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Lck/PLCγ control migration and proliferation of interleukin (IL)-2-stimulated T cells via the Rac1 GTPase/glycogen phosphorylase pathway

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ABSTRACT

Recently, we have reported that the IL-2-stimulated T cells activate PKCθ in order to phosphorylate the serine residues of αPIX-RhoGEF, and to switch on the Rac1/PYGM pathway resulting in T cell migration and proliferation. However, the molecular mechanism connecting the activated IL-2-R with the PKCθ/αPIX/Rac1/PYGM pathway is still unknown. In this study, the use of a combined pharmacological and genetic approach identified Lck, a Src family member, as the tyrosine kinase phosphorylating PLCγ leading to Rac1 and PYGM activation in the IL-2-stimulated Kit 225 T cells via the PKCθ/αPIX pathway. The PLCγ tyrosine phosphorylation was required to activate first PKCθ, and then αPIX and Rac1/PYGM. The results presented here delineate a novel signalling pathway ranking equally in importance to the three major pathways controlled by the IL-2-R, i.e. PI3K, Ras/MAPK and JAK/STAT pathways. The overall evidence strongly indicates that the central biological role of the novel IL-2-R/Lck/PLCγ/PKCθ/αPIX/Rac1/PYGM signalling pathway is directly related to the control of fundamental cellular processes such as T cell migration and proliferation.

1. Introduction

Tyrosine phosphorylated IL-2-R downstream signalling controls not only the JAK/STAT, MAPK and PI3K pathways, the three best described signal transduction pathways in IL-2-stimulated T lymphocytes [1,2] but it also involves complex interactions with other signalling networks, here typified by signalling molecules such as tyrosine kinases Lck and BTK [3], PLCγ, [4,5], serine/threonine kinase PKCθ [6] and the small GTPases of the Rho family RhoA [7] and Rac1 [6,8]. In fact, small GTPases of the Rac subfamily, and more specifically Rac1, have been gaining in relevance in T cell biology [9,10]. Like other small GTPases, Rac functions as a molecular switch that cycles between an inactive GDP-bound and an active GTP-bound state. The transition between the inactive to the active state is regulated by guanine nucleotide exchange factors (GEFs) [11–13]. Recently, we have identified αPIX (also known as ARH-GEF6 or Cool-2) as the Rac1-specific GEF in IL-2-stimulated T cells [6]. Upon IL-2/IL-2-R ligation and receptor activation, the exchange activity of this Rac1-GEF, like other GEFs of the Dbl family activating Rac1 [14–18], is directly regulated by phosphorylation [6]. In fact, in IL-2-stimulated T cells, αPIX must be phosphorylated at serine residues 225 and 488 by the PKCθ, in order to turn on its GDP/GTP nucleotide exchange activity [6].

In response to IL-2, Rac1 active form (Rac1-GTP) interacts with downstream effector molecules to promote a variety of biological responses, such as control of the actin cytoskeleton reorganization [19], and/or T cell migration and proliferation [6,8]. Importantly, IL-2-stimulated T cell migration and proliferation responses depend on the activation of the glycogen phosphorylase muscle isoform (PYGM) via the αPIX/Rac1 route [6].

PLCγ is a key intracellular signalling molecule that requires recruitment to the membrane for its subsequent tyrosine phosphorylation and activation [20]. This hydrolase regulates the intracellular concentration of its substrate phosphatidylinositol-4,5-bisphosphate (PIP_2) by generating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) that control the intracellular calcium mobilization and PKCs activation, respectively [21,22]. Given that the activated IL-2-R heterotrimer formed by the ligand binding has no intrinsic kinase activity, the

Abbreviations: PYGM, glycogen phosphorylase muscle isoform; Lck, lymphocyte-specific protein tyrosine kinase; PLCγ, phospholipase; PI3K, phosphatidylinositide 3-kinase; AKs, Janus kinases; STAT, signal transducer and activator of transcription; GEF, Guanine Nucleotide Exchange Factor; IL-2, Interleukin 2; IL-2-R, Interleukin 2 receptor; RBD, Rac binding domain; TCR, T cell receptor; BCR, B cell receptor; DMSO, dimethyl sulfoxide.

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receptor initiates the intracellular signalling process by inducing the activation of IL-2-R β and γ chains that are constitutively associated with the JAK family (JAK1 and JAK3) of tyrosine kinases [23]. However, these enzymes are not the only tyrosine kinases linked to the IL-2-R involved in T cell signalling. IL-2 is capable of activating several different tyrosine kinases, although the relationship amongst them is not fully clarified. One of the activated tyrosine kinase that associates itself with the IL-2-R β subunit is the Lck tyrosine kinase [24–26].

In this study, Lck was identified as a tyrosine kinase signalling upstream of Rac1 in the IL-2-stimulated T cells. More importantly, the connection between the activated IL-2-R and the PKCθ/αPIX/Rac1/PYGM signalling pathway mediating T cell migration and proliferation responses is established via the molecular tandem Lck/PLCγ, independently of JAK tyrosine kinase activity and intracellular calcium release. Finally, further evidence is provided that PLCγ must be tyrosine phosphorylated by Lck in order to activate the PKCθ/αPIX/Rac1/PYGM pathway and thereby regulate T cell migration and proliferation.

Taken together our results reveal a novel early intracellular signalling cascade playing a central role in the control of the T lymphocytes migration and proliferation stimulated with IL-2. This novel signalling pathway composed by IL-2-R/Lck/PLCγ/PKCθ/αPIX/Rac1/PYGM may be as specifically and functionally significant as the three canonical pathways already described in IL-2-stimulated T lymphocytes.

2. Materials and methods

2.1. Reagents

Lck inhibitor (7-cyclopentyl-5-(4-phenoxyphenyl)-7H-pyrrolo[2,3-*d*]pyrimidin-4-ylamine), the JAK1/3 inhibitor (Tofacitinib citrate), PKC inhibitors Gö6976 and Rottlerin, mouse monoclonal anti-phosphoSerine (clone PSR-45), monoclonal anti-Glutathione-S-transferase (GST) antibodies, MISSION® *esiRNA* targeting EGFP (Reference EHUEGFP), MISSION® *esiRNA* targeting human LCK (Reference EHU064811) and MISSION® *esiRNA* targeting human PLCγ1 (Reference EHU069301) were from Sigma-Aldrich. Mouse monoclonal anti-HA antibody was from Covance. Mouse monoclonal anti-Rac1 (clone 23A8) antibody was from Millipore, and the enhanced chemiluminescence (ECL) reagent was from GE Healthcare. IL-2 was kindly provided by the "AIDS Research and Reference Reagent Program," Division of AIDS (NIAD, National Institutes of Health), USA.

2.2. Cell culture and DNA/esiRNA transfection

The Kit 225 T cells were cultured as described by Hori et al. in the presence of 16 U/ml recombinant human IL-2 [27]. Kit 225 T cells are a human T cell line established from a patient with T cell chronic lymphocytic leukaemia, this cell line expresses IL-2 receptor constitutively and it depends exclusively on IL-2 for cellular proliferation [27]. Kit 225 T cells are synchronized in G0/G1 when deprived of IL-2 for 48 h and subsequent stimulation with IL-2 allows cell cycle entry and progression. This feature represents a key advantage for IL-2-stimulated signalling studies. For transient transfections, cells were cultured in complete RPMI 1640 medium in the absence of IL-2 for 24 h. Thereafter, cells were washed and re-suspended in 200 μl of serum-free medium, and placed in an electroporation cuvette (0.4 mm Sigma-Aldrich) containing 10–20 μg of the different plasmids, or 15 ng *esiRNAs*. The electroporation was carried out in a Gene Pulser Xcell Electroporator (Bio-Rad) at 260 V and 950 μF [8]. The cuvette content was collected into 10 ml of complete RPMI 1640 medium and cultured in the absence of IL-2 for another 24 h.

2.3. Agonists and inhibitors

The Kit 225 T cells were maintained in the absence of IL-2 for 48 h and subsequently stimulated with 500 U/ml IL-2 at 37 °C. In some experiments, the Kit 225 T cells were pretreated with the Lck inhibitor (10 μM) [28] or the JAK1/3 inhibitor (250 nM) [29] for 1 h prior to IL-2 stimulation.

2.4. Site-directed mutagenesis

The pCI-neoPLCγ^{H335Q} single mutated construct were generated according to the manufacturer's instructions (QuickChange Lightning Site-Directed Mutagenesis Kit, Stratagene). Oligonucleotides used for mutating the H^{335} for Q^{335} were as follows: 5′-ATCTCCTCCTCGCAGAACACGTACCTG-3′ (forward) and 5′-CAGGTACGTGTTCTGCGAGGAGGAGAT-3′ (reverse).

