁹⁸), and PKCθ (PKCθΔ¹⁴⁸⁶) were incubated in the presence or absence of 500 units/ml of IL-2 for 10 min at 37 °C and washed twice with cold PBS. Thereafter, cells were lysed with lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 10 µg/ml of aprotinin, 1 mM PMSF, 10% glycerol, and 1% (v/v) Triton X-100) and lysates were clarified by centrifugation for 10 min at 12,300 × g at 4 °C. PKCs were immunoprecipitated with specific antibodies and immunocomplexes were recovered using Gamma Bind Plus-Sepharose beads (GE Healthcare). The immunocomplexes were washed twice with cold lysis buffer, twice with cold washing buffer (100 mM HEPES, pH 7.4, 100 mM NaCl, 20 µg/ml of aprotinin, and 0.5% Igepal-360) and twice with reaction buffer (20 mM Tris, pH 7.4, 20 mM NaCl, 1 mM DTT, 10 mM MgCl₂, and 1 mM MnCl₂). 500 ng of purified recombinant GST-αPIX²⁰⁴–₅₃₂, which encompasses the two potential serine phosphorylation sites (Ser²²⁵ and Ser⁴⁸⁸) of αPIX (33), and 20 µM ATP was then added to the reaction mixture. The in vitro kinase reaction was carried out for 30 min at 30 °C after which it was stopped by adding 30 µl of 2× loading buffer. Proteins were separated by SDS-PAGE, followed by Western blot. Immunoreactive bands were visualized with anti-phosphoserine antibody and ECL.

Cell Migration Assay—esiRNA-transfected cell suspensions (2.5 × 10⁶ cells in a 100-µl volume) were placed into the upper chamber, whereas 600 µl of medium with or without IL-2 (500 units/ml) was introduced into the lower chamber. Both chambers were incubated overnight at 37 °C in 5% CO₂ and 95% air. Cells in the upper and bottom chamber were recovered separately into equal volumes for cell counting. The percentage of migrating cells was determined as follows: [number of cells migrating (lower chamber)/total number of cells (cells in the lower chamber + remaining cells in the upper chamber)]. Assay was performed using pore filters (8 µm, Corning® Costar Transwell and cell culture inserts were from Sigma) and cell counts were done in triplicate.

Cell Proliferation Measurement—esiRNA-transfected cells were seeded in 24-well plates in complete RPMI (10⁶ cells/ml), and maintained in the absence of IL-2 for 48 h. Subsequently, cells (10⁶) were incubated with 4 µM PKH26 following the manufacturer’s instructions (Sigma). A sample (10⁴ cells) was taken as the start control, another sample (10⁴ cells) was left untreated, and the remaining cells were incubated in the presence of IL-2 (16 units/ml). Fluorescence was measured every 24 h for 3 days to the monitor cell division rate on a FACSCalibur (BD Biosciences) flow cytometer. Data obtained were analyzed using ModFit LT 3.0.

Statistical Analysis—Student’s t test for the mean of two-paired samples was used to determine the significance between data means (**, p < 0.05; ***, p < 0.001).

RESULTS

αPIX-Rho-GEF Leads to PYGM Activation—To test if αPIX could be a link between IL-2 receptor and Rac 1 leading to the activation of PYGM, Kit 225 T cells were transfected with pMT2-HA or pMT2-HA-αPIX and stimulated or not with 500 units/ml of IL-2 for 10 min, lysed, and PYGM activity was determined as described under “Experimental Procedures.” As shown in Fig. 1A (first closed bar), IL-2 stimulated robust PYGM activity of empty vector-transfected Kit 225 T cells. This IL-2 stimulation of PYGM activity was already maximal and it was not further increased in αPIX-overexpressing cells (Fig. 1A, second closed bar compared with first closed bar). However, αPIX overexpression in the absence of IL-2 resulted in a significant increase of the PYGM activity when compared to control cells (Fig. 1A, second empty bar compared with first empty bar). Western blot shows αPIX expression levels (Fig. 1A, upper panel).
PKCδ Regulates αPIX/Rac 1/PYGM Pathway

FIGURE 1. IL-2 stimulates glycogen phosphorylase activity in Kit 225 T cells through αPIX Rho-GEF. Kit 225 T cells deprived of IL-2 for 24 h were transfected with plasmids coding for: A, pMT2-HA (empty vector) and pMT2-HA-αPIX; B, pcDNA3-HA (empty vector), pEF-Vav, pcDNA3-HA-Tiam-1, and pcDNA3-HA-STEF; and C, αpix(esRNA) and egfp(esRNA). As a control, 24 h post-transfection cells were stimulated (+) or not (−) with 500 units/ml of IL-2 for 10 min and lysed. Cell extracts from unstimulated or stimulated cells were used to measure glycogen phosphorylase activity, as described under “Experimental Procedures.” Western blot (W.B.) analysis of protein expression levels was carried out using specific antibodies, as indicated. Results show the mean of three independent experiments ± S.D. and statistical analysis shows a significant difference (***, p < 0.001).

