Rac1 Protein Regulates Glycogen Phosphorylase Activation and Controls Interleukin (IL)-2-dependent T Cell Proliferation*

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Background: Rac1 has a relevant role in signal transduction pathways in T lymphocytes.

Results: Rac1 GTPase associates with glycogen phosphorylase and modulates its enzymatic activity to trigger T cell proliferation.

Conclusion: This study reveals a new role for Rac1 GTPase in cellular physiology, coordinating metabolism and proliferation.

Significance: This new Rac1/PYGM pathway might be essential for an appropriate immune response.

Small GTPases of the Rho family have been implicated in important cellular processes such as cell migration and adhesion, protein secretion, and/or gene transcription. In the lymphoid system, these GTPases participate in the signaling cascades that are activated after engagement of antigen receptors. However, little is known about the role that Rho GTPases play in IL-2-mediated responses. Here, we show that IL-2 induces Rac1 activation in Kit 225 T cells. We identified by mass spectrometry the muscle isoform of glycogen phosphorylase (PYGM) as a novel Rac1 effector molecule in IL-2-stimulated cells. The interaction between the active form of Rac1 (Rac1-GTP) and PYGM was established directly through a domain comprising amino acids 191–270 of PYGM that exhibits significant homology with the Rac binding domain of PAK1. The integrity of this region was crucial for PYGM activation. Importantly, IL-2-dependent cellular proliferation was inhibited upon blocking both the activation of Rac1 and the activity of PYGM. These results reveal a new role for Rac1 in cell signaling, showing that this GTPase triggers T cell proliferation upon IL-2 stimulation by associating with PYGM and modulating its enzymatic activity.

Small GTPases of the Rho family, whose best characterized members include RhoA, Rac1, and Cdc42, are key players in the complex signaling networks that control the normal activity in all cell types (1). Like other small GTPases, Rho GTPases function as molecular switches that cycle 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. Signals emanating from a large variety of membrane receptors positively regulate Rho GTPase activities. Examples include receptors with intrinsic tyrosine kinase activity such as EGF receptor (2–4) and PDGF receptor (5, 6) or other types such as G protein-coupled receptors (7, 8). In their active configuration, GTPases interact with downstream effector molecules to promote a variety of biological responses (9). GTPases of the Rho family are best known for controlling the appropriate actin cytoskeleton reorganization in response to extracellular signals, although their implication in additional biological processes such as gene expression regulation, cell polarity, and cell migration has also been reported (1).

In the last few years, GTPases of the Rac subfamily (composed of Rac1, Rac2, Rac3, and RhoG) have gained increasing relevance in T cell biology (10, 11). It has been reported that T cell receptor engagement by antigen promotes the rapid activation of these GTPases together with an array of other signaling molecules (12, 13). Subsequently, Rac proteins transduce signals through intermediate signaling molecules, most notably PKCθ (14), phosphatidylinositol 3-kinase (PI3K) (5), calcium (15), and PLCγ1 (13). Importantly, Rac1 controls the translocation of RasGRP1 (a guanine nucleotide exchange factor for Ras) to the actin juxtamembrane structures to facilitate Ras/ERK pathway activation (12). The integration of these signaling events reaches the nucleus via AP-1, nuclear factor of activated T cells, NF-kB, and JNK. These transcription factors are known to actively participate in the transcription of the genes coding for IL-2 and the a chain of the IL-2 receptor, thus contributing to the clonal expansion of T cells (16–18).

In contrast to its well established participation in the T cell receptor-mediated activation program, the role of Rac1 in IL-2 signaling has not been clearly elucidated. IL-2 is a cytokine that plays a crucial role in the clonal proliferation of T lymphocytes...
(19). The binding of IL-2 to its high affinity receptor (IL-2R) triggers multiple signaling pathways, including the Janus kinase (Jak)/STAT, PI3K, and Ras/Raf/MAPK pathways, which are essential for cell cycle progression and inhibition of apoptosis (19). The JakS have been well documented for initiating the signaling from the activated IL-2R. According to the current IL-2 receptor signaling model, IL-2-activated JakS recruit critical Src homology 2 (SH2)-containing signaling mediators, leading to signal propagation in the cytoplasm. Tyrosine phosphorylation of STAT3 and STAT5 is mediated by Jak1 and Jak3 and leads to STATS dimerization followed by nuclear translocation and DNA binding (20–22). Additionally, IL-2 has been shown to mediate activation of the PI3K/AKT (protein kinase B) pathway, which regulates downstream signaling molecules such as p70S6K and mammalian target of rapamycin required for activation of the cell cycle regulator E2F and subsequent cell cycle progression (23).

The role of Ras in the signaling cascades initiated by IL-2 is also well established. Upon IL-2/IL-2R ligation, the adapter protein Shc is anchored to the phosphorylated IL-2R β chain (24, 25). Subsequently, Shc becomes tyrosine phosphorylated, allowing the recruitment of the Grb2-Son of Seven less complex, which mediates the activation of the Ras/Raf/MAPK pathway. Activation of this cascade leads to phosphorylation and activation of transcription factors such as AP-1, Elk-1, and Myc, which in turn regulate the expression of genes involved in cell proliferation (26–29). To accomplish this cellular response, T cells possibly require not only the Ras/MAPK pathway but also a complex cooperation with other signaling networks, including some GTPases of the Rho family. In fact, it has been reported that RhoA cooperates with the ERK-dependent signaling pathways to transcribe c-fos in response to IL-2 (30). Moreover, Rac1 has been found to participate in IL-2-induced actin cytoskeleton rearrangement in a murine T cell line (31). However, the relevance of this Rac1-mediated response in T cell proliferation is still unclear.