2.5. Activity assay for glycogen phosphorylase

The glycogen phosphorylase activity assay was performed as previously described [6,8]. Briefly, cells were washed twice with cold PBS and resuspended in 500 μl of TES buffer (20 mM Tris, pH 7.4, 1 mM EDTA, 225 mM sucrose, 2.5 mM DTT, 0.1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin). Samples were sonicated and centrifuged at 13,500 rpm for 10 min at 4 °C. The total protein (100 μg) was used to measure the PYG activity in the assay buffer (50 mM KH_2PO_4 pH 7.5, 10 mM $MgCl_2$, 5 mM EDTA pH 8, 0.5 mM $NADP^+$, 1.5 U/ml glucose-6-phosphate dehydrogenase, 1 units/ml phosphoglucomutase, 0.1 mg/ml glycogen (all from Sigma-Aldrich). The assay buffer containing 300 μl of TES, without $NADP^+$, glycogen, phosphoglucomutase, and glucose-6-phosphate dehydrogenase, was added to 100 μg of the 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. The sample absorbance was detected at 340 nm in a spectrophotometer (Ultrospec 3100 pro, Amersham Biosciences). The amount of NADPH formed was determined using a standard curve of known NADPH concentrations (Sigma-Aldrich).

2.6. Rac1 activation assay

The Rac1 pull-down assay was performed using a GST fusion protein containing the Rac1 binding domain of PAK1 (GST-RBD-PAK1). The transfected and the non-transfected cells kept in the absence of IL-2 for 48 h were stimulated with IL-2 for 10 min and lysed, as previously described [6,8]. Cell lysates were centrifuged at 13,500 rpm for 10 min at 4 °C and incubated for 1 h at 4 °C with 50 μg GST-RBD-PAK1 fusion protein coupled to glutathione-Sepharose beads. The precipitated proteins were eluted from beads using the 2 x loading buffer (12 mM Tris, pH 6.8, 5% glycerol, 0.4% SDS, 140 mM 2-mercaptoethanol, 0.02% bromophenol blue), separated by SDS-PAGE, and analysed by immunoblot with specific monoclonal antibodies. The immunoreactive bands were visualized using ECL.

2.7. Immunoprecipitation assay

The Kit 225 T cells were transfected with pMT2-HA-αPIX, pCI-neoPLCγ or the empty vectors (pMT2-HA, pCI-neo) or the *esiRNAs* to knock down PKCθ and PLCγ1 or *esiRNA* EGFP, as previously described, and cells were treated or not with 500 U/ml IL-2 for 10 min. Cells were washed three times in ice-cold PBS and lysed in the RIPA buffer (50 mM Tris pH 7,4, 150 mM NaCl, 1% IGEPAL, 0.25% Na-Deoxicolate, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/ml aprotinin, 1 μg/ml leupeptin). HA-αPIX was immunoprecipitated at 4 °C for 2 h with the anti-HA antibody. The immune complexes were recovered using Gamma Bind Plus Sepharose beads (GE Healthcare, Pittsburgh, PA, USA), washed and eluted from beads and resolved electrophoretically by SDS-PAGE and analysed by Western blot with the anti-phosphoserine or the anti-HA antibodies. The immunoreactive bands were visualized using ECL.

2.8. In vitro kinase assay

The Kit 225 T cells were pretreated or not with the tyrosine kinase inhibitors, as indicated, for 1 h and incubated in the presence or in the absence of 500 U/ml IL-2 for 10 min at 37 °C and washed twice with cold PBS. Moreover, the Kit 225 T cells transfected with the *esiRNA* PLCγ1 to knock down PLCγ1 or the *esiRNA* EGFP, as previously described, were stimulated or not with 500 U/ml IL-2 for 10 min at 37 °C and washed twice with cold PBS. Thereafter, cells were lysed with the 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 aprotinin, 1 mM PMSF, 10% glycerol and 1% v/v Triton X-100) and the lysates were clarified by centrifugation for 10 min at 13,500 rpm at 4 °C. PKC_θ was immunoprecipitated with a specific antibody and the immunocomplexes were recovered using Gamma Bind Plus Sepharose beads (GE Healthcare, Pittsburgh, PA, USA). The immunocomplexes were washed twice with the cold lysis buffer, twice with the cold washing buffer (10 mM HEPES (pH 7.4), 100 mM NaCl, 20 μg/ml aprotinin and 0.5% IGEPAL-360 and twice with the reaction buffer (20 mM Tris (pH 7.4), 20 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$ and 1 mM $MnCl_2$). 500 ng of the purified recombinant GST-$\alpha PIX^{204–532}$ encompassing the two potential serine phosphorylation sites (S225 and S488) of αPIX and ATP (20 μM) were then added to the reaction mixture. The in vitro kinase reaction was carried out for 30 min at 30 °C, after that it was stopped by adding 30 μl of the 2× loading buffer. Proteins were separated by SDS-PAGE, followed by Western blot. The immunoreactive bands were visualized with the anti-phosphoserine antibody and ECL.

2.9. Cell viability assay

Kit 225 T cells were seeded at 10^4 cells per well in a 96-wellplate, IL-2-deprived cells for 48 h, were pretreated (+) or not _(−) with 2.5 μM Rottlerin for 1 h, prior to IL-2 stimulation for 24 h. Cell viability was measured with CellTiter 96 Non-Radioactive Cell Proliferation Assay (MTT) (Promega, Madison, WI, USA), according to the manufacturer's instructions.

2.10. Cell migration assay

The *esiRNA* transfected cell suspensions (2.5×10^5 cells in 100 μl) were placed into the upper chamber, whereas 600 μl of medium with or without IL-2 (500 U/ml) was introduced into the lower chamber. Both chambers were incubated overnight at 37 °C in 5% CO_2 and 95% air. Cells in the upper and in the bottom chamber were recovered separately into equal volumes for cell counting. The percentage of migrating cells was determined as follows: [the number of cells migrating (the lower chamber) / the total number of cells (the cells in the lower chamber + the remaining cells in the upper chamber)]. The assay was performed using the pore filters (8 μm, Corning® Costar® Transwell® and the cell culture inserts were from Sigma-Aldrich) and the cell counts were done in triplicate.

2.11. Cell proliferation measurement

The *esiRNA* transfected cells were seeded in 24-well plates in complete RPMI (10^6 cells/ml), and maintained in the absence of IL-2 for 48 h. Subsequently, cells (10^6) were incubated with 4 μM PKH26 following the manufacturer's instructions (Sigma-Aldrich). A sample (10^4 cells) was taken as the start control and the remaining cells were treated with 16 U/ml IL-2 every 24 h for 3 days. Fluorescence was measured every 24 h for three days to monitor the cell division rate on a FACSCalibur (Becton & Dickinson) flow cytometer. The data obtained were analysed using the flow cytometric analysis program ModFit LT 3.0 (Verity Software House, Topsham, ME). This program uses a nonlinear least squares analysis to iteratively find the best fit to the raw data by changing the position, height, and coefficient of variation of each Gaussian. The area under each generational Gaussian is taken as a measure of the relative number of cells in that generation and the sum of all Gaussians corresponds to the relative number of cells in the total population.

2.12. Statistical analysis

The Student's *t*-test for the mean of two-paired samples was used to determine the significance between data means ($^*p < 0.05$,$^{***}p < 0.001$).

3. Results

3.1. PLCγ1 leads to PYGM activation

To examine whether PLCγ1 could be a link between IL-2 receptor and PYGM, Kit 225 T cells were transfected with pCI-neoHA (mock control), pCI-neoHAPLCγ1 (wt), pCI-neoHAPLCγ1-palm (constitutively active form of PLCγ1) or pCI-neoHAPLCγ1^{H335Q} (an inactive mutant of PLCγ1) and stimulated or not with 500 U/m1 IL-2 for 10 min, lysed and PYGM activity was determined as described in Materials and methods, Section 2.5. As shown in Fig. 1A, IL-2 induced robust PYGM activity in Kit 225 T cells transfected with the empty vector. This IL-2 stimulation of PYGM activity was already maximal and it was not increased any further either in cells overexpressing PLCγ1 or the PLCγ1 constitutively active form. In fact, PLCγ1 constitutively active mutant overexpression reached the maximal PYGM activity in the absence of IL-2. In contrast, transfection with PLCγ1^{H335Q} completely abolished PYGM activity both with or without IL-2 stimulation. Western blots show PLCγ1 endogenous and ectopic expression levels of all forms of PLCγ1 and an equivalent amount of tubulin was used in each of the conditions analysed (Fig. 1A).

Next, in order to corroborate that PLCγ1 was specifically regulating the PYGM activity in IL-2-stimulated T cells, *plcγ1* was knocked down in Kit 225 T cells. To this end, Kit 225 T cells were transfected with *esiRNA* human PLCG1 (PLCγ1) or *esiRNA* targeting EGFP, as a negative control. 24-h post-transfection PYGM activity was determined in both Kit 225 T cells stimulated with IL-2 for 10 min and unstimulated. As shown in Fig. 1B, IL-2 stimulated robust PYGM activity in *egfp*(*esiRNA*)-transfected Kit 225 T cells. *plcγ1*-knockdown (*plcγ1*(*esiRNA*)) cells stimulated by IL-2 for 10 min did not show any PYGM activity. Western blots show, on the one hand PLCγ1 expression levels after *esiRNA* transfection, and on the other hand an equivalent amount of tubulin was used in each of the conditions analysed (Fig. 1B).