Next, we aimed to determine whether other GEFs such as Vav, Tiam-1, and/or STEF/Tiam-2 that preferentially activate Rac 1 could also modulate the activity of the PYGM in our cellular system. For that purpose, following the same procedure described above, Kit 225 T cells were transfected with pcDNA3-HA (empty vector), pEF-Vav, pcDNA3-HA-Tiam-1, and pcDNA3-HA-STEF, and 24 h post-transfection PYGM activity was determined in Kit 225 T cells unstimulated and stimulated with IL-2 for 10 min. As expected, IL-2 significantly increased PYGM activity compared with unstimulated cells (Fig. 1B, first and second bars). This maximal increase in the PYGM activity induced by IL-2 also occurred in the presence of Vav and TEF (Fig. 1B, fourth and eighth bars compared with the second bar). However, in contrast to the αPIX outcome, Vav or STEF overexpression per se did not modify PYGM activity (Fig. 1B, third and seventh bars). In sharp contrast, Tiam-1 overexpression dramatically blocked PYGM activity (Fig. 1B, sixth bar). Western blots show Vav, STEF, and Tiam-1 expression levels (Fig. 1B, upper panel).

Finally, to corroborate that αPIX was specifically regulating the glycogen phosphorylase activity in IL-2-stimulated T cells, αpix was knocked down in Kit 225 T cells. To this end, Kit 225 T cells were transfected with esiRNA human ARHGEF6 (αPIX) or esiRNA targeting EGFP, as negative control. 24 h post-transfection PYGM activity was determined in Kit 225 T cells unstimulated and stimulated with IL-2 for 10 min. As shown in Fig. 1C, IL-2 stimulated robust PYGM activity in egfp(esRNA)-transfected Kit 225 T cells. αpix knockdown (αpix(esRNA)) cells stimulated by IL-2 for 10 min did not show any PYGM activity (Fig. 1C, second closed bar compared with first closed bar). Furthermore, in the absence of IL-2 αpix knockdown, cells also displayed no PYGM activity, in contrast to αPIX overexpressing cells (Fig. 1C, second empty bar compared with A, second empty bar). Small aliquots of cell lysate from each condition were stored, electrophoretically resolved by SDS-PAGE, and followed by Western blot. Immunoreactive bands were visualized with specific antibodies as indicated. Fig. 1C, first and second panels show αPIX expression levels after esiRNA transfection and tubulin, respectively. The γ-tubulin Western blot result shows that an equivalent amount of protein was used in each of the conditions analyzed.

Rac 1/PYGM Pathway Activation Depends on the Integrity of αPIX Serine 225 and 488 Residues—To demonstrate that αPIX functions as a Rac-activating molecule in Kit 225 T cells, αPIX was knocked-down with αpix(esRNA), as we described above and the endogenous active Rac 1 was measured by the pulldown assay. As shown in Fig. 2A, in the absence of αPIX (knockdown), IL-2 was unable to stimulate Rac 1 activation (fourth lane compared with second lane). αPIX expression levels in the presence of esiRNA control (egfp) or αpix(esRNA) were determined by Western blot (Fig. 2A, upper panel). Rac 1 detected in whole cell lysates shows that the total loaded proteins are equivalent in all lanes (Fig. 2A, third panel).

Directed mutagenesis was used to investigate a putative role of serine 225 and 488 residues in activation of the Rac 1/PYGM pathway. To this end, the following single αPIX mutants were
PKC\(\theta\) Regulates \(\alpha\)PIX/Rac 1/PYGM Pathway

using the fusion protein GST-RBD of PAK 1 and visualized as described under “Experimental Procedures.” As shown in Fig. 2B (first panel), IL-2 stimulated Rac 1 activation (lane 2) in control transfected cells. \(\alpha\)PIX wt overexpression induced massive Rac 1 activation, which was not further increased by IL-2 stimulation (Fig. 2B, upper panel, lanes 3 and 4). In contrast, both \(\alpha\)PIX single and double mutants dramatically blocked Rac 1 activation stimulated by IL-2 (Fig. 2B, upper panel, sixth, eighth, and tenth lanes). Furthermore, overexpression of these \(\alpha\)PIX mutants in unstimulated cells did not induce any Rac 1 activation as it was observed with the \(\alpha\)PIX wild type form (Fig. 2B, upper panel, fifth, seventh, and ninth lanes compared with third lane).