Here we show that Rac1 is rapidly activated in Kit 225 cells, an IL-2-dependent human T cell line, following exposure to IL-2. GST pulldown assays using a constitutively active form of Rac1 (Rac1G12V) followed by mass spectrometry analysis led to the identification of the muscle isoform of glycogen phosphorylase (PYGM) as a new specific effector molecule for the active form of Rac1 (Rac1-GTP) in IL-2-activated cells. We characterized the interactive domain of PYGM with Rac1. This domain exhibits significant homology with the interactive domain of PKA1, an effector molecule for the active forms of Rac1 and Cdc42. Furthermore, Rac1-GTP (active form)/PYGM association was crucial for PYGM activation and subsequent cellular proliferation. These results show an unsuspected connection between Rac1 GTPase and glycogen metabolism through PYGM and imply that PYGM may function downstream of Rac1 in a novel signal transduction pathway regulating IL-2-dependent T cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Rac1 inhibitor NCS23766 N-[N-(3,5-difluorophenacetyl-1-α-α)-S-phenylglycine t-butyl ester and glycogen phosphorylase inhibitor (1-(3-(3-(2-chloro-4,5-difluorobenzoyl)ureido)-4-methoxyphenyl)-3-methy]urea) were obtained from Calbiochem. PKA inhibitor H-89 dihydrochloride and adenylate cyclase activator forskolin were obtained from Sigma-Aldrich. Mouse monoclonal anti-AU5 and anti-HA antibodies were obtained from Covance, mouse monoclonal anti-FLAG were from Sigma-Aldrich, mouse monoclonal anti-Cdc42 and anti-Rac1 antibodies were from Upstate, mouse monoclonal anti-GFP antibodies were from Molecular Probes, rabbit polyclonal anti-RhoA and anti-PYGM (U-23) antibodies were from Santa Cruz Biotechnology, Inc., and enhanced chemiluminescence (ECL) reagent was from GE Healthcare. IL-2 cytokine was kindly provided by the “AIDS Research and Reference Reagent Program,” Division of AIDS (NIAD, National Institutes of Health).

**Cell Culture and DNA Transfection**—Kit 225 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 220 mg/liter sodium pyruvate, 2 mM l-glutamine, 100 ng/ml streptomycin, and 100 units/ml of penicillin (all from Invitrogen) in the presence of 16 units/ml recombinant human IL-2 (32). For transient transfection assays, cells were washed twice with PBS and cultured in complete RPMI 1640 medium in the absence of IL-2 for 24 h. After this period, cells (2 × 10^7) were washed twice, resuspended in 200 µl of serum-free medium, and placed in an electroporation cuvette (0.4 mm’ Sigma-Aldrich) containing 10–20 µg of DNA. The cells and DNA mixture were electroporated at 260 V, 950 microfarads in a Gene Pulser Xcell Electroporator (Bio-Rad). The cuvette content was collected into 10 ml of complete RPMI 1640 medium and cultured in the absence of IL-2 for an additional 24 h.

**Agonists and Inhibitors**—Kit 225 cells were maintained in the absence of IL-2 for 48 h and subsequently stimulated with 500 units/ml IL-2 at 37 °C as indicated under “Results” (33). In some experiments, Kit 225 cells were pretreated with 50 µM NCS23766 (Rac1 inhibitor) (34) or 10 µM glycogen phosphorylase inhibitor (GPI) (35) for 16 h and with 50 µM H89 (PKA inhibitor) (36) for 1 h prior to IL-2 or 10 µM forskolin (37) stimulation.

**Plasmid Constructs**—pCEFL-HA-PYGM-FL and pCEFL-HA-PYGMABRD constructs were generated by PCR amplification from pCR4-TOPO-PYGM (Gene Service, UK) and cloned into pCEFL-HA vector. PCR amplifications were carried out with Elongase® enzyme mixture following the manufacturer’s instructions (Invitrogen). Amplified DNA was purified by agarose gel electrophoresis and extracted using the QIAquick extraction kit from Qiagen. The oligonucleotides used for full-length PYGM (PYGM-FL) amplification were as follows: 5’-ATGAAATTCATGTCGCCGGCCCCCTGTCAGAC-3’ (forward) and 5’-AGTTACGATCATGATGCCCTCATC-CCGGGCT-3’ (reverse), which contained EcoRI and XbaI restriction sites (underscored), respectively. The EcoRI/XbaI