3.2. PLCγ1 regulates Rac1/PYGM pathway activation via nPKCs

In order to demonstrate that PLCγ1 functions as a positive regulator molecule upstream of Rac1 in Kit 225 T cells, PLCγ1 was knocked-down with *plcγ1*(*esiRNA*), as we described above, and the endogenous active Rac1 was measured by the pull-down assay. As shown in Fig. 2A, in the absence of PLCγ1 (knockdown) IL-2 was unable to stimulate Rac1 activation. PLCγ1 expression levels in the presence of *esiRNA* control (*egfp*) or *plcγ1*(*esiRNA*) were determined by Western blot. The Rac1 detected in whole cell lysates shows that the total loaded proteins are equivalent in all lanes (Fig. 2A).

PKC inhibitors were used to investigate whether PLCγ1 required any PKCs activity in order to control the Rac1/PYGM pathway activation. To this end, Kit 225 T cells deprived of IL-2 for 24 h were transfected with empty vector (mock control) or PLCγ1-palm (constitutively active form of PLCγ1). 24-h post-transfection, cells were pretreated or not with 100 nM Gö6976 (an inhibitor of classic PKCs, mainly α and β) and 2.5 μM Rottlerin (which was initially described as PKCθ inhibitor) [30,31], followed or not by stimulation with 500 U/ml IL-2 for 10 min and the Rac1 activation was measured by the pull-down assay. PLCγ1-palm overexpressing cells, as well as control stimulated cells, exhibited

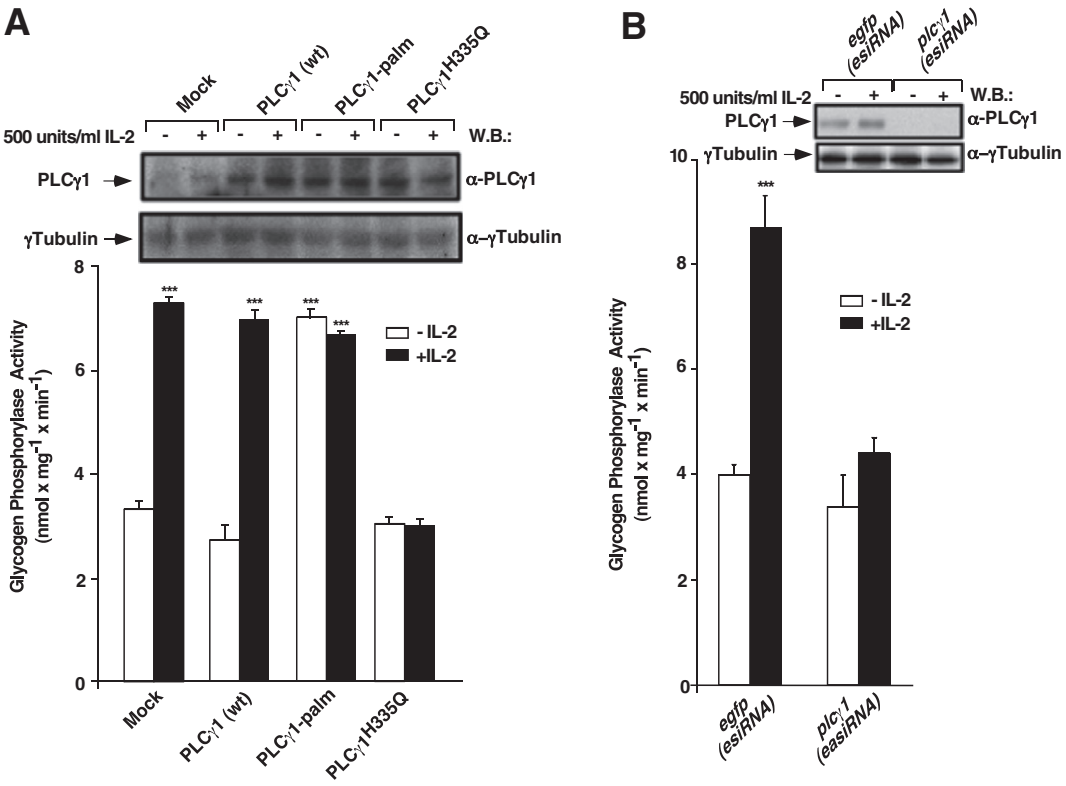

Fig. 1. IL-2 modulates the glycogen phosphorylase activity in Kit 225 T cells via PLCγ1. The Kit 225 T cells deprived of IL-2 for 24 h were transfected with plasmids encoding: (*A*) pCIneo-HA (mock control), pCIneo-HA-PLCγ1wt, pCIneo-HA-PLCγ1-palm (the constitutively active mutants) and pCIneo-HA-PLCγ1^{H335Q} (the constitutively inactive mutant), and (*B*) *plcγ1*(*esiRNA*) and *egfp*(*esiRNA*) as controls. 24 h post-transfection, cells were stimulated (+) or not (−) with 500 U/ml for 10 min and lysed. The cell extracts from unstimulated and stimulated cells were used to measure the glycogen phosphorylase activity, as described under Experimental Procedures. The protein expression levels were analysed by Western blotting using specific antibodies, as indicated. The results show the mean of the three independent experiments ± S.D. and the statistical analysis shows a significant difference (***, $p < 0.001$).

a maximal Rac1 activation even in the absence of IL-2 stimulation (Fig. 2B). Inhibition of classic PKCs did not affect Rac1 activation in PLCγ1-palm overexpressing cells; notwithstanding, Rottlerin efficiently prevented PLCγ1-palm mediated Rac1 activation (Fig. 2B).

Next, the effect of these PKC inhibitors on PYGM activity in PLCγ1-palm overexpressing Kit 225 T cells was also examined. As shown in Fig. 2C, IL-2-stimulated PYGM maximal activity in Kit 225 T cells transfected with the empty vector control was equivalent to the PYGM activity reached in Kit 225 T cells transfected with PLCγ1-palm. Expectedly, the classic PKC inhibitor (Gö6976) did not affect PYGM activity of either empty vector control or PLCγ1-palm overexpressing cells. In contrast, PYGM activity was completely blocked in PLCγ1-palm-overexpressing Kit 225 T cells treated with the novel PKC inhibitor, Rottlerin (Fig. 2C). However, this compound alone or in the presence of IL-2 did not alter the viability of Kit 225 T cells (Supplementary Fig. 1S).

3.3. PLCγ1 regulates αPIX-RhoGEF serine phosphorylation via PKCθ

To find out whether or not PLCγ1 played a role in the control of the αPIX phosphorylation/activation upstream of PKCθ, Kit 225 T cells deprived of IL-2 for 24 h were co-transfected with pMT2-HA-αPIX and pCIneo-HA (empty vector) or pCIneo-HA-PLCγ1-palm (the constitutively active mutant). As shown in Fig. 3A, IL-2 stimulated αPIX serine phosphorylation and it was blocked by Rottlerin. When the effect of PLCγ1-palm overexpression on αPIX phosphorylation was examined, the phosphorylation signal measured was stronger than that found in control cells stimulated by IL-2. IL-2 stimulation of PLCγ1-palm overexpressing cells did not increase the phosphorylation signal any further than that of unstimulated PLCγ1-palm overexpressing Kit 225 T cells. In contrast, Rottlerin forcefully blocked αPIX serine phosphorylation in both the unstimulated and the stimulated PLCγ1-palm-overexpressing Kit 225 T cells. To determine the amount of immunoprecipitated αPIX, membranes that were used to examine αPIX serine phosphorylation were stripped and reblotted with the anti-HA antibody. PLCγ1-palm expression in whole cell lysates was also examined by SDS-PAGE and followed by Western blot. Immunoreactive bands were visualized using anti-HA antibody (Fig. 3A).