Next, the effect of these \(\alpha\)PIX mutants on PYGM activity was also examined. As shown in Fig. 2C, IL-2-stimulated PYGM maximal activity of both empty vector and \(\alpha\)PIX wt transfected Kit 225 T cells (Fig. 2C, second and fourth bars, respectively). \(\alpha\)PIX overexpression without IL-2 stimulation also induced a significant increase in PYGM activity in comparison to control cells (Fig. 2C, third bar compared with first bar). In contrast, PYGM activity was completely blocked by both the single serine and double serine mutants with or without IL-2 stimulation (Fig. 2C, fifth right bars).

nPKCs Regulate Rac 1/PYGM Pathway Activation—To search for additional kinases also involved in IL-2 early signaling leading to PYGM activation, the possible involvement of PKA, PI3K, and/or PKCs was examined. To this end, Kit 225 T cells deprived of IL-2 for 48 h were pretreated for 1 h with vehicle, 10 \(\mu\)M H-89 (a PKA inhibitor), 20 \(\mu\)M LY294002 (a specific inhibitor of PI3K), 100 \(\mu\)M Gö6976 (an inhibitor of classical PKCs, mainly \(\alpha\) and \(\beta\)), or 5 \(\mu\)M Rottlerin (which was initially described as a selective inhibitor of the novel PKC isoform \(\delta\) (46)) and was subsequently described to inhibit also PKC\(\theta\) (47, 48), followed by stimulation or not with 500 units/ml of IL-2 for 10 min. PYGM activity was determined as described above. Inhibition of PKA, PI3K, or classic PKCs did not affect PYGM activity stimulated by IL-2 (Fig. 3A, fourth, sixth, and eighth bars compared with second bar); notwithstanding, Rottlerin efficiently blocked PYGM activity stimulated by IL-2 (Fig. 3A, tenth bar) in a concentration-dependent manner (Fig. 3B). 2.5 \(\mu\)M Rottlerin was the minimal concentration leading to the maximal blockage (Fig. 3B).

To verify a putative connection between nPKC and PYGM in Kit 225 T cells, PMA, a natural DAG analog, was used to directly activate PKC. Kit 225 T cells deprived of IL-2 for 48 h were stimulated with 500 units/ml of IL-2 for 10 min or 1 \(\mu\)M PMA for 15 min. PMA was able to stimulate maximal PYGM activity; a response equivalent to the one produced by IL-2 (Fig. 3C, second and third bars). Accordingly, IL-2- and PMA-stimulated PYGM activity was blocked in Rottlerin (2.5 \(\mu\)M, for 1 h) pretreated cells (Fig. 3C, fifth and sixth bars). Next, the impact of PKCs inhibition on Rac 1 activation stimulated by IL-2 was examined. Results presented here confirmed that IL-2 stimulation correlated with an increase of the Rac 1 active form (Fig. 3D, first panel, second lane). IL-2 stimulated Rac 1 activation was unaffected when classical PKCs were inhibited by 100 \(\mu\)M Gö6975 (Fig. 3D, first panel, fourth lane). However, 2.5 \(\mu\)M Rottlerin almost completely blocked IL-2-stimulated Rac 1 activa-

---

**FIGURE 2. IL-2-stimulated Rac 1/PYGM pathway activation requires intact serine residues 225 and 488 of \(\alpha\)PIX-Rho-GEF.** Kit 225 T cells deprived of IL-2 for 24 h were transfected with \(\alpha\)PIX wt (\(\alpha\)PIX), \(\alpha\)PIX wt (\(\alpha\)PIX), or \(\alpha\)PIX wt (\(\alpha\)PIX) and \(\alpha\)PIX wt (\(\alpha\)PIX). 24 h post-transfection cells were stimulated (+) or not (−) with 500 units/ml of IL-2 for 10 min and lysed. A and B, cell lysates were used to measure Rac 1 activation by affinity precipitation assay. Precipitated active Rac 1 (Rac1-GTP, total Rac1), and \(\alpha\)PIX expression levels of the wild type and the mutant forms were analyzed by Western blot using anti-Rac1 and anti-HA antibodies, respectively. Results are representative of four independent experiments. C, cell extracts from unstimulated and stimulated cells were used to measure glycogen phosphorylase activity, as described under “Experimental Procedures.” Western blot (W.B.) analysis of protein expression levels was carried out using specific anti-HA antibody. Results show the mean of three independent experiments ± S.D. and statistical analysis shows a significant difference (***, \(p < 0.001\)).