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*The abbreviations used are: PYGM, muscle isoform of glycogen phosphorylase; CRIB, Cdc42/Rac1 interactive binding domain; PYGL, liver-specific PYG isoform; RBD, Rho/Rac binding domain; YWHAZ, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide; IL-2R, IL-2 receptor; GPI, glycogen phosphorylase inhibitor; PYGM-FL, full-length PYGM; WASP, Wiskott–Aldrich syndrome protein; EGFP, enhanced GFP; PKA, p21-activated kinase.*
PYGM fragment was subcloned into the pCEFL-HA vector digested with the same enzymes (Fermentas). The pCEFL-HA-PYGM*RRDD* construct was amplified in two steps. First, the N-terminal fragment (nucleotides 817–1359) was amplified (the forward oligonucleotide was the same as for PYGM-FL; the reverse oligonucleotide was 5′-AAGATATCGTGGCGTAGGAAAGCCATGTCAG-3′, which contained an EcoRV (underscored) restriction site). The EcoRI/EcoRV N-terminal fragment was subcloned into pCEFL-HA vector digested with the same restriction enzymes to generate pCEFL-HA-PYGM-Nt. Next, the C-terminal fragment (nucleotides 1761–3346) of PYGM was amplified (the forward primer was 5′-GAAGATATCGTGGCGTAGGAAAGCCATGTCAG-3′, which contained an EcoRV (underscored) restriction site; the reverse primer was the same as that for PYGM-FL). The EcoRV/XbaI fragment was subcloned into the pCEFL-HA-PYGM-Nt vector digested with the same enzymes to generate the pCEFL-HA-PYGM*RRDD* construct. The pEGFP-PYGM and pEGFP-PYGM*RRDD* constructs were inserted into the pCEFL-HA constructs into the pEGFP-C2 backbone using EcoRI and XbaI restriction enzymes. The fusion protein GST-Rac1 binding domain (RBD)-like domain of PYGM (nucleotides 1360–1760) was generated by PCR amplification from pCR4-TOPO-PYGM (the forward oligonucleotide was 5′-GGAGAATCTGACTG-GCTCGTCTGACCGCAA-3′, and the reverse oligonucleotide was 5′-ATCGAATTCATGACGGATTCCATAGGATG-3′, each harboring EcoRI restriction sites (underscored)). The EcoRI RBD-like fragment was subcloned into pGEX-4T3 (GE Healthcare).

RNA Isolation, RT-PCR, and TaqMan Gene Expression Assays—Total RNA isolation was performed using TRIzol extraction (Invitrogen), and cDNA synthesis was carried out using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer’s instructions. TaqMan gene expression assays for the liver-specific PYG isoform (PYGL) (Hs00958093_m1), PYGM (Hs00989942_m1), and control gene YWHAZ (Hs00237047_m1) were carried out following the manufacturer’s instructions for 40 cycles (95 °C for 15 s and 60 °C for 1 min) after an initial incubation at 95 °C for 10 min in the 7000 Fast Real-Time PCR System (Applied Biosystems) and analyzed with the ABI Prism 7000 SDS software.

RhoA, Rac1, and Cdc42 Activation Assays—Effector molecules for Rho family GTPases contain within their amino acid sequence a region called PBD (p21-Rho binding domain) that binds specifically to the active conformation of GTPases (38). A RhoA pulldown assay was performed using a GST fusion protein containing the Rho binding domain of Rothekin (GSTMARDD-RTKN). A Rac1 pulldown assay was performed using a GST fusion protein containing the Rac1 binding domain of PAK1 (GSTMARDD-PAK1). A Cdc42 pulldown assay was performed using a GST fusion protein containing the Cdc42/Rac1 interactive binding domain of WASP (GSTMARDD-WASP). Cells kept in the absence of IL-2 for 48 h were stimulated with IL-2 for the indicated times and lysed as described previously (39). Cell lysates were centrifuged at 13,500 rpm for 15 min at 4 °C and incubated for 1 h at 4 °C with 50 μg of GST fusion proteins coupled to glutathione-Sepharose beads. Precipitated proteins were eluted from beads using 2× Laemmli 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 immunoblot with specific antibodies. Immune-reactive bands were visualized using ECL.

Affinity Precipitation of Proteins Bound to Active Rac1—Unstimulated or IL-2-stimulated cells were lysed in cold cell lysis buffer (10 μM Tris, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 10 mM MgCl2, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin). Cell lysates were centrifuged at 13,500 rpm for 10 min at 4 °C and subsequently incubated for 1 h at 4 °C with GST-Rac1G12V as indicated above. Precipitated proteins were washed three times, resuspended in 2× Laemmli buffer, and separated by resolutive SDS-PAGE. Gels were fixed with a solution containing 50% methanol, 10% acetic acid for 1 h. After three washes with ultrapure water, gels were stained using the ProQ Diamond phosphospecific fluorescent gel stain (Molecular Probes, Invitrogen) for 1 h and 30 min under gentle agitation. Gels were washed three times in 20% acetic alcohol, 50 mM sodium acetate, pH 4 for 30 min each time. After two washes of 5 min each with ultrapure water, stained proteins were visualized in a Typhoon Trio 9210 scanner (GE Healthcare).

Tryptsin Digestion and Mass Spectrometry Identification of Peptides—Protein bands were manually excised and identified by the Proteomics Facility of CIC bioGUNE (Derio, Spain). Briefly, tryptic digestion was performed using Proteineer dp (Bruker Daltonics), and recovered peptides were purified prior to MALDI analysis by reverse phase material nanocolumns casted on site (POROS R2, Applied Biosystems). Peptides were eluted directly from columns onto the MALDI target for peptide mass fingerprinting or peptide fragment fingerprinting on a Bruker Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics).

