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SRC-dependent signalling regulates actin ruffle formation induced by glycerophosphoinositol 4-phosphate

Beatrice Maria Filippi [1], Stefania Mariggiò, Teodoro Pulvirenti [2], Daniela Corda *

Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Via Nazionale 8/A, 66030 Santa Maria Imbaro, Chieti, Italy

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ABSTRACT

The glycerophosphoinositols are diffusible phosphoinositide metabolites reported to modulate actin dynamics and tumour cell spreading. In particular, the membrane permeant glycerophosphoinositol 4-phosphate (GroPIns4*P*) has been shown to act at the level of the small GTPase Rac1, to induce the rapid formation of membrane ruffles. Here, we have investigated the signalling cascade involved in this process, and show that it is initiated by the activation of Src kinase. In NIH3T3 cells, exogenous addition of GroPIns4*P* induces activation and translocation of Rac1 and its exchange factor TIAM1 to the plasma membrane; in addition, in in-vitro assays, GroPIns4*P* favours the formation of a protein complex that includes Rac1 and TIAM1. Neither of these processes involves direct actions of GroPIns4*P* on these proteins. Thus, through the use of specific inhibitors of tyrosine kinases and phospholipase C (and by direct evaluation of kinase activities and inositol 1,4,5-trisphosphate production), we show that GroPIns4*P* activates Src, and as a consequence, phospholipase Cγ and Ca^{2+}/calmodulin kinase II, the last of which directly phosphorylates TIAM1 and leads to TIAM1/Rac1-dependent ruffle formation.

1. Introduction

Although the membrane phosphoinositides represent a minor, but essential, fraction of the total membrane phospholipids, they have crucial regulatory roles as docking molecules for protein-and lipid-binding domains [1–3] and as precursors of signalling molecules [4,5]. They are thus the substrates of different cellular enzymes, including lipid kinases, phosphatases and phospholipases, which catalyze the formation of active metabolites that are involved in the regulation of different cellular functions [6,7].

We have previously investigated the metabolism and physiological functions of a specific class of the phosphoinositide metabolites, the glycerophosphoinositols [7,8]. These are water-soluble products of phospholipase A_2 (PLA_2) and lysolipase activities that were originally identified in Ras-transformed epithelial cells [9], and were then characterized in a number of normal and transformed cell systems [7,10,11]. Recently, we have shown that in thyroid epithelial cells, the glycerophosphoinositols are specifically produced by a single enzyme, the PLA_2 IVα isoform, which possesses both PLA_2 and lysolipase activities [8]. In these cells, this pathway, and more specifically the formation of glycerophosphoinositol (GroPIns), is involved in the control of thyrotropin-independent cell proliferation [8]. In fibroblasts, glycerophosphoinositol 4-phosphate (GroPIns4*P*) has a role in promoting actin cytoskeleton reorganization, through the activation of the small GTPases of the Rho family [12]. Thus, in serum-starved Swiss 3T3 cells, exogenously added GroPIns4*P* stimulates the rapid formation of membrane ruffles, followed at later times by the formation of stress fibres [12]. This GroPIns4*P* effect in intact cells involves the activation of Rac1, as determined by an increased fraction of GTP-bound Rac1 and by a rapid translocation of green-fluorescent-protein (GFP)-tagged Rac1 into ruffles [12]. No information is available, however, on the mechanism by which GroPIns4*P* activates Rac1.

In addition to the effects reported above, GroPIns4*P* is involved in other processes in which cytoskeletal rearrangements are also essential, such as T-cell chemotaxis [13]. Through its ability to inhibit adenylyl cyclase activity [14], GroPIns4*P* enhances chemokine-induced chemotactic responses in Jurkat T-cells and in peripheral blood lymphocytes [13]. This GroPIns4*P* activity is due to the activation of the Rac1 guanine-nucleotide-exchange factor (GEF) Vav, which in turn regulates actin polymerization in these systems [13,15]. Moreover, when exogenously added to breast carcinoma and melanoma cell cultures, both GroPIns and GroPIns4*P* decrease the ability of these cells to degrade, and thus invade, the extracellular matrix, indicating the potential of these compounds in the control of tumour spreading [16].