---

generated: \(\alpha\)PIX\(^{225A}\), \(\alpha\)PIX\(^{488A}\), and the \(\alpha\)PIX double mutant, \(\alpha\)PIX\(^{225A,488A}\). The effects of these \(\alpha\)PIX mutants on IL-2-stimulated Rac 1 activation were examined in Kit 225 T cells previously transfected with \(\alpha\)PIX wt, \(\alpha\)PIX\(^{225A}\), \(\alpha\)PIX\(^{488A}\), \(\alpha\)PIX\(^{225A,488A}\), or the empty vector (pMT2- HA) and stimulated or not with 500 units/ml of IL-2 for 10 min. The active form of Rac 1 present in whole cell lysates was pulled down.
PKCδ Regulates αPIX/Rac1/PYGM Pathway

FIGURE 3. nPKCs regulate Rac1/PYGM activation in IL-2-stimulated T cells. A−C, Kit 225 T cells were treated with inhibitors or vehicle (dimethyl sulfoxide) for 1 h and subsequently unstimulated (empty bars) or stimulated (closed bars) with 500 units/ml of IL-2 for 10 min (A and B) or 1 μM PMA for 15 min (C) as indicated. Cells were lysed and extracts were used to measure glycogen phosphorylase activity. Results show the mean of three independent experiments ± S.D., and statistical analysis shows a significant difference (***, p < 0.001). D and E, Kit 225 T cells were treated with PKC inhibitors or vehicle (dimethyl sulfoxide) for 1 h and stimulated or not with 500 units/ml of IL-2 for 10 min (D) or 1 μM PMA for 15 min (E) as indicated and lysed. Cell extracts were used to measure Rac1 activation by affinity precipitation assays. Precipitated active Rac1 (Rac1-GTP) and total Rac1 from cell lysates were analyzed by Western blot (W.B.) using anti-Rac1 specific antibody. Results are representative of three independent experiments.

To further characterize the involvement of nPKCs in signaling pathways stimulated by IL-2 leading to Rac1/PYGM activation, Kit 225 T cells were transiently transfected with pcDNA3 (empty vector) or with cDNAs encoding the α, ε, and δ constitutively active isoforms of PKCs for 24 h, and Rac1 activation was analyzed. As shown in Fig. 4A, transfection of the constitutively active form of PKCδ activated Rac1 to a level comparable with those obtained by IL-2 stimulation. The potent stimulating effect of PKCδ was specific given that neither activated PKCα nor PKCε had any effect on Rac1 activation. In agreement with this neither PKCα nor PKCε increased the basal level of PYGM activity, whereas constitutively active PKCδ induced PYGM activity to levels comparable with that stimulated by IL-2 (Fig. 4B).

To confirm the involvement of PKCδ in the Rac1/PYGM pathway, Rac1 and PYGM activation was determined in Kit 225 T cells overexpressing PKCδ (wt), PKCδA148E (constitutively active form of PKCδ), and PKCδK409R (dominant-negative form of PKCδ) with or without IL-2 stimulation. First, immunoblotting showed that all forms of PKCδ were equally expressed (Fig. 4, C, third panel, and D, upper panel). As shown in Fig. 4C, transfection of the PKCδ constitutively active form was found to activate Rac1 to levels comparable with those stimulated by IL-2. In fact, IL-2 stimulation of cells overexpressing PKCδ (wt) or PKCδA148E did not increase Rac1 activation any further (Fig. 4C, fourth and sixth lanes compared with second). In contrast, transfection with PKCδK409R completely abolished Rac1 activation either with or without IL-2 stimulation (Fig. 4C, seventh and eighth lanes). Similar results were obtained when PYGM activity was measured. PKCδA148E overexpression alone was enough to stimulate maximal PYGM activity; equivalent to PYGM activity stimulated by IL-2 (Fig. 4D, fifth bar compared with second, fourth, and sixth bars). Finally, PKCδK409R overexpression completely blocked PYGM activity either with or without IL-2 stimulation (Fig. 4D, seventh and eighth bars).

To confirm results obtained with PKCδ demonstrating that this serine-threonine kinase functions as a Rac1/PYGM-activating molecule in Kit 225 T cells, PKCδ was knocked down with pckδ esiRNA, as described above. The endogenous Rac1 active state was measured by the pulldown assay. As shown in Fig. 4E, in the absence of PKCδ (knockdown) IL-2 stimulation was unable to induce Rac1 activation (fourth lane compared with second lane). The PKCδ expression level in cells transfected with esiRNA control (egfp) or pckδ(esiRNA) was determined by Western blot (Fig. 4E, upper panel). Rac1 detected in whole cell lysates shows that the loaded proteins were equivalent in all lanes (Fig. 4E, third panel). When PYGM activity in
PKCθ Regulates αPIX/Rac 1/PYGM Pathway

PKCθ knockout Kit 225 T cells stimulated with IL-2 was examined, it was observed in the absence of PKCθ IL-2 was unable to stimulate PYGM activation (Fig. 4F). Small aliquots of whole cell lysate from each condition were stored, electrophoretically separated on SDS-PAGE, and followed by Western blot. Immunoreactive bands were visualized with specific antibodies as indicated. Fig. 4F (first and second panels) shows PKCθ expression levels after esiRNA transfection and γ-tubulin, respectively. γ-Tubulin blot analysis indicates that equivalent amounts of protein were used in SDS-PAGE analysis.