To test whether or not PLCγ1 controlled αPIX serine phosphorylation residues through PKCθ, IL2-deprived Kit 225 T cells were co-transfected with pMT2-HA-αPIX and esiRNAs (*egfp*, *plcγ1*, *pkc$_\theta$*) or pMT2-HA-αPIX with PLCγ1-palm (PLCγ1 constitutively active mutant) and *pkc$_\theta$*(*esiRNA*) or pMT2-HA-αPIX with PKCθ^{A148E} (PKCθ constitutively active mutant) and *plcγ1*(*esiRNA*), as indicated in Fig. 3B. After 24 h, cells were stimulated or not with IL-2 for 10 min and lysed. As shown in Fig. 3B, IL-2 stimulated αPIX serine phosphorylation that was completely blocked in the absence of either PLCγ1 or PKCθ expression. Combining the overexpression of the constitutively active form of PLCγ1 (PLCγ1-palm) along with PKCθ knock-down resulted in inhibition of αPIX serine phosphorylation either in the presence or in the absence of IL-2. Conversely, Kit 225 T cells overexpressing PKCθ constitutively active form (PKC$_\theta^{A148E}$) and PLCγ1 knockdown showed that αPIX serine was phosphorylated independently of any stimuli, and comparably, αPIX showed a phosphorylation signal equivalent to that of the control cells. To determine the amount of immunoprecipitated αPIX, membranes that were used to examine αPIX serine phosphorylation were stripped and reblotted with the anti-HA antibody. Ectopic HA-PLCγ1-palm and HA-PKCθ^{A148E} expression, and endogenous PLCγ1 and PKCθ expression in whole cell lysates were also examined by SDS-PAGE and followed by Western blot. The immunoreactive

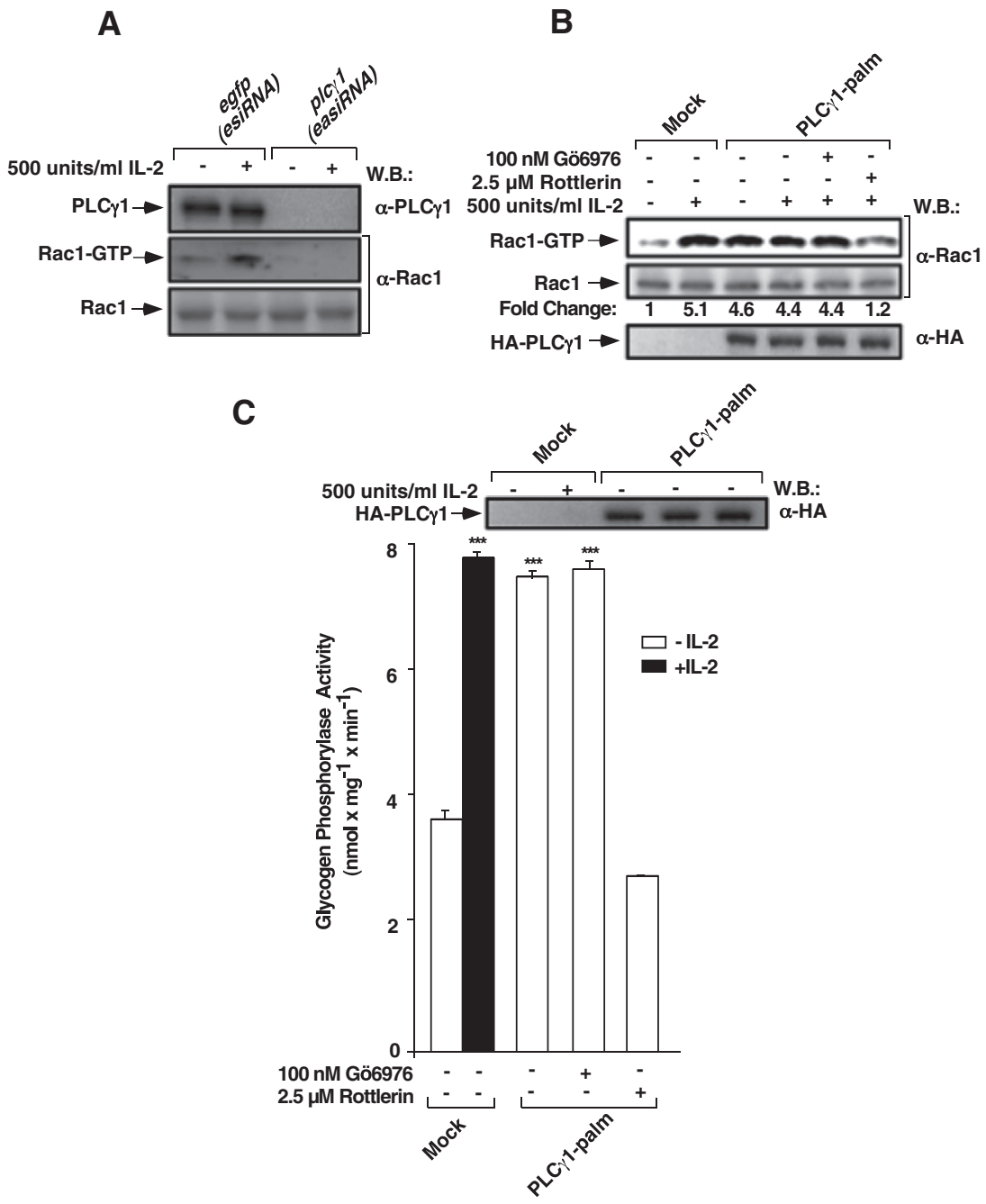

Fig. 2. The IL-2-stimulated Rac1 and glycogen phosphorylase activation in Kit 225 T cells requires PLCγ1. The Kit 225 T cells deprived of IL-2 for 24 h were transfected with *egfp*(*esiRNA*), *plcγ1*(*esiRNA*) empty vector (the mock control) and pCIneo-HA-PLCγ1-palm (the constitutively active mutant). 24 h post-transfection, the cells were treated with the PKC inhibitors or the vehicle (DMSO) for 1 h and stimulated (+) or not (−) with 500 U/ml IL-2 for 10 min and lysed. (*A* and *B*) The cell lysates were used to measure the Rac1 activation by the affinity precipitation assay. The precipitated active Rac1 (Rac1-GTP) and the total Rac1 from the cell lysates were analysed by Western blotting using an anti-Rac1 specific antibody. The expression levels of endogenous and ectopic PLCγ1 were visualized using anti-PLCγ1 and anti-HA antibodies, as indicated. The results shown are representative of the three independent experiments. (*C*) The cell extracts were used to measure the glycogen phosphorylase activity, as described under Experimental Procedures. The protein expression levels were analysed by Western blotting using a specific anti-HA antibody. The results show the mean of the three independent experiments ± S.D. and the statistical analysis shows a significant difference (***, $p < 0.001$).

bands were visualized using specific antibodies, as indicated. Finally, an equivalent amount of tubulin was used in each of the conditions analysed (Fig. 3B).

In order to confirm PLCγ1 involvement in PKCθ-mediated phosphorylation of αPIX serine residues 225 and 488, endogenous PKCθ activity was examined by an in vitro *kinase* assay using a GST-αPIX$^{204–532}$ fusion protein as an exogenous substrate for PKCθ. Briefly, Kit 225 T cells were transfected with *plcγ1*(*esiRNA*) to knockdown PLCγ1 or *egfp*(*esiRNA*) as negative control and stimulated or not with IL-2 for 10 min. PKCθ was immunoprecipitated from cell lysates and immunocomplexes were incubated with ATP and GST-αPIX$^{204–532}$. Subsequently, proteins were resolved by SDS-PAGE followed by Western blot and GST-αPIX$^{204–532}$ serine phosphorylation was visualized using an anti-phosphoserine antibody. As illustrated in Fig. 3C, in the absence of PLCγ1 expression, IL-2 was unable to stimulate αPIX serine phosphorylation. To determine the amount of immunoprecipitated PKCθ, the membrane that was used to examine the GST-αPIX$^{204–532}$ serine phosphorylation was stripped and reblotted with anti-PKCθ antibody. The same membrane was