Protein identification was attained by searching a non-redundant protein database with an in-home Mascot search engine (Matrix Science). Protein identities were obtained by searching against Swiss-Prot (51.3) non-redundant databases selected for human taxonomy. Up to two missed cleavages were allowed, oxidation of methionine was set as a variable modification, and carboxyamidomethylation of cysteine was set as a fixed modification. External mass calibration was performed by default, achieving a typical mass accuracy of <50 ppm. When possible, internal mass calibration was performed by using trypsin autodigestion peptides, allowing a typical increase of mass accuracy lower than 30 ppm. For protein positive identification, besides considering the Mascot scores from protein mass fingerprinting (40), other criteria such as sequence coverage and the delta score to the second protein match were taken into account. For peptide fragment fingerprinting, 50 or 30 ppm (depending on the calibration) for precursor ion and 0.6 Da for fragment ion mass accuracies were chosen. Peptides were excluded if their masses corresponded to human keratins and porcine trypsin fragments after internal calibration.

Activity Assay for Glycogen Phosphorylase—A glycogen phosphorylase activity assay was performed as indicated (41, 42) with some modifications. 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
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sonicated and centrifuged at 13,500 rpm for 10 min at 4 °C. Total protein (100 μg) was used to measure PYGM activity in assay buffer (50 mM K2HPO4, pH 7.5, 10 mM MgCl2, 5 mM EDTA pH 8, 0.5 mM NADP+, 1.5 units/ml glucose-6-phosphate dehydrogenase, 1 units/ml phosphoglucomutase, 0.1 mg/ml glycogen (all from Sigma-Aldrich). 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. The metabolic activity assay was carried out by incubating the mixture at 37 °C for 20 min. The reaction was stopped by placing samples on ice. Sample absorbances were 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).

ClustalW Alignments—Multiple sequence alignments were performed with ClustalW2 (43) using default parameters of KTUP and gap opening and gap extension penalties. The presence of identical amino acids in a given position is marked by an asterisk (*), the presence of amino acids belonging to the same “weak” group is marked by a colon (:), and the presence of a “strong” group is marked by a single dot (.). Strong groups are given a score of >0.5 (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, and FYW), weak groups are given a score of ≤0.5 (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLM, and HFY).

Cell Proliferation Measurement—Cells were seeded in 24-well plates in complete RPMI 1640 medium (106 cells/ml) and maintained in the absence of IL-2 for 48 h to synchronize them in G0/G1. After this period, cells (106) were incubated with PKH26 (4 μM) following the manufacturer’s instructions (Sigma-Aldrich). 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 absence of IL-2. The inhibitors GPI (10 μM) and NCS23677 (50 μM) were added every 24 h. For EGFP-expressing transfectants, 40 × 106 cells were transfected with EGFP-expressing constructs as mentioned before. The day after transfection, live cells were purified by Ficoll gradient following the manufacturer’s instructions (GE Healthcare). Purified cells were maintained in the absence of IL-2 for 48 h after which they were incubated with PKH26 as explained above. A sample was taken from the non-stimulated control, and the rest of the cells were grown in the presence of IL-2. Fluorescence was measured every day for 4 days or 48 h in EGFP-construct transfected cells to monitor the cell division rate on a FACSCalibur (BD Biosciences) flow cytometer. Data obtained were analyzed using ModFit LT 3.0.

Determination of Glycogen Content—Cells were cultured in 24-well plates in complete RPMI 1640 medium (106/ml) in the absence of IL-2 for 48 h and subsequently treated with vehicle (0.08% DMSO) or GPI (10 μM) every 24 h in the presence of IL-2 (16 units/ml). Cells were counted every 48 h, and glycogen content was determined as described (44). Briefly, cells were centrifuged, resuspended in 0.4 ml of 30% KOH, and incubated for 15 min at 95 °C to extract glycogen. Afterward, 0.2 ml of 2% Na2SO4 was added, and glycogen was precipitated with 1.2 ml of ethanol. The precipitate was resuspended in 1 ml of H2O/ 

RESULTS

Cytokine IL-2 Leads to Rac1 Activation—To examine IL-2-activated signaling pathways, we used Kit 225 cells, a human T cell line established from a patient with T cell chronic lymphocytic leukemia. Kit 225 cells express IL-2R constitutively and depend exclusively on IL-2 for cellular proliferation (32). This feature represents an important advantage for IL-2-mediated signaling studies. To test whether engagement of IL-2 with its receptor stimulated Rac1 activation, Kit 225 cells were synchronized in G0/G1 by depriving them of IL-2 for 48 h and stimulated subsequently with 500 units/ml IL-2 to allow for entry and progression into the cell cycle. At several time points, cells were lysed, and lysates were mixed with a GST-RBD of PAK1 (GST-RBD-PAK1). Pulled down Rac1-GTP was visualized by immunoblotting using anti-Rac1 antibodies. As shown in Fig. 1A, a significant increase in the active form of Rac1 was observed 1 min after IL-2 stimulation, reaching maximal activation after 5 min of cytokine addition. This activation level was maintained for at least 10 min after stimulation (Fig. 1A).