PKCθ Controls αPIX Phosphorylation in Vivo and in Vitro—To test whether or not nPKCs could phosphorylate αPIX serine residues when Kit 225 T cells were stimulated by IL-2, HA-αPIX overexpressing cells were pretreated or not with 2.5 μM Rottlerin for 1 h, and stimulated or not with 500 units/ml of IL-2 for 10 min. HA-αPIX present in cell lysates was immunoprecipitated using anti-HA antibody, as described under “Experimental Procedures.” As shown in Fig. 5A (first left panel, fourth lane), IL-2 stimulated αPIX serine phosphorylation, which was blocked by Rottlerin (Fig. 5A, first right panel, fourth lane). Given that these experiments were performed in αPIX overexpressing cells, endogenous αPIX phosphorylation was also examined. Kit 225 T cells were transfected with pckθ (esiRNA) to knockdown PKCθ and cell lysates were immunoprecipitated using anti-αPIX antibody, as described under “Experimental Procedures.” As shown in Fig. 5B (fourth lane 4 compared with second lane), in the absence of PKCθ expression, IL-2 was unable to induce αPIX serine phosphorylation. To determine the amount of immunoprecipitated αPIX, membranes that were used to examine the αPIX serine phosphorylation were stripped and reblotted with anti-αPIX antibody (Fig. 5B, second panel). PKCθ expression levels in the presence of esiRNA control (egfp) or pckθ (esiRNA) present in whole cell lysates were also examined by SDS-PAGE and followed by Western blot. Immunoreactive bands were visualized using anti-PKCθ antibody (Fig. 5B, third panel). γ-Tubulin shows that
PKCα Regulates αPIX/Rac 1/PYGM Pathway

total protein was equivalent in all assay conditions (Fig. 5B, fourth panel). These results suggest that αPIX serine phosphorylation stimulated by IL-2 requires PKCα protein expression.

As shown in Fig. 2, αPIX serine residues 225 and 488 are required for Rac 1 and PYGM activation in IL-2-stimulated Kit 225 T cells. Therefore, to determine whether or not PKCα could phosphorylate the αPIX region comprising both serines, an in vitro kinase assay was performed using a GST-αPIX204–532 fusion protein as an exogenous substrate for PKCα. The GST moiety was fused to the αPIX region spanning from 204 to 532 residues, as described under “Experimental Procedures.” Briefly, to carry out the in vitro kinase assay PKCα (constitutive active form), PKCα (wild type and mutants) were immunoprecipitated from cell lysates derived from stimulated or unstimulated Kit 225 T cells and immunocomplexes were incubated with ATP and GST-αPIX204–532. Subsequently, proteins were resolved by SDS-PAGE followed by Western blot and GST-αPIX204–532 serine phosphorylation was visualized using an anti-phosphoserine antibody. As illustrated in Fig. 5C, PKCα (constitutively active) induced robust GST-αPIX204–532 serine phosphorylation and it was not modified by IL-2 stimulation (fifth and sixth lanes). However, serine phosphorylation was not detectable either in the presence of PKCα (dominant-negative) or PKCε (Fig. 5C, from first to fourth lanes).

To confirm PKCα involvement in the αPIX204–532 region serine phosphorylation, Kit 225 T cells were transiently transfected with pcDNA3 (empty vector) or with cDNAs encoding PKCα (wt), PKCα (constitutively active form), and PKCα (dominant-negative form). Equal expression levels of these PKCα forms in Kit 225 T cells were confirmed by immunoblotting (Fig. 5D). As shown in Fig. 5D (second lane), IL-2 stimulates GST-αPIX204–532 serine phosphorylation in Kit 225 T cells overexpressing PKCα (wt). When the effect of PKCα A148E overexpression on GST-αPIX204–532 phosphorylation was examined, a stronger level of exogenous substrate phosphorylation was observed than that found in cells overexpressing PKCα (wt) and stimulated by IL-2 (Fig. 5D, third lane compared with second lane). IL-2 stimulation of PKCα A148E overexpressing cells did not increase the phosphorylation level further than that of unstimulated PKCα A148E overexpressing cells (Fig. 5D, third and fourth lanes). In contrast, transfection with PKCα K409R (PKCα, dominant-negative) completely blocked GST-αPIX204–532 phosphorylation independently of IL-2 stimulation (Fig. 5D, fifth and sixth lanes).