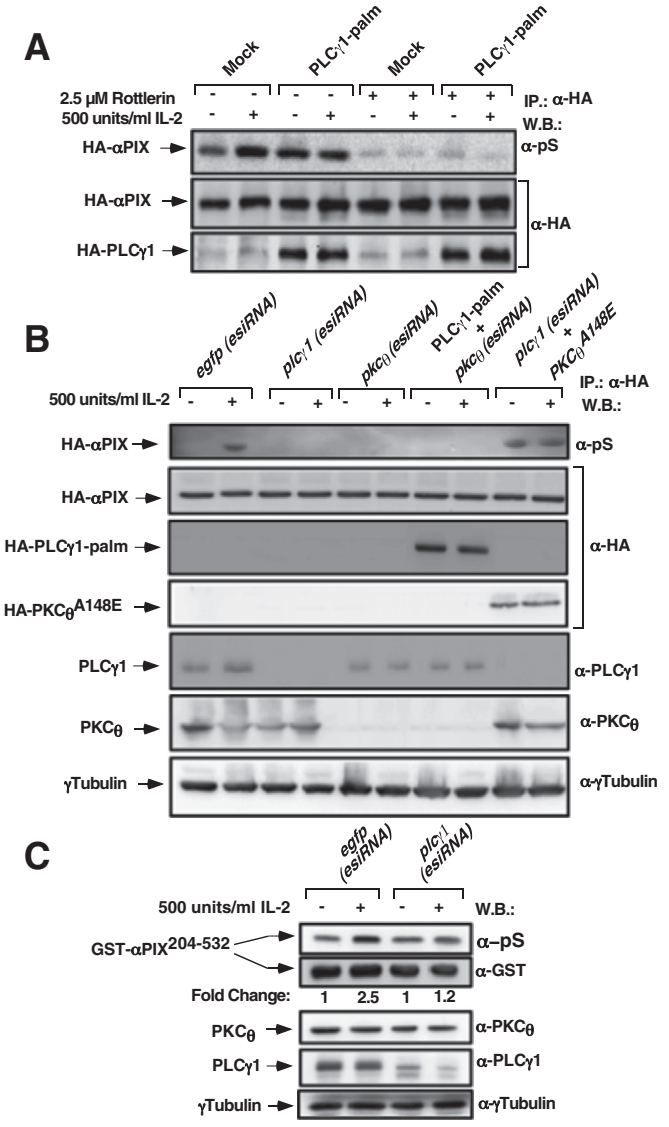

Fig. 3. The αPIX serine phosphorylation depends on PLCγ1 and PKCθ in wild type Kit 225 T cells. The Kit 225 T cells deprived of IL-2 for 24 h were transfected with the indicated constructs. (*A*) 24 h post-tranfection, the Kit 225 T cells were pretreated with 2.5 μM Rottlerin or the vehicle (DMSO) for 1 h. Subsequently, cells were stimulated or not with 500 U/ml IL-2 for 10 min and lysed. (*B*) 24 h post-tranfection, cells were stimulated (+) or not (−) with 500 U/ml IL-2 for 10 min and lysed. The cell lysates were subjected to immunoprecipitation with an anti-HA antibody (*A* and *B*) and the immunoreactive bands were visualized using anti-phosphoserine, anti-HA, anti-PLCγ1, anti-PKCθ and anti-γ−tubulin antibodies. The results are representative of the four independent experiments. (*C*) As the negative control, the Kit 225 T cells deprived of IL-2 for 24 h were transfected with *plcγ1*(*esiRNA*) and *egfp*(*esiRNA*). 24 h post-transfection, cells were stimulated (+) or not (−) with 500 U/ml IL-2 for 10 min and lysed. The cell extracts were immunoprecipitated with the anti- PKCθ antibody and the immunocomplexes activities were analysed by the in vitro kinase assay followed by SDS-PAGE and Western blotting. The immunoreactive bands were visualized using an anti-phosphoserine (pS) antibodies. The amount of GST-αPIX$^{204–532}$ in the lysates were analysed by Western blotting using an anti-GST antibody. The expression levels of PKCθ, PLCγ1, and γ−tubulin were visualized using anti-PKCθ, anti-PLCγ1 and anti-γ−tubulin antibodies, as indicated. The results shown are representative of the three independent experiments.

reblotted once again, but this time with anti-GST antibody and served to determine the amount of GST-αPIX$^{204–532}$ fusion protein added to in the *vitro kinase* reaction. Endogenous PLCγ1 expression in whole cell lysates was also examined by SDS-PAGE and followed by Western blot. Immunoreactive bands were visualized using anti-PLCγ1 antibody. Tubulin Western blot analysis shows that equal amounts of protein were used (Fig. 3C).

3.4. Src family member, lck, connects the IL-2R and the PLCγ1/PKCθ/αPIX/Rac1 pathway

To examine whether or not JAK1/3 and/or Lck participated in tyrosine phosphorylation of PLCγ1 in Kit 225 T cells, IL-2-deprived cells were pretreated with potent and selective inhibitors of Lck [28] or JAK1/3 [29] or vehicle and subsequently stimulated with IL-2. As shown in Fig. 4A, IL-2 stimulated PLCγ1 tyrosine phosphorylation that was completely blocked by the Lck inhibitor. However, the JAK1/3 inhibitor did not alter IL-2-stimulated tyrosine phosphorylation of PLCγ1 (Fig. 4A). This result prompted us to verify the efficiency of this inhibitor in our experimental conditions by demonstrating that the JAK1/3 inhibitor was active and able to completely block IL-2-stimulated AKT phosphorylation (Fig. 4B).

Next we investigated whether or not the absence of non-receptor tyrosine kinase activity impacted on the PKCθ-mediated phosphorylation of αPIX serine residues 225 and 488. To this end, PKCθ activity present in the immunocomplexes was measured by an in vitro *kinase* assay using a GST-αPIX$^{204–532}$ fusion protein as described above. As shown in Fig. 4C, IL-2 stimulated robust αPIX serine phosphorylation that was unaffected by the JAK1/3 inhibitor (Fig. 4C). However, the Lck inhibitor completely blocked IL-2-stimulated αPIX serine phosphorylation (Fig. 4C). To determine the amount of immunoprecipitated PKCθ, the membrane that was used to examine the serine phosphorylation of GST-αPIX$^{204–532}$ was stripped and reblotted with an anti-PKCθ antibody (Fig. 4C). The same membrane was reblotted once again but this time with an anti-GST antibody to determine the amount of GST-αPIX$^{204–532}$ fusion protein added to the in vitro *kinase* reaction (Fig. 4C).

To further characterize the involvement of Lck and/or JAK1/3 in the signalling pathways stimulated by IL-2 leading to Rac1 activation, Kit 225 T cells that were deprived of IL-2 for 48 h and pretreated with 10 μM Lck inhibitor, 250 nM JAK1/3 inhibitor or the vehicle for 1 h and stimulated or not with 500 U/ml IL-2 for 10 min. Subsequently, Rac1 activation was analysed. As shown in Fig. 4D, the JAK1/3 inhibitor did not interfere with IL-2-stimulated Rac1 activation. In contrast, the Lck inhibitor completely blocked Rac1 activation stimulated by IL-2 (Fig. 4D). To confirm this result, Lck was knocked down with *lck*(*esiRNA*), as described above, and the active endogenous Rac1 was measured by the pull-down assay. In the absence of Lck (knockdown), IL-2 was unable to stimulate Rac1 activation (Fig. 4E). Lck expression level in cells transfected with *esiRNA* control (egfp) or *lck*(*esiRNA)* was determined by Western blot. Rac1 detected in whole cell lysates shows that the loaded proteins were equivalent in all lanes (Fig. 4E).

In order to further investigate the hypothesis that Lck signals downstream from the IL-2-R to the PLCγ1/PKCθ/αPIX/Rac1 pathway, Kit 225 T cells that were deprived of IL-2 for 24 h were transfected with empty vector (mock control), or pCIneo-Lck^{Y505F} (Lck constitutively active form) or cotransfected pCIneo-Lck^{K273R} (Lck dominant negative mutant) together with PLCγ1 constitutively active form (pCIneo-PLCγ1-palm) or cotransfected pCIneo-Lck^{Y505F} together with pCIneo-PLCγ1-^{H355Q} (dominant negative mutant of PLC γ1). After 24 h, cells were pretreated with 100 nM Gö6976 and 2.5 μM Rottlerin or the vehicle for 1 h followed by stimulation or not with 500 U/ml IL-2. Rac1 activation was measured by the pull-down assay, as described previously. As shown in Fig. 4F, IL-2 stimulated Rac1 activation and it was not increased any further in either unstimulated or in IL-2-stimulated Lck-^{Y505F}overexpressing cells. Likewise, inhibition of classic PKCs with Gö6976 did not affect Rac1 activation either in unstimulated or in IL-2-stimulated Lck^{Y505F}overexpressing cells (Fig. 4F). However, Rottlerin efficiently inhibited Rac1 activation stimulated by overexpression of Lck constitutively active form, both in unstimulated as well as in IL-2-stimulated cells (Fig. 4F). When Rac1 activation in Kit 225 T cells overexpressing Lck^{K273R}/PLCγ1-palm was examined, it was observed that PLCγ1-palm (PLCγ1 constitutively active form) was enough to stimulate Rac1 activation, independently of both stimuli and overexpression of Lck inactive form (Fig. 4F). Nonetheless, this mechanism is reversed