Activated Rac1 Associates with PYGM—To isolate potential effector molecules for Rac1 after IL-2 stimulation, lysates of untreated or IL-2-stimulated Kit 225 cells were incubated with GST-Rac1G12V (a constitutively active form of Rac1 that mimics the Rac1-GTP configuration) and subsequently precipitated with glutathione-Sepharose. The affinity-precipitated proteins were separated by one-dimensional resolutive SDS-PAGE and visualized using the ProQ Diamond fluorescent dye. We observed several intense bands in the extracts from IL-2-stimulated cells compared with nonstimulated cells (Fig. 1B). We focused our attention on a band with an apparent molecular mass of 97 kDa (Fig. 1B, see arrow). This band was excised from the acrylamide gel followed by in-gel trypsin digestion. The resulting peptides were subjected to mass spectrometry (Fig. 1C). Mascot software was used to compare the mass per charge (m/z) ratios observed by peptide mass fingerprinting analysis against the databases of non-redundant protein sequences theoretically digested with trypsin (SwissProt 51.3). Peptides derived from the digestion of the selected gel-excised band (Fig. 1B) were identified as the metabolic enzyme glycogen phosphorylase, in particular PYGM (EC 2.4.1.1) (Fig. 1D). The same result was obtained in three independent biological replicates.

Mascot defines a significant identification match using the Mowse algorithm (40). The total score of a protein is the absolute probability that the overlap between experimental masses and protein masses listed in the database is a random event. Given these constraints, a probabilistic limit for nonspecific protein was set at 67 (p < 0.05). Under these conditions, a value of 115 was obtained. Another piece of evidence that supports
our finding is that the molecular mass of the identified enzyme PYGM (97.1 kDa) is very similar to the one predicted for the excised band. And finally, the 12 peptides identified in the mass spectrometry analysis could be assigned to the sequence of PYGM, covering up to 12% of the full sequence of PYGM (Fig. 1, C and D). Additionally, we confirmed that the identified protein was PYGM and not the liver or the brain isofor because the peptide EIWGVEPSR (amino acids 824–832) generated by trypsin digestion and sequenced by peptide fragment fingerprinting is specific to the muscle isofrom (Fig. 1 C, inset). These results suggest that PYGM may participate in Rac1-mediated signaling upon IL-2 stimulation.

It has been reported that human leukocytes express mainly PYGL with only trace amounts of PYGM and the brain isofrom (PYGB) (45, 46). Given that the isofrom associated to Rac1 identified by mass spectrometry was the muscle-specific PYG, we verified its endogenous expression levels in Kit 225 cells. A TaqMan-based expression assay was performed for PYGL and
PYGM genes in mRNA samples derived from Kit 225 cells deprived of IL-2 for 48 h or activated with 16 units/ml IL-2 for 24 h after synchronization (Fig. 1E). Gene expression levels were normalized with a housekeeping gene (YWHAZ) whose expression remains unchanged in Kit 225 cells regardless of the activation status. Quantitative reverse transcription-PCR analysis showed that Kit 225 cells express PYGM in addition to PYG. In fact, the expression of PYGM is substantially higher than that of PYGL. Furthermore, this result showed that PYGM expression is not regulated by IL-2 stimulation.

**PYGM Is Specific Effector Molecule for Active Form of Rac1—** We searched for a possible sequence homology between PYGM and PAK1, a known Rac1-interacting molecule. Sequence alignment was performed using the ClustalW2 alignment tool (43). Inspection of the ClustalW2 alignments led to the identification of a 79-amino acid stretch in PYGM (amino acids 191–270) with nearly a 40% identity with the RBD of PAK1 (Fig. 2B, upper alignment). This homology indicates that this region could be interacting directly with Rac1. This possibility was further supported by the observation that 4 of the 7 amino acids of PAK1 that interact directly with GTPases are also present in PYGM (47) (Fig. 2B, upper alignment, underscored and with an asterisk). The level of similarity between PYGM and PAK1 is substantially higher than that exhibited by other Rho GTPase effector molecules relative to the RBD of PAK1 (20% in the case of WASP and 15% in the case of Rhotekin) (48, 49). When the 79-amino acid stretch of PYGM was compared with other Rho effector molecules, sequence similarity dropped considerably (8% in the case of RBD of Rhotekin and 21% in the case of C-terminus of RhoA) (Fig. 2B, middle and lower alignments). Nevertheless, despite the lower level of similarity with these effector molecules, 5 of the 7 crucial amino acids of WASP that interact directly with Cdc42 (50) were also conserved in PYGM (Fig. 2B, lower alignment, underscored and with an asterisk). Moreover, in the 79-amino acid stretch of PYGM, 4 of the 8 core amino acids of the consensus sequence of the Cdc42/Rac1 interactive binding motif of PAK1 are conserved (EKAPEFPLVH-PYGHVEHTS) (38). Together, these results suggest that PYGM may be an effector molecule for Rho family GTPases.

To clarify whether the amino acid stretch between positions 191 and 270 of PYGM could represent an effective binding domain for Rac1 and other Rho GTPases, a GST fusion protein with this region was generated (GST-RBD-like PYGM) (38). Together, these results suggest that PYGM activity in response to IL-2, we pretreated the cells with H89 (50 μM), a pharmacological inhibitor of PAK (36), and stimulated cells for 10 min with IL-2 or forskolin (10 μM; an activator of adenylate cyclase) (37). Forskolin induced glycolysis and phosphorylation activation via the PAK-mediated canonical activation pathway, which was blocked by the pretreatment with H89 (Fig. 3B, gray bars). However, IL-2-induced glycolysis phosphorylation activation did not seem to be mediated by PAK-induced signals as H89 treatment did not block the activation (Fig. 3B, black bars). These results suggested an alternate activation mechanism for PAG in which the active form of Rac1 could have a determinant role.