PKCα and αPIX Are Needed for IL-2-induced Chemotaxis and Proliferation of Kit 225 T Cells—To investigate the role of PKCα and αPIX in IL-2-stimulated Kit 225 T cell migration, cells were transfected with egfp (as control), pkcα, or αpix (esiRNAs) and their effects on the IL-2-induced T cell chemotaxis through polyethylene terephthalate membranes were examined. As shown in Fig. 6A, IL-2 stimulated robust migration of Kit 225 T cells (first solid bar). On the other hand, lack of either PKCα or αPIX expression abolished Kit 225 T cells migration stimulated by IL-2 (Fig. 6A, second and third solid bars). At the end of the experiment, cells in the upper and lower chambers from each transfected condition were mixed. Cells were lysed and whole cell lysates were separated by SDS-PAGE followed by Western blot. The expression levels of αPIX and PKCα were visualized using specific antibodies as indicated in Fig. 6A (first and second panels). γ-Tubulin Western blot analysis shows that equal amounts of protein were used (Fig. 6A, third panel).

FIGURE 5. αPIX serine phosphorylation depends on PKCα in intact Kit 225 T cells. Kit 225 T cells deprived of IL-2 for 24 h were transfected with (A), empty vector (pMT2-HA) or pMT2-HA-αPIX (wt), and (B) with egfp (esiRNA) and pkcα (esiRNA). A, 24 h post-transfection an aliquot of cells were pretreated with 2.5 μM Rottlerin or vehicle (dimethyl sulfoxide) for 1 h. Subsequently, cells were stimulated or not with 500 units/ml of IL-2 for 10 min and lysed. B, 24 h post-transfection cells were stimulated or not with 500 units/ml of IL-2 for 10 min and lysed. Cell lysates were subjected to immunoprecipitation with anti-HA (A) or anti-αPIX (B) antibodies and immunoreactive bands were visualized using anti-phosphoserine, anti-HA, anti-αPIX, anti-PKCα, and anti-γ-tubulin antibodies. Results are representative of three independent experiments. C, Kit 225 T cells deprived of IL-2 for 24 h were transfected with empty vector or with the constitutively active mutants of PKCα, PKCε, and PKCθ. D, Kit 225 T cells deprived of IL-2 for 24 h were transfected with cDNAs encoding PKCα (wt), PKCα A148E (constitutively active form), and PKCα K409R (dominant-negative form). 24 h post-transfection cells were stimulated or not with 500 units/ml of IL-2 for 10 min and lysed. Cell extracts were immunoprecipitated with anti-PKCα and anti-HA antibodies (C) and anti-HA antibody (D). Immunocomplex activities were analyzed by an in vitro kinase assay followed by SDS-PAGE and Western blot. Immunoreactive bands were visualized using anti-phosphoserine (pS) antibodies. In addition, to determine the amount of GST-αPIX204–532 aliquots of lysates were analyzed by Western blot (W.B.) using anti-GST antibody. The expression levels of PKCα A148E, PKCα A159E, PKCα (wt), PKCα A148E, and PKCα K409R were visualized using anti-PKCα to detect PKCα A25E and anti-HA antibody to detect PKCα. Results are representative of three independent experiments.
To evaluate the role of PKCθ and αPIX in IL-2-stimulated cell proliferation, esiRNAs for egfp, pkcθ, and apix were used. Cell proliferation was analyzed after flow cytometry by monitoring the decrease in fluorescence of dye PKH6 incorporated in cell membrane, which is diluted approximately 2-fold with each cell division. PKH26-labeled cells were treated with 16 units/ml of IL-2 every 24 h for 3 days. In control cells (egfp (esiRNA) transfected cells) cultured for 3 days, IL-2 stimulation resulted in approximately a 2-fold increase in cell number (Fig. 6B, closed circles) compared with IL-2-unstimulated cells (Fig. 6B, open circles). Remarkably, IL-2-stimulated cell proliferation was significantly reduced with either pkcθ (esiRNA) (Fig. 6B, closed triangles) or with apix(esiRNA) (Fig. 6B, closed diamonds) knockdown. At the beginning of the experiment, 3 × 10⁵ cells from each transfected condition were taken and lysed. Total protein in lysates was separated by SDS-PAGE followed by Western blot. The expression level of αPIX and PKCθ was visualized using specific antibodies as indicated in Fig. 6B (first and second panels). γ-Tubulin Western blot analysis indicates that equal amounts of protein were used in the analysis (Fig. 6B, third panel).

**DISCUSSION**

Small GTPases of the Rho family actively participate in the immune response after antigenic stimulation, allowing for appropriate actin cytoskeleton reorganization and regulation of transcription factors activity (53). Cooperation between transcription factors such as NFAT, NF-κB, and JNK is key to guarantee an adequate T cell clonal proliferation; in part by regulating transcription of IL-2 and the α chain of the IL-2Rα (49). Recently, we reported that upon IL-2R activation in Kit 225 T cells, the Rac 1 GTPase active form binds to and activates the metabolic enzyme PYGM, leading to cell proliferation (13). In the present study, we show that the IL-2/IL2-R engagement signals to the Rac 1/PYGM pathway through PKCθ and the GEF αPIX. More importantly, we identify αPIX as a Rac 1-specific GEF in IL-2-stimulated T cells and provide novel evidence demonstrating that αPIX requires serine phosphorylation by PKCθ to control the Rac 1/PYGM pathway, and thereby regulate T cell migration and proliferation.