Following on from the above, the present study was undertaken to define the molecular pathways involved in GroPIns4*P*-dependent ruffle formation in fibroblasts. This is relevant to the general remodelling of actin-based structures that are involved in

* Corresponding author. Tel.: +39 0872 570 353; fax: +39 0872 570412.
E-mail address: corda@negrisud.it (D. Corda).
[1] Current address: MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dundee, Scotland.
[2] Current address: Cell Biology Program, Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA.

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processes such as cell adhesion, division and motility [17,18], and that are controlled by a number of signalling molecules and protein cofactors, including lipid kinases, serine/threonine and tyrosine kinases [19–22], phospholipases [23,24], scaffold proteins (such as IRSp53, filamin and Abl) [25–27], WASP family proteins [28] and the GTPases of the Rho family [29,30]. In addition, lipids, and more specifically the membrane phosphoinositides phosphatidylinositol 4,5-bisphosphate (PtdIns4,5P_2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns3,4,5P_3), have been shown to be relevant for actin cytoskeleton organization in three specific ways: (i) direct regulation of the activities and targeting of actin regulatory proteins, such as exchange factors, WASP family proteins and profilin [31–33]; (ii) stimulation of actin nucleation and the subsequent actin polymerization through the dissociation of capping proteins, such as CapZ and gelsolin, from the actin filaments [34]; and (iii) formation of cytoskeleton-plasma-membrane connections to form stable, bundled actin fibres [35,36].

Using biochemical and morphological approaches, we now show that GroPIns4*P* activates Src kinase, and thus initiates a phosphorylation cascade that leads to the activation of the specific GEF of Rac1, T-cell lymphoma invasion and metastasis protein-1 (TIAM1). This pathway is responsible for Rac1 activation, and finally for the formation of the membrane ruffles induced by GroPIns4*P*.

2. Materials and methods

2.1. Reagents

NIH3T3, HEK293T and SYF cells were from the American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle's Medium (DMEM), OptiMEM, calf serum (CS), penicillin, streptomycin, trypsin-EDTA, L-glutamine and the Lipofectamine/plus reagent were from Gibco BRL (Grand Island, NY, USA). Foetal calf serum (FCS) was from Biochrom KG (Berlin, Germany). The TIAM1- and wild-type-Src-expressing constructs (see below) were kindly provided by I. Fleming (University of Dundee, Scotland, UK) and S. Gutkind (NIH, Bethesda, USA), respectively. The pEGFP-Rac1 was from our laboratory [12], while pEGFP was from Molecular Probes (Eugene, OR, USA). The constructs expressing the myc-tagged constitutively active forms of Rac1 (myc-L61Rac1), RhoA (myc-L63RhoA) and Cdc42 (myc-L61Cdc42) were kindly provided by A. Hall (Sloan-Kettering Institute for Cancer Research, NY, USA). TRITC- and FITC-labelled phalloidin were from Sigma-Aldrich (St. Louis, MO, USA), and Fluo3-AM and the Alexa 488-and Alexa 546-conjugated goat anti-rabbit and anti-mouse antibodies from Molecular Probes. For the antibodies: anti-HA was from Babco (Richmond, CA, USA), anti-p-Src (phosphorylated on tyrosine 416, p-Tyr416) from Upstate (Chicago, IL, USA), anti-PLCβ1, anti-PLCγ1, anti-TIAM1 (C-16), anti-c-Src were from Santa Cruz (Santa Cruz, CA, USA), the polyclonal anti-TIAM1 antibody was kindly provided by J.G. Collard (The Netherlands Cancer Institute), monoclonal anti-*c-myc* Cy3 conjugate clone 9E10 from Sigma-Aldrich and monoclonal antiphosphotyrosine clone 4G10 from Upstate. GroPIns4*P* was prepared by deacylation of phosphatidylinositol 4-phosphate (Avanti Polar Lipids, Alabaster, AL, USA), following [37]. KN-93, U73122, U73433, PP2, SU6656, GroPIns, mowiol and the secondary antibodies conjugated to horse-radish peroxidase and directed against mouse and rabbit IgGs were from Calbiochem (La Jolla, CA, USA). Ionomycin, ATP, genistein and 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,-*N*',*N*'-tetraacetic acid tetraacetoxy-methyl ester (BAPTA-AM) were from Sigma-Aldrich. PDGF, CaMKII, Src, the "CaMKII activity assay" kit and the "Src assay kit" were from Upstate. The "D-myo-inositol 1,4,5-trisphosphate [^{3}H]-Biotrak assay system" and the ECL reagents were from Amersham Pharmacia (Piscataway, NJ, USA). [γ^{32}P]-ATP (3,000 Ci/mmole), Filter Count and Ultima Gold scintillation fluids were from Perkin Elmer Life Sciences (Boston, MA, USA). All other reagents were of the highest purities from standard commercial sources.