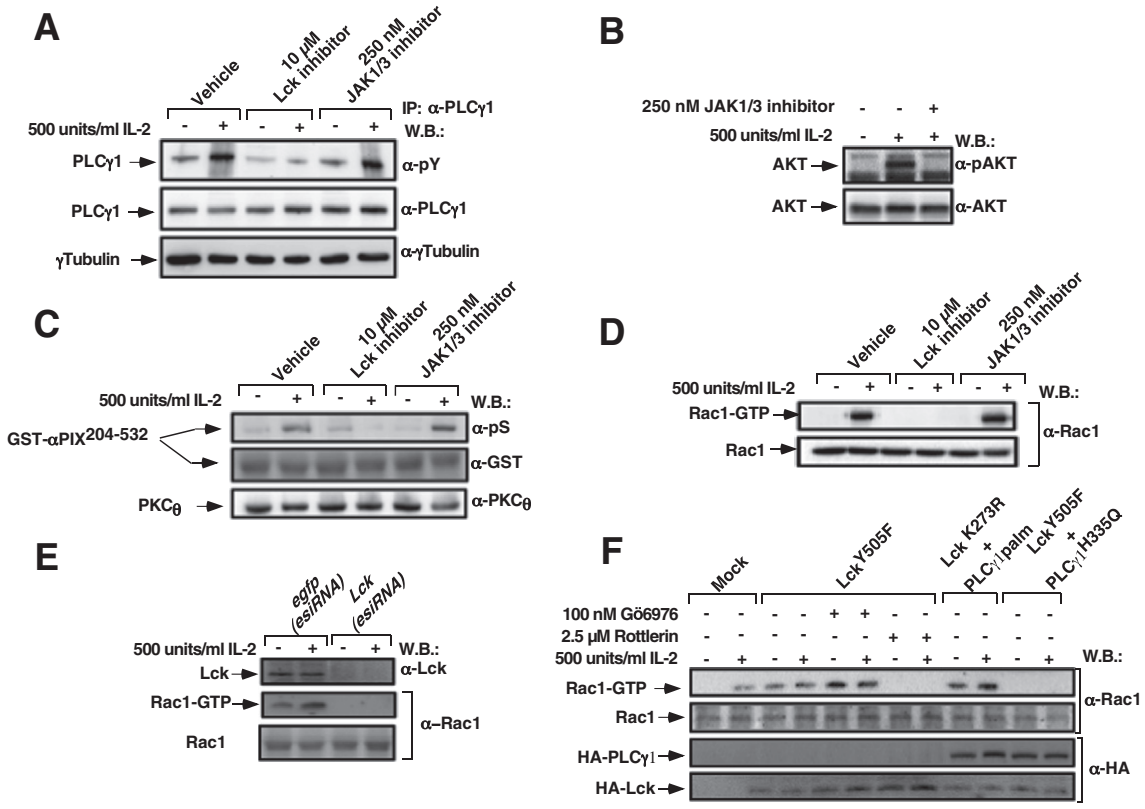

Fig. 4. Lck regulates the PKCθ activity and the Rac1 activation via PLCγ1 in Kit 225 T cells. The Kit 225 T cells deprived of IL-2 for 48 h were pretreated with the Lck inhibitor (10 μM), the JAK1/3 inhibitor (250 nM) or the vehicle (DMSO) for 1 h. Subsequently, the cells were stimulated or not with 500 U/ml IL-2 for 10 min and lysed. (*A*) The cell lysates were immunoprecipitated with an anti-PLCγ1 antibody and the immunoreactive bands were visualized using anti-phosphotyrosine, anti-PLCγ1 and anti-γ−tubulin antibodies, as indicated. The results are representative of four independent experiments. (*B*) The cell extracts were analysed by Western blot using an anti-phospho AKT antibody. The AKT amounts present in the samples analysed were examined with an anti-AKT antibody. The results are representative of the three independent experiments. (*C*) The cell extracts were immunoprecipitated with an anti-PKCθ antibody. The immunocomplexes activities were analysed by the in vitro kinase assay followed by SDS-PAGE and Western blotting. The immunoreactive bands were visualized using an anti-phosphoserine (pS) antibody. The amount of GST-αPIX$^{204-532}$ in lysates was analysed by Western blot using an anti-GST antibody. The expression levels of PKCθ were visualized using an anti-PKCθ antibody. The results shown are representative of the three independent experiments. (*D*) The cell extracts were used to measure the Rac1 activation by affinity precipitation assay. The precipitated active Rac1 (Rac1-GTP), the total Rac1 in the cell lysates were analysed by Western blot using an anti-Rac1 specific antibody. The results are representative of the three independent experiments. (*E*) The Kit 225 T cells deprived of IL-2 for 24 h were transfected with *Lck*(*esiRNA*) and *egfp*(*esiRNA*) as negative control. 24 h post-transfection, cells were stimulated (+) or not (−) with 500 U/ml IL-2 for 10 min and lysed. The cell lysates were used to measure Rac1 activation by affinity precipitation assay. The precipitated active Rac1 (Rac1-GTP), the total Rac1 from the cell lysates were analysed by Western blotting using an anti-Rac1 specific antibody. The expression levels of the endogenous Lck were visualized using an anti-Lck antibody. The results are representative of the four independent experiments. (*F*) the Kit 225 T cells deprived of IL-2 for 24 h were transfected with the empty vector (mock control), Lck^{Y505F} (the Lck constitutively active mutant) or cotransfected LckK^{273R} (the Lck inactive mutant) together with PLCγ1-palm (the PLCγ1 constitutively active mutant) or Lck^{Y505F} (the Lck constitutively active mutant) cotransfected together with PLCγ1^{H335Q} (the PLCγ1 inactive mutant). 24 h post-transfection, Kit 225 T cells were pretreated with the PKCs inhibitors, as indicated, or the vehicle (DMSO) for 1 h. Subsequently, cells were stimulated or not with 500 U/ml IL-2 for 10 min and lysed. The cell extracts were used to measure Rac1 activation by affinity precipitation assay. The precipitated active Rac1 (Rac1-GTP), the total Rac1 from the cell lysates were analysed by Western blotting using an anti-Rac1 specific antibody. The expression levels of ectopic expression of the Lck and PLCγ1 mutants were visualized using anti-HA antibodies. The results shown are representative of the three independent experiments.

in Lck^{Y505F}/PLCγ1^{H335Q} overexpressing cells, where Lck constitutively active mutant was unable to activate Rac1 in the presence of PLCγ1^{H335Q} (PLCγ1 inactive mutant), both in the presence or absence of IL-2 stimulation (Fig. 4F). HA-PLCγ1 and HA-Lck mutants' overexpression levels were determined by Western blot (Fig. 4F). Rac1 detected in the whole cell lysates shows that the loaded proteins were equivalent in all lanes (Fig. 4F).

3.5. Lck controls PYGM activation in Kit 225 T cells stimulated with IL-2

When PYGM activity was examined in 225 T cells treated with Lck or JAK1/3 inhibitors and stimulated by IL-2, it was observed that inhibition of JAK1/3 did not affect IL-2-stimulated PYGM activity (Fig. 5A). In contrast, Lck inhibitor efficiently blocked IL-2-stimulated PYGM activity (Fig. 5A).

In order to confirm the involvement of Lck in regulating PYGM activity, PYGM activation was determined in Kit 225 T cells overexpressing Lck (wt), Lck^{Y505F} (constitutively active form of Lck) and Lck^{K273R} (dominant negative form of Lck) with or without IL-2 stimulation. As shown in Fig. 5B, Lck (wt) overexpression alone was enough to partially stimulate PYGM activity. Additionally, Lck constitutively active mutant overexpression was found to activate PYGM to levels comparable to those reached by IL-2 stimulation. In fact, IL-2 stimulation of cells overexpressing Lck (wt) or Lck^{Y505F} did not increase PYGM activation any further. In contrast, Lck^{K273R} transfection completely abolished PYGM activation with or without IL-2 stimulation (Fig. 5B). Immunoblotting show Lck endogenous and ectopic expression levels of all forms of Lck. An equivalent amount of tubulin was used in each of the conditions analysed (Fig. 5B).

To confirm results obtained with Lck demonstrating that this tyrosine kinase functions also as a PYGM-activating molecule in Kit 225 T cells, Lck was knocked down with *lck*(*esiRNA*), as described above, and IL-2-stimulated PYGM activity in Lck-knockdown Kit 225 T cells was examined. The results presented in Fig. 5C show that IL-2 was unable to stimulate PYGM activation in the absence of Lck expression. Lck expression levels after *esiRNA* transfection is also shown. Tubulin blot analysis