Signals emanating from membrane receptors such as TCR, BCR, and IL-2R leading not only to the activation of protein-tyrosine kinases, but also of serine/threonine kinases, can positively regulate downstream effector molecules, including small GTPases of the Ras superfamily (18, 50). However, little is known about which Rho-GEF activates Rho GTPases after IL-2 receptor activation. Kit 225 T cells express IL-2R constitutively and depend exclusively on IL-2 for cellular proliferation (41). This feature represents an important advantage for IL-2-stimulated signaling studies and this cellular system has emphasized the importance of RhoA (51) and Rac 1 (12, 13) in T cell biology regulated by IL-2.

Given that tyrosine phosphorylation of cellular proteins is one of the most important and characteristic events in early cell signaling stimulated by IL-2, Osinalde et al. (35) used high resolution mass spectrometry, combined with phosphotyrosine immunoprecipitation and stable isotope labeling by amino acids in cell culture (SILAC) to identify 172 tyrosine-phosphorylated target proteins; among which is αPIX-RhoGEF. This result prompted us to examine the potential participation of αPIX in PYGM activation. Overexpression experiments with αPIX demonstrated a significant increase in PYGM activity in the absence of any stimuli. αPIX overexpression promotes its own spontaneous dimerization in the absence of any stimuli, and in this configuration αPIX activates Rac 1 (52). Furthermore, in the presence of IL-2, αPIX-transfected cells reached a maximum level of PYGM activity. However, these results do not exclude the possibility that other GEFs capable of undergoing tyrosine phosphorylation may be involved in this signaling

---

**FIGURE 6. PKCθ/αPIX pathway mediates IL-2-stimulated Kit 225 chemotaxis and proliferation.** A, esiRNA (egfp, pkcθ, apix) transfected Kit 225 T cells migration was studied in a Transwell assay. Data representing the percentage of migrating cells are expressed as the mean of three independent experiments ± S.D. and statistical analysis shows a significant difference (**, p < 0.001). At the end of the assay, cells were recovered, lysed, and cell lysates were analyzed by Western blot (W.B.) using specific antibodies as indicated. B, esiRNA (egfp, pkcθ, αpix)-transfected Kit 225 T cells were stained with PKH26. Fluorescence was analyzed before IL-2 stimulation (− IL-2 (0 h) and after every 24-h incubation with IL-2 (+ IL-2) for 3 consecutive days. Results represent the mean of three independent experiments ± S.D. and the statistical analysis showed a significant difference (**, p < 0.05 and ***, p < 0.001). 3 × 10⁵ Kit 225 T cells were taken for each transfection condition, lysed, and cell lysates were analyzed by Western blot using specific antibodies as indicated.
PKCθ Regulates αPIX/Rac 1/PYGM Pathway

pathway stimulated by IL-2. In fact, following this hypothesis we proposed that Rac 1 might also require Vav Rho-GEF to bind to and activate PYGM. Vav is the main GEF for Rac 1 in the hematopoietic system and its GEF function targeting the Rho family of GTPases is modulated by tyrosine phosphorylation at residue 174 (26, 27). Evans et al. (53) also reported that in peripheral blood lymphocytes, Vav is tyrosine phosphorylated after IL-2 stimulation. However, in Vav overexpressing Kit 225 T cells there is no increase of PYGM activity. In our view, this was quite an expected result. This is because specifically Vav Tyr174 must be phosphorylated (26, 27) to release its DH domain from its inhibitory configuration and thus gain access to and activate Rac 1 (27). When the effect of IL-2 on Tyr174 phosphorylation was examined, it was observed that this cytokine was not able to stimulate Tyr174 phosphorylation (data not shown). These results do not contradict the observations reported by Evans et al. (53), given that this group described general tyrosine phosphorylation of Vav occurring mostly in its SH2 domain and located at Vav carboxy-terminal region; whereas, Tyr174 is located between Vav CH and DH domains at the amino-terminal region (26, 27).

From the start our results showed that αPIX, and not Vav, mediated PYGM activation in IL-2-stimulated Kit 225 T cells. Although, additional GEFs from the Dbl family of exchange factors such as Tiam-1 and STEF are capable of activating Rac 1 (28, 54), these GEFs have not been reported as being activated by tyrosine phosphorylation, but rather Tiam-1 was reported being activated by threonine phosphorylation (30), whereas STEF was reported being activated by serine/threonine phosphorylation (31). Even so, their effects on PYGM activity were evaluated. When these GEFs were individually overexpressed in Kit 225 T cells, it was observed that neither of them increased PYGM activity. Moreover, overexpression of Tiam-1 seems to have a dominant-negative effect on PYGM activity. Furthermore, when we examined the effects of αPIX knockdown with αPIX(esriRNA) on either PYGM activity or Rac 1 activation, it was observed that the absence of the αPIX-Rho-GEF expression was enough to block activation of the Rac 1/PYGM pathway. Collectively, these results strongly suggest that αPIX is the only GEF responsible for activation of the Rac 1/PYGM pathway in Kit 225 T cells stimulated by IL-2.