2.2. Cell culture, transfection and treatments

Cells were grown in DMEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10% CS (mouse fibroblasts, NIH3T3 cells) or FCS (human cell lines, HEK293T cells, mouse embryo fibroblasts, SYF cells). For immunofluorescence experiments, cells were seeded onto glass coverslips in 24-well plates at a concentration suitable for 70% confluence without transfection or 50% confluence in the case of Lipofectamine-based cell transfection. In the latter, about 24 h after seeding, NIH3T3 and SYF cells were transiently transfected with different plasmids (pEGFP-Rac1 encoding Rac1-GFP; pcDNA-C1199-TIAM1 encoding TIAM1-HA, pEGFP-C1199-TIAM1 encoding TIAM1-GFP, pRK5-L61Rac1 encoding myc-L61Rac1, pRK5-L63RhoA encoding myc-L63RhoA, pRK5-L61Cdc42 encoding myc-L61Cdc42 and pSM-c-Src encoding c-Src) with the Lipofectamine/plus reagent, following the manufacturer instructions. Before treatments, NIH3T3 and SYF cells were serum starved in DMEM with 2 mM glutamine, 1 U/ml penicillin and streptomycin for 24 h and with 0.1% FCS for 12 h, respectively. Then the cells were treated with the different stimuli and/or inhibitors, as indicated in the text and/or Figure legends.

2.3. Immunofluorescence analysis for membrane ruffling and protein localization

For ruffle assessment after treatments, the cells (NIH3T3 or SYF) were fixed with 4% (w/v) paraformaldehyde in PBS for 12 min, permeabilised in blocking solution (0.05% saponin, 0.5% BSA, 50 mM NH_4Cl, in PBS) for 20 min, and then incubated with 0.1 μg/ml TRITC- or FITC-labelled phalloidin for 45 min for filamentous actin visualization [12]. The samples (independent experiments in duplicate; 200 cells per sample) underwent blinded morphological scoring for ruffle formation, under an Axiophot microscope using a 100×1.3 objective (Carl Zeiss, Jena, Germany), as: absence, 0; partial response, 1; full response, 2 (see also [12]). This provided a maximum score of 400, with the data given as percentages of each response with respect to the respective control.

For protein localization, the effects of different agents were also analysed in NIH3T3 and SYF cells: the cells were fixed and permeabilized with blocking solution (see above), and then incubated with the specified primary and fluorescent-probe-(Alexa 488 or Alexa 546)-conjugated secondary antibody diluted in blocking solution and stained for actin (see above and [12]). To determine the specificity of GFP-tagged constructs, the same experiments were also performed with cells overexpressing GFP. This always showed a nuclear and cytoplasmic localization for GFP that did not change upon treatment. The samples were then analyzed with blinded quantification under an LSM 510 confocal microscope equipped with a 63× objective (Zeiss, Germany). Optical confocal sections were taken at 1 Airy unit with a resolution of 512×512 pixels and exported as TIFF files. The quantitative evaluation of the immuno-staining patterns was performed on at least 50 cells per sample, in at least three independent experiments, each in duplicate. The data are expressed as percentages of cells displaying a given pattern.

The endogenously expressed TIAM1 was also followed in NIH3T3 cells using a polyclonal anti-TIAM1 antibody kindly received from J.G. Collard (The Netherlands Cancer Institute) (see Results), however the high level of background labelling prevented a good resolution of the specific signal.

2.4. GroPIns4P-induced translocation of Rac1 and TIAM1, and Vav1 activation

For the membrane translocation of Rac1 and TIAM1, NIH3T3 cells were serum starved in DMEM with 2 mM glutamine, 1 U/ml penicillin and 0.1 mg/ml streptomycin for 24 h, stimulated, lysed and processed