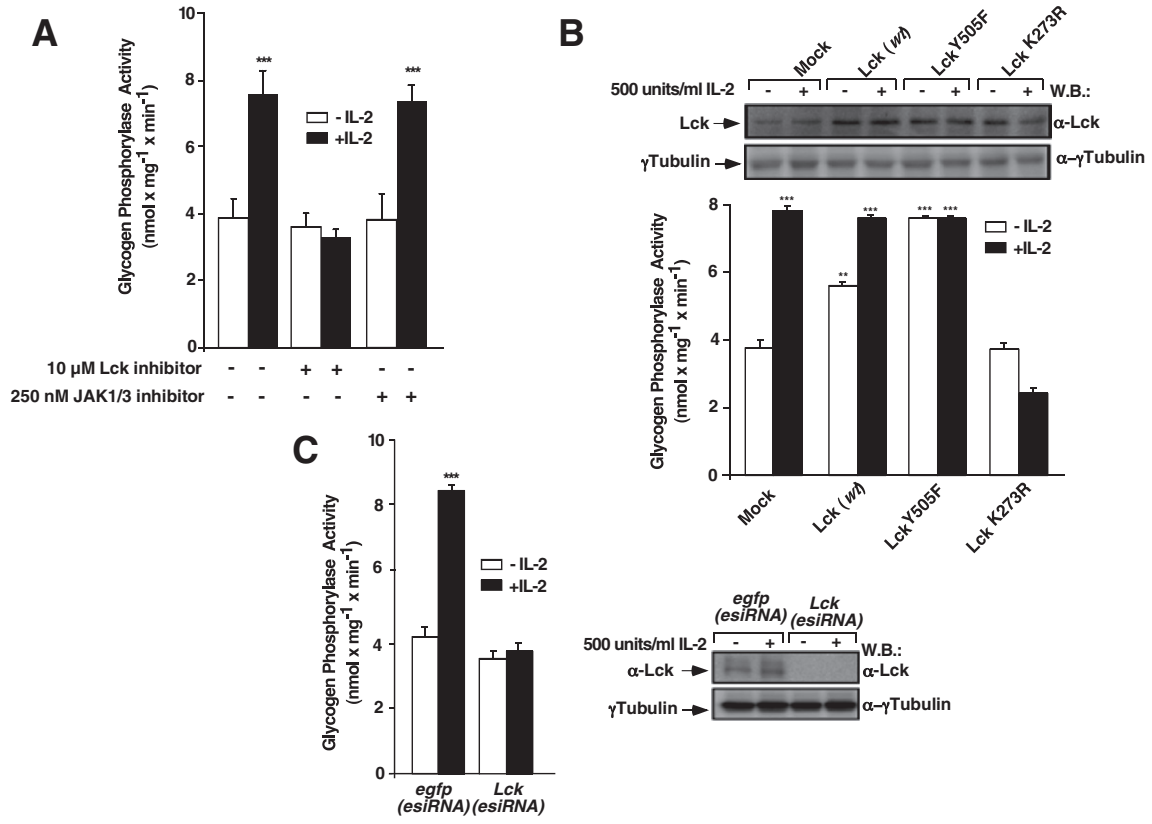

Fig. 5. Lck stimulates the glycogen phosphorylase activation in the IL-2 activated T cells. (*A*) The Kit 225 T cells deprived of IL-2 for 48 h were pretreated with the Lck inhibitor (10 μM), the JAK1/3 inhibitor (250 nM) or the vehicle (DMSO) for 1 h. Subsequently, cells were stimulated or not with 500 U/ml IL-2 for 10 min and lysed. The cell extracts from the unstimulated and the stimulated cells were used to measure the glycogen phosphorylase activity. The results show the mean of the three independent experiments ± S.D. and the statistical analysis shows a significant difference (***, $p < 0.001$). (*B*, *C*) The Kit 225 T cells deprived of IL-2 for 24 h were transfected with (*B*) the empty vector (mock control), or the different forms of Lck (i.e. wt, the constitutively and inactive mutants) and (*C*) *Lck*(*esiRNA*) and *egfp*(*esiRNA*) as control. 24 h post-transfection, cells were stimulated (+) or not (−) with 500 U/ml for 10 min and lysed. The cell extracts from the unstimulated and the stimulated cells were used to measure the glycogen phosphorylase activity. The protein expression levels were analysed by Western blotting using specific antibodies, as indicated. The results show the mean of the three independent experiments ± S.D. and the statistical analysis shows a significant difference (***, $p < 0.001$).

indicates that equivalent amounts of protein were used in SDS-PAGE analysis (Fig. 5C).

3.6. Lck and PLCγ1 are required for IL-2-stimulated chemotaxis and proliferation of Kit 225 T cells

To investigate the role of Lck and PLCγ1 in IL-2-stimulated Kit 225 T cell migration and proliferation, cells were transfected with *egfp* (negative control), *lck* or *plcγ1*(*esiRNAs*). In order to verify the efficiency of the RNA expression silencing, a cell sample from each condition was lysed and the whole cell lysates were separated by SDS-PAGE followed by Western blot. The expression levels of Lck and PLC γ1 were visualized using specific antibodies as indicated in Fig. 6A. Tubulin Western blot shows that equal amounts of protein were used in the analysis.

Another cell sample was used to examine the effects of *lck* and *plcγ1* expression silencing on the chemotaxis of IL-2-stimulated T cell through Polyethylene Terephthalate (PT) membranes. As shown in Fig. 6B, IL-2 stimulated robust migration of Kit 225 T cells. In contrast, lack of either Lck or PLCγ1 expression abolished IL-2-stimulated Kit 225 T cells migration. Finally, a third cell sample was used to evaluate the role of either Lck or PLCγ1 in IL-2-stimulated cell proliferation. The cellular response was analysed after flow cytometry by monitoring the decrease in fluorescence of the PKH6 dye incorporated into the cell membranes, which is diluted approximately 2-fold with each cell division. PKH26-labelled cells were treated with 16 U/ml IL-2 every 24 h for 3 days. As shown in Fig. 6C, IL-2 stimulation of control cells (*egfp*(*esiRNA*) transfected cells) cultured for 3 days resulted in approximately a 3-fold increase in the cell number compared to unstimulated cells. Remarkably, IL-2-stimulated cell proliferation was dramatically reduced with either *lck*(*esiRNA*) or with *plcγ1*(*esiRNA*) expression knockdown (Fig. 6C).

4. Discussion

This study identifies Lck as a tyrosine kinase upstream of Rac1 in IL-2-stimulated T cells and provides novel evidence demonstrating that PLCγ requires tyrosine phosphorylation by Lck in order to control the PKCθ/αPIX/Rac1/PYGM pathway, and thereby regulate T cell migration and proliferation.

Protein tyrosine phosphorylation/dephosphorylation cycles are one of the major events in early intracellular signalling. In order to activate STAT, MAPK and PI3K; the three major and best characterized signalling cascades (Fig. 7, grey layout) and control transcriptional activity regulating cell survival and T cell proliferation [1], receptors that do not have intrinsic tyrosine-kinase activity, like the IL-2 receptor, require binding to non-receptor tyrosine kinases, such as Janus Kinase family members 1 and 3 (JAK1/3) [32,33]. This canonical signalling network regulated by JAK1/3 is not unique. In fact, other tyrosine kinases linked to the IL-2-R are involved in T cell signalling, such as Lck [34–36]. Indeed, IL-2-stimulated T cells proliferation mediated by Lck via a MAP kinase-independent pathway was reported by Brockdorff et al. [3]. The findings presented here point in the same direction, i.e. pharmacological inhibition of JAK1 and 3 did not affect PLCγ1 and αPIX tyrosine- and serine-phosphorylation, respectively, and Rac1/PYGM-activation in IL-2-stimulated Kit 225 T cells. However, Lck activity inhibition blocked not only PLCγ1 tyrosine phosphorylation, but it also blocked αPIX-