Even if the αPIX predicted phosphorylation sites were the serines phosphorylated at residues 225 and 488 rather tyrosine residues (35), this prediction does not contradict experimental evidence obtained from SILAC-based quantitative phosphoproteomics data (35), because a protein enriched by immunoprecipitation with antiphosphotyrosine antibody upon IL-2 stimulation does not necessarily mean that it has been tyrosine phosphorylated in response to that cytokine. Any non-tyrosine-phosphorylated proteins may also be enriched if it is bound to a tyrosine-phosphorylated protein. The prediction that αPIX serine residues 225 and 488 may be susceptible to being phosphorylated in Kit 225 T cells (35) is consistent with the phosphoproteomic data obtained from ES and iPS cells (36), KG1 AML cells (37), and colorectal cancer cells (38).

From a functional point of view, phosphorylation/dephosphorylation cycles are major early events in intracellular signaling cascades. Therefore, the importance of these two αPIX-RhoGEF residues (Ser225 and Ser488) on Rac 1 and the subsequent PYGM activation was examined. The results obtained are compelling: both residues are essential to control Rac 1 activation and PYGM enzymatic activity. In addition, by pharmacological and genetic (either by PKCθ loss or gain of function) approaches we were able to find out that PKCθ was in control of αPIX phosphorylation and therefore activation of the Rac 1/PYGM pathway in Kit 225 T cells. The involvement of PKCθ in this cellular model is a novel finding although not a surprising one; given that its expression is restricted to certain tissues and cell types, including T cells (55). Since the relevance of this serine/threonine kinase in regulating the dynamics of the immunological synapse was described (56–58), many additional functions have been discovered, such as the control of NF-κB, AP-1, and NFAT transcription factors activation, which regulate the expression of proinflammatory cytokines and anti-apoptotic molecules Bcl-XL (59). Moreover, the adhesive capacities of T lymphocytes (60) is the mechanism through which stable adhesion between T cells and antigen presenting cells is achieved (55, 61, 62). More recently, a new role for PKCθ in T cell physiology was described; i.e. PKCθ participates in the CCR7 downstream signaling driving T cell migration (63). Therefore, the present findings are in agreement with those reported by Cannon et al. (63) that not only stimulated IL-2 T cell proliferation but also T cell migration is regulated by the PKCθ/αPIX axis upstream of Rac 1/PYGM.

In conclusion, our findings reveal the mechanism through which IL-2 stimulates Rac 1 activation in Kit 225 T cells. We identified αPIX as the GEF that specifically activates Rac 1 and consequently regulates PYGM activation, as well as the molecular mechanism of αPIX activation of the Rac1/PYGM pathway. Regulation of this novel metabolic pathway requires that PKCθ phosphorylates αPIX serine residues participating in the control of T cell migration and/or proliferation. Mechanistically, the specific molecular players connecting the activated IL-2R and the PKCθ/αPIX/Rac 1/PYGM pathway, and also PYGM downstream signaling targets are still unknown. Future studies will allow us to characterize the signaling molecules upstream of PKCθ and effector molecules that participate in this signal transduction pathway.

Acknowledgments—We thank the AIDS Research and Reference Program, Division of AIDS (NIAD, National Institutes of Health) for the generous gift of recombinant IL-2, Dr. Kerstin Kutsche (Universitätsklinikum Hamburg-Eppendorf, Germany) for the generous gift of plasmid encoding for αPIX, and Dr. Gottfried Baier (Medical University of Innsbruck, Austria) for the generous gift of plasmids encoding for the constitutively active and dominant-negative forms of PKCα.

REFERENCES
PKC\(\theta\) Regulates \(\alpha\)PIX/Rac 1/PYGM Pathway

Acta 1570, 53–62


Guanine Nucleotide Exchange Factor αPIX Leads to Activation of the Rac 1 GTPase/Glycogen Phosphorylase Pathway in Interleukin (IL)-2-stimulated T Cells
Francisco Llavero, Bakarne Urzelai, Nerea Osinalde, Patricia Gálvez, Hadriano M. Lacerda, Luis A. Parada and José L. Zugaza

doi: 10.1074/jbc.M114.608414 originally published online February 18, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M114.608414

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 63 references, 24 of which can be accessed free at http://www.jbc.org/content/290/14/9171.full.html#ref-list-1