To examine the possibility that Rac1 could be activating PYGM upon IL-2 stimulation, we co-transfected Kit 225 cells with an expression construct carrying full-length PYGM (pCEFL-HA-PYGM-FL) and wild type Rac1 (AU5-Rac1). Another set of cells was co-transfected with pCEFL-HA-PYGM-FL and a dominant negative form of Rac1 (FLAG-Rac1T17N). To establish the kinetics of PYGM activation, cells were stimulated with IL-2 at several time points (0, 10, 15, 20, 30, and 60 min) after 48 h of IL-2 deprivation. As shown in Fig. 3C (closed squares), PYGM exhibited a peak of maximal activity

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after 10 min of IL-2 stimulation, coincident with Rac1 activation. Subsequently, it returned to base-line levels after 15 min. Conversely, the co-expression of HA-PYGM-FL with FLAG-Rac1T17N produced no change in PYGM activity at any of the time points measured (Fig. 3C, open squares). This result suggests that Rac1 needs to be activated to modulate PYGM activ-
ity after IL-2 stimulation in Kit 225 cells. Because Rac1 can activate several signaling cascades simultaneously (54), we searched for possible intermediary effector pathways leading to PYG activation. EGFP-tagged Rac1 WT, Rac1T17N, a constitutively active form of Rac1 (Rac1Q61L), and two constitutively active Rac1 effector mutants (Rac1F37A/Q61L and Rac1Y40C/Q61L) that can discriminate between different signaling routes were transfected into Kit 225 cells. Rac1F37A/Q61L is able to activate PAK and JNK but cannot signal to the cytoskeleton, and it is not involved in cell transformation processes.

**FIGURE 3.** IL-2 modulates glycogen phosphorylase activity in Kit 225 cells through Rac1. A, extracts from unstimulated (−) or stimulated cells (+) (500 units/ml IL-2 for 10 min) were used to measure glycogen phosphorylase activity as described under “Experimental Procedures.” Results show the mean of four independent experiments ± S.D. (**, p < 0.01). B, Kit 225 cells were pretreated with H89 (50 μM) for 1 h and stimulated with forskolin (10 μM; gray columns) or IL-2 (500 units/ml; black columns) for 10 min or left non-stimulated (white column). Cell extracts were used to measure glycogen phosphorylase activity as described under “Experimental Procedures.” Results show the mean of three independent experiments ± S.D. (*, p < 0.05; **, p < 0.01). C, Kit 225 cells were co-transfected with plasmids coding for HA-PYGM-FL, AU5-Rac1 WT, or FLAG-Rac1T17N and HA-PYGM RBD with AU5-Rac1 WT. Glycogen phosphorylase activity was measured in non-stimulated cells or cells stimulated with IL-2 for the indicated times. Expression levels of transfected constructs were analyzed by Western blot using antibodies specific for HA, FLAG, and AU5 epitopes as indicated. The results show the mean of three independent experiments ± S.D., and the statistical analysis showed a significant difference (*, p < 0.05). D, Kit 225 cells were transfected with plasmids coding for EGFP-Rac1 WT, EGFP-Rac1T17N, EGFP-Rac1F37A/Q61L, EGFP-Rac1Y40C/Q61L, or EGFP-Rac1T17N. Glycogen phosphorylase activity was measured in non-stimulated cells or cells stimulated with IL-2 for 10 min. The results show the mean of three independent experiments ± S.D. (**, p < 0.05; ***, p < 0.01). As a control, the expression levels of transfected constructs were analyzed by Western blot using GFP-specific antibodies.
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The other hand, Rac1Y40C+Q61L can control cytoskeletal changes and tumorigenesis without mediating PAK or JNK kinase activation (55, 56). Expression of EGFP-Rac1T17N produced no change in PYG activity either in control or in IL-2-stimulated cells. However, expression of Rac1Q61L resulted in a significant increase in PYG activity compared with the control cells that was in the same range of activity as the positive control (cells co-expressing wild type Rac1 and stimulated with IL-2) (Fig. 3D). Finally, the effects of Rac1F37A+Q61L and Rac1Y40C+Q61L on PYG activity were comparable with those displayed by Rac1Q61L (Fig. 3D). Taken together, these results suggest that neither PAK nor classical downstream Rac1 pathways such as PAK kinases and/or cytoskeletal reorganization are necessary to regulate IL-2-stimulated PYG activation.

In parallel experiments, Kit225 cells were co-transfected with an expression construct carrying a deleted fragment of pygm lacking the RBD-like domain (pCEFL-HA-PYGMΔRBD) and wild type AU5-Rac1 and analyzed under the same conditions as described above. Cells co-transfected with these constructs were unable to induce glycogen phosphorylase activity (Fig. 3C, closed circles), implying that the integrity of the RBD-like domain of PYGM is necessary for the induction of its enzymatic activity.