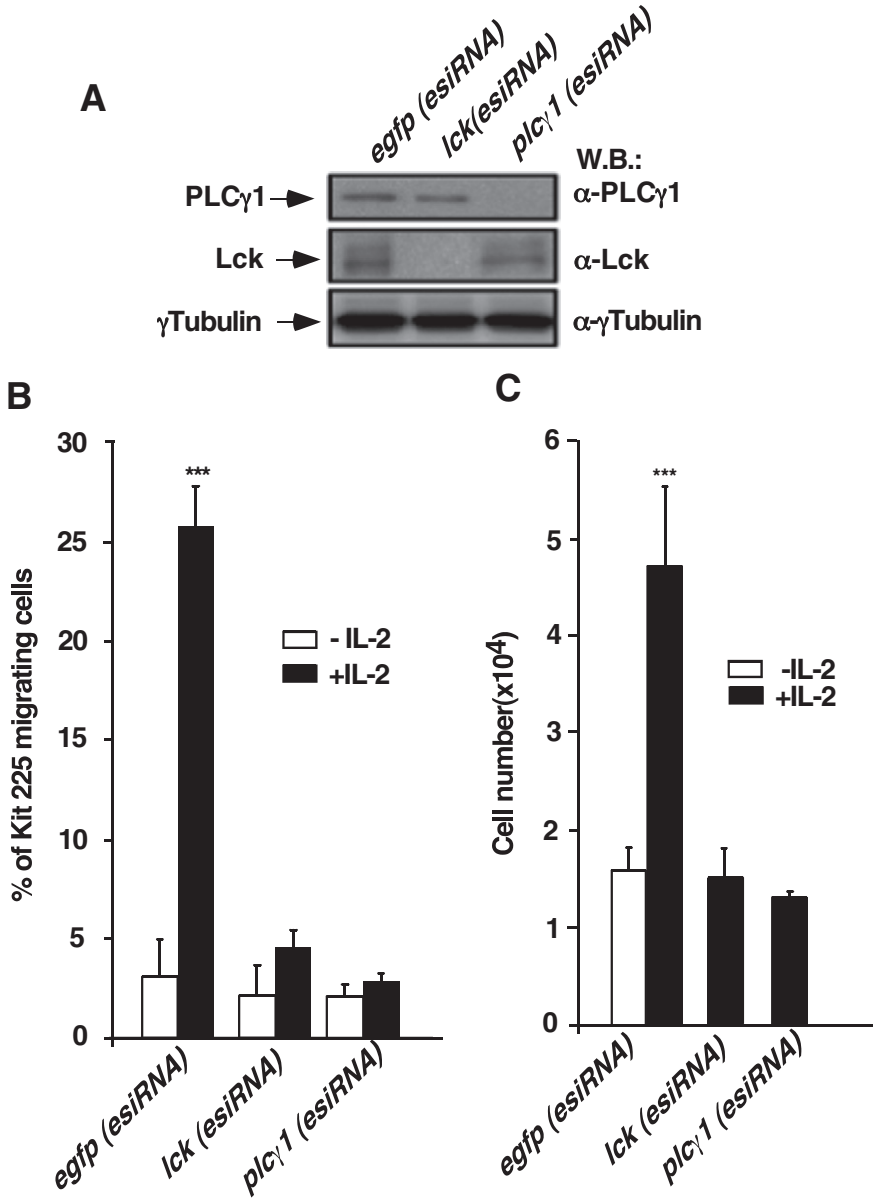

Fig. 6. The Lck/PLCγ1 pathway mediates the IL-2-stimulated Kit 225 chemotaxis and proliferation. (*A*) 3×10^5 Kit 225 T cells were taken for each transfection condition with *esiRNA* (i.e. *egfp*, *Lck*, *plcγ1*), lysed and the cell lysates were analysed by Western blot using specific antibodies, as indicated. (*B*) the *esiRNA* (*egfp*, *Lck*, *plcγ1*) transfected Kit 225 T cell migration was studied using a Transwell assay. The data representing the percentage of migrating cells is expressed as the mean of the four independent experiments ± S.D. and the statistical analysis shows a significant difference (***, $p < 0.001$). The cell lysates were analysed by Western blotting using specific antibodies, as indicated. (*C*) the *esiRNA*(*egfp*, *Lck*, *plcγ1*) transfected Kit 225 T cells were stained with PKH26. The fluorescence was analysed before the IL-2 stimulation (at 0 h and after the 72 h incubation or not with IL-2). The results represent the mean of the three independent experiments ± S.D. and the statistical analysis showed a significant difference (***, $p < 0.001$).

mediated Rac1/PYGM activation. The expression knockdown of either Lck or PLCγ1 strengthen these findings even further.

It is well established that signals emanating from receptors linked to tyrosine kinases such as TCR [37,38], BCR [39] and IL-2-R [40] actively regulate the enzymatic activation of PLCγ1 by tyrosine phosphorylation [34–36]. In fact, Veri et al. reported that in activated lymphocytes, Lck activation stimulated PLCγ1 tyrosine phosphorylation [41]. PLCγ1 controls IP_3 generation and intracellular calcium mobilization and ensuing PKC activation [21,22]. Notably, we reported previously that IL-2-R activation signals to the Rac1/PYGM pathway via PKCθ [6] in IL-2–stimulated T cells. Here a combined pharmacological and genetic approach demonstrates that PKCθ activation is mediated by PLCγ.

For some authors [42,43] but not all [24,44], the IL-2-stimulated cellular proliferation and migration depend on intracellular calcium mobilization. It is noteworthy that PKCθ serine threonine kinase belongs to the family of novel PKCs that are activated in a DAG-dependent and calcium-independent manner [45] and yet, it regulates the dynamics of the immunological synapse [46–48] and other functions, including: the control and clustering of integrin LFA-1 on the surface of T cells [49] facilitating stable adhesion between T cells and APCs [50–52], and T cell migration and proliferation [6].

Regardless the controversy, several lines of evidence indicate that IL-2-stimulated intracellular calcium mobilization is not a component of the signalling cascade presented here. Our data demonstrates that the IL-2/IL-2R engagement signals to the PKCθ/αPIX/Rac1/PYGM pathway via Lck tyrosine kinase activation independently of calcium mobilization or JAK1/3 activation in Kit 225 T cells.

Finally, taken our recent results demonstrating that PKCθ mediates Rac1/PYGM activation via αPIX phosphorylation in IL-2-stimulated Kit 225 T cells [6] together with the findings described in this study, we

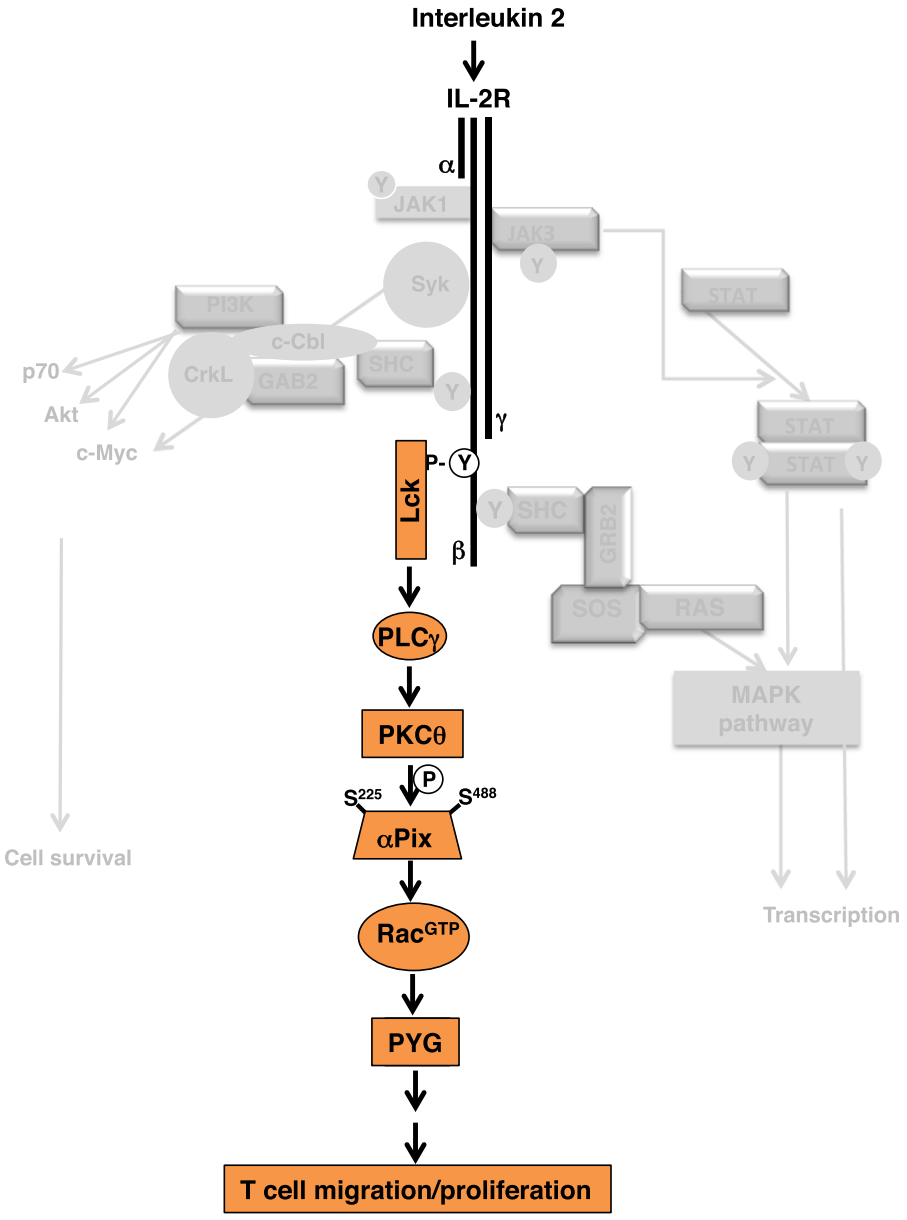

Fig. 7. The model of T cell migration and proliferation stimulated by the IL-2 receptor. The stimulation of IL-2 receptor leads to the activation of the three canonical pathways: PI3K, Ras/MAPK and STAT (in grey) controlled by JAK1/3 and with specific biological functions. The results presented here document another signalling pathway also stimulated by IL-2, independently of the three canonical pathways, constituted by Lck/PLCγ1/PKCθ/αPIX/Rac1/PYGM and regulating the T cell migration and proliferation.

propose a novel signalling pathway constituted by Lck/PLCγ1/PKCθ/αPIX/Rac1/PYGM and also governed by IL-2. The overall evidence indicates that this pathway participates in the control of T cell migration and proliferation (Fig. 7, orange layout), independently of the canonical pathways JAK/STAT, MAPK and PI3K. We hypothesize that the biological significance of this pathway, whose main element is the tandem constituted by Rac1/PYGM, could be attributed to the functioning of an early intracellular signalling pathway that regulates reversible glycosylation (O-GlcNAc) by serine and threonine residues in proteins, rather than ATP generation or synthesis of macromolecules [53]. The equilibrium between O-glycosylation/phosphorylation of proteins constitutes a nutrient sensor that modulates intracellular signalling, transcription and actin cytoskeleton modifications [54]. The imbalance between the levels of phosphorylation and glycosylation underlies pathologies such as human neurodegenerative diseases, Type 2 Diabetes, cancer, infectious diseases [55] and perhaps even some rare diseases like McArdle's disease. Future studies will allow us to characterize the signalling molecules downstream of PYGM that participate in this signal transduction pathway and its relevance to health and disease.

In conclusion, our findings open new prospects of modulating the control mechanisms of cell migration and proliferation in IL-2-stimulated T cells by targeting molecules of the Rac1 GTPase/PYGM pathway.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cellsig.2016.07.014.

Disclosure statement

The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions

F.L., H.M.L., LAP and JLZ conceived experiments; F.L. and A.A. performed the experiments; F.L., H.M.L., LAP and JLZ analysed the data;

J.L.Z. wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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