Role of Rac1/PYGM Pathway in Kit 225 Cell Proliferation—We next considered the possibility that the Rac1/PYGM pathway could be implicated in the regulation of IL-2-stimulated cell proliferation. First, the effect of the Rac1 inhibitor NCS23766 (34) on PYGM activity was tested. For these experiments, the PYGM inhibitor GPI was also used (35) as a control. Kit 225 cells that were deprived of IL-2 for 32 h were treated with vehicle (DMSO), 50 μM NCS23766, or 10 μM GPI for 16 h in the absence of IL-2. After this time, cells were stimulated with 50 units/ml IL-2 for 10 min at 37 °C. Cells lysates (100 μg of total protein/sample) were used to measure PYGM activity as described under “Experimental Procedures.” Remarkably, the Rac1 inhibitor NCS23766 was able to efficiently block PYGM activity to the same extent as GPI (Fig. 4A, histogram). In parallel control experiments, Rac1-GTP was pulled down using GST-RBD of PAK1, and results confirmed that NCS23766 inhibited Rac1 activation induced by IL-2 (Fig. 4A, upper panel, lane 4).

Once the effect of Rac1 and PYGM inhibitors was tested, Kit 225 proliferation induced by IL-2 in the presence of these inhibitors was examined. Cell proliferation was analyzed after flow cytometry by monitoring the decrease in fluorescence of the incorporated membrane dye PKH26, which is diluted 2-fold with each cell division. PKH26-labeled cells were treated with 16 units/ml IL-2 every 48 h for 4 days. The inhibitors GPI (10 μM) and NCS23677 (50 μM) were added every 24 h. In control cultures, IL-2 addition resulted in close to a 6-fold increase in glycogen synthase activity in these cells. Interestingly, inhibition of PYGM by GPI treatment resulted in a nearly 2-fold increase in cytoplasmic glycogen accumulation compared with non-treated cells (Fig. 4B), suggesting an increase in glycogen synthase activity in these cells.

To evaluate the role of Rac1-PYGM interaction in cellular proliferation expression vectors for Rac1, PYGM WT and the PYGMΔRBD as well as the RBD domain of PAK1 were utilized. These constructs were tagged with EGFP to allow us to select GFP+ cells by flow cytometry. Cells overexpressing Rac1 and PYGM WT proliferated at a slightly higher rate than IL-2-stimulated control cells expressing EGFP alone (Fig. 4D). Nevertheless, cells overexpressing the RBD-like deletion mutant of PYGM (PYGMΔRBD) exhibited a significantly reduced proliferation capacity after IL-2 stimulation, indicating that Rac1-PYGM interaction through the RBD-like domain of PYGM is necessary to mediate Kit 225 cell proliferation induced by IL-2. We also examined whether occupation of the Rac1 effector domain that signals to PAK affected cell proliferation induced by IL-2. Cells overexpressing a construct carrying the RBD domain of PAK1 exhibited a proliferation rate comparable with that of control cells expressing EGFP alone (Fig. 4D), implying that occupation of the Rac1 effector domain that signals to PAK does not affect cell proliferation induced by IL-2. Taken together, these results suggest a new role for Rac1 in the direct activation of a metabolic pathway involving glycogen phosphorylase, which participates in the proliferative response stimulated by IL-2.

DISCUSSION

The functional specificity of small GTPases of the Rho family in intracellular signaling pathways depends mainly on the cellular system, type of stimuli, and their intracellular localization. Regarding the lymphoid system, GTPases of the Rho family participate actively in the proper lymphocyte activation after antigenic stimulation, but their role in IL-2-mediated stimulation is less clear (16–18). In the present study, we show that IL-2/IL-2R engagement induced a rapid and robust Rac1 activation, triggering a signaling cascade that results in cellular proliferation. Importantly, we identified PYGM as a specific effector molecule for Rac1 in IL-2-stimulated cells and provide novel evidence supporting the relevance of the Rac1/PYGM axis in T cell proliferation induced by IL-2.

Signals emanating from membrane receptors frequently lead to the activation of more than one GTPase, thus ensuring a rapid and efficient pleiotropic response (9). However, the kinetics of activation of these GTPases often differs significantly from one another. For instance, a sequential activation has been described during actin polymerization in fibroblasts whereby Cdc42 operates upstream of Rac1, which is followed by RhoA (57). By contrast, integrin-mediated adhesion signaling results in an early activation of Rac1 and Cdc42 and a later activation of RhoA (58). Our results demonstrate a rapid and robust activation of Cdc42 upon IL-2 stimulation that was concomitant with Rac1 activation. In contrast, no RhoA activation was observed in our cells after a short stimulation with IL-2. However, we cannot exclude the possibility of RhoA activation with longer stimulation in these cells as RhoA is involved in the transcription of c-fos in Kit 225 cells (30). Despite the similarity in the
early activation of Rac1 and Cdc42 in Kit 225 cells, it is clear that their downstream pathways diverge very shortly given that only Rac1 is able to interact with PYGM and modulate its activity.

It is believed that the functional specificity of the GTPases is provided by the interaction that they establish with specific guanine nucleotide exchange factors and effector molecules (9). Thus, there is an increasing interest to identify the protein partners with which GTPases associate. Many of these protein partners have been identified by non-hypothesis-driven proteomics approaches (59, 60). The proteomics methodology used in this work to identify PYGM involves affinity precipitation followed by mass spectrometry (shotgun proteomics) and has emerged as a powerful method to screen for putative protein-protein interactions because of its sensitivity (61). Moreover, we consistently identified the presence of GST-RBD-PYGM™, in the three biological replicates that were analyzed by Western blot. The cell fluorescence level was analyzed on a FACS Calibur cytometer, and data were analyzed using ModFit LT 3.0 software. The curve represents cell number over time (days). Results show the mean of three independent experiments ± S.D. D, proliferation analysis of transfected cells with the vital dye PKH26. Cells transfected with the empty vector pEGFP-C2 or the different gene-expressing vectors, pEGFP-RBD-PAK1, pEGFP-Rac1, pEGFP-PYGM, and pEGFP-PYGM™, were maintained in the absence of IL-2 for 48 h and stained with PKH26. Fluorescence was analyzed before adding IL-2 (− IL-2 (0 h)) and after 48-h incubation with IL-2 (+ IL-2 (48 h)). Results show the mean of three independent experiments ± S.D., and the statistical analysis showed a significant difference (*, p < 0.05). WB, Western blot.
from the consensus sequence of the minimum Cdc42/Rac1 interactive binding motif, we found that 4 of the amino acids were also conserved in PYGM. The main evidence is provided by the GST pulldown experiments using this region of PYGM, which was only able to bind to active Rac1 and not to active Cdc42 or inactive Rac1. Taken together, our results argue that PYGM is a novel Rac1-specific effector molecule, thus linking Rac1 activity and glycogen metabolism.

There are three glycogen phosphorylase isoforms: liver, muscle, and brain (also known as fetal) (45, 62). They share 80% sequence homology, although the muscle and brain isoforms seem to be evolutionarily closer (63). In the present study, Mascot software analysis defined PYGM as the isoform that associated with constitutively active Rac1. This identification was based on the fact that the peptide EIWGVEPSR identified by the GST pulldown experiments using this region of PYGM, were also conserved in PYGM. The main evidence is provided from the consensus sequence of the minimum Cdc42/Rac1 interaction pathway after IL-2 stimulation as we determined using the C-terminal position of PYGM replaced with aspartic acid, which cannot be cleaved by trypsin. Here we show that the muscle isoform was expressed at higher levels than the liver isoform in Kit 225 cells at least at the mRNA level. This is in contrast to previous studies reporting simply liver isoform expression in human leukocytes (45, 46). However, the detection methods used in these reports were based on polyacrylamide gel electrophoresis experiments, which are less sensitive than PCR.

From a functional point of view, there is a substantial difference between the three PYG isoforms. The liver isoform in combination with glucose-6-phosphatase allows the release of glucose from the liver into the bloodstream, supplying this sugar to the other tissues. By contrast, the muscle and brain isoforms release glucose intracellularly to provide an energy source and to regulate cellular responses (64). In addition, the activation mechanism is also an important difference among the PYG isoforms: the liver isoform is only activated by reversible phosphorylation on serine at position 14, whereas the other two isoforms can also be regulated by allosteric modification by the binding of energy availability sensors such as AMP or glucose (52, 53). Rac1 does not appear to control PYG activity by the canonical cAMP/PKA/phosphorylase kinase phosphorylation pathway after IL-2 stimulation as we determined using the PKA inhibitor H89. By contrast, we have shown here that the interaction between Rac1 and PYGM occurred through a region that is homologous to the RBD of PAK1, suggesting that Rac1 regulates PYGM activity by allosteric modification in a manner similar to PAK1 activation mediated by Rac1 binding (65). PYGM activation is transient after IL-2 stimulation of T cells, and it depends on the active form of Rac1 and on the integrity of the RBD-like interacting domain of PYGM.

Proliferating cells require the coordination of signaling networks that drive various cellular responses such as gene activation and DNA synthesis. How these networks are linked to the metabolic pathways is still a subject of intensive study. In the present work, we observed a significant decrease in cell proliferation when Rac1 activation or PYGM activity was inhibited. We propose that the active form of Rac1 associates with PYGM, and this signaling pathway participates in the control of T cell proliferation stimulated by IL-2. Thus, in addition to its well-known role in actin cytoskeleton reorganization (1), Rac1 may also directly control metabolic pathways associated with cellular proliferation. In this context, Rac1-mediated PYGM activation after IL-2 stimulation would rapidly trigger downstream signals to provide the cell with energy and macromolecules required for cellular proliferation (66). Intriguingly, the intracellular glycogen content was increased when the PYG pathway was blocked. A similar effect has been described in BHK-21 fibroblasts where addition of insulin to cells cultured in low serum conditions favors the incorporation of glucose into glycogen, thereby increasing the cytoplasmic glycogen content without inducing cell proliferation (67). Glycogenesis is initiated by the synthase, which activates glycogen synthase after release of an inhibitory phosphate group (68). Synthase inactivated can be inhibited by glycogen phosphorylase, thus blocking glucose incorporation into glycogen (68).

Based on the results obtained, we hypothesize that in the presence of GTP the synthase phosphorylates receives no inhibitory signal from PYG, and thus, activation of glycogen synthase leads to an increase in intracellular glycogen. This increase could be linked to a block in the cellular response to the mitogenic stimulus induced by IL-2 perhaps due to a reduction in available free intracellular glucose.

In conclusion, our findings demonstrate that IL-2 induces PYGM activation in Kit 225 T cells by binding to the active form of Rac1, and this interaction controls PYGM enzymatic activity leading to cell proliferation. Thus, Rac1/PYGM association constitutes a novel metabolic pathway leading to T cell proliferation induced by IL-2. The mechanism by which Rac1 delivers proliferative signals through PYGM is still unknown. Future studies will allow us to characterize the effectors and signaling molecules that participate in this signal transduction pathway.

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