Nerve growth factor- and epidermal growth factor-stimulated translocation of the ADP-ribosylation factor-exchange factor GRP1 to the plasma membrane of PC12 cells requires activation of phosphatidylinositol 3-kinase and the GRP1 pleckstrin homology domain

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ADP-ribosylation factors (ARFs) are small GTP-binding proteins that are regulators of vesicle trafficking in eukaryotic cells. GRP1 is a member of a family of ARF guanine-nucleotide-exchange factors that binds *in vitro* the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3]. In order to study the effects of PtdIns(3,4,5)P_3 on the function of GRP1, we have cloned the human homologue of GRP1, encoding for a protein which is 98.8 % identical to mouse brain GRP1. Human GRP1 binds, via its pleckstrin homology (PH) domain, the inositol head group of PtdIns(3,4,5)P_3, inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)P_4], with high affinity (K_d 32.2 ± 5.2 nM) and inositol phosphate specificity [K_d values for Ins(1,3,4,5,6)P_5, $InsP_6$, Ins(1,3,4)P_3 and Ins(1,4,5)P_3: 283 ± 32, >10000, >10000 and >10000 nM, respectively). Furthermore, GRP1 can accommodate addition of glycerol or diacetylglycerol to the 1-phosphate of Ins(1,3,4,5)P_4, data that are consistent with its proposed role as a putative PtdIns(3,4,5)P_3 receptor. To address whether GRP1 binds PtdIns(3,4,5)P_3 *in vivo*, we have expressed a chimaera of green fluorescent protein (GFP) fused to the N-terminus of GRP1 in PC12 cells and, using confocal microscopy, examined its resultant localization in live cells. Stimulation with either nerve growth factor or epidermal growth factor (both at 100 ng/ml) results in a rapid, PH-domain dependent, translocation of GFP–GRP1 from the cytosol to the plasma membrane, which occurs with a time course that parallels the production of PtdIns(3,4,5)P_3. This translocation is dependent on the activation of phosphatidylinositol 3-kinase, since it is inhibited by wortmannin (100 nM), LY294002 (50 μM) and by the co-expression with dominant negative p85. Taken together these data strongly suggest that GRP1 interacts *in vivo* with plasma membrane-located PtdIns(3,4,5)P_3 and hence constitutes a true PtdIns(3,4,5)P_3 receptor.

INTRODUCTION

Many cell-surface receptor tyrosine kinases, as well as certain G-protein-linked receptors, acutely activate phosphatidylinositol 3-kinase (PI 3-kinase), leading to the generation of 3′-polyphosphoinositides [1,2]. Phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3] is the primary product *in vivo* of the receptor-stimulated PI 3-kinase, resulting from phosphorylation of PtdIns(4,5)P_2 [1,2]. PtdIns(3,4,5)P_3, which subsequently undergoes dephosphorylation by a specific phosphatase to yield PtdIns(3,4)P_2, has been suggested to function as an ubiquitious second messenger. A large number of biological processes have been implicated as targets of this particular pathway [3], including certain intracellular membrane-trafficking systems, such as mast-cell secretion [4] and insulin-sensitive GLUT4 glucose transporter translocation to the plasma membrane in muscle and adipocytes [5].

Recently, work from a number of groups has identified a family of potential target proteins for 3-phosphorylated inositol lipids, which includes GRP1 (general receptor for 3-phosphoinositides), ADP-ribosylation factor (or ARF) nucleotide-binding-site opener (ARNO) and cytohesin-1 [6–9]. All of these proteins contain a C-terminal pleckstrin homology (PH) domain that binds PtdIns(3,4,5)P_3 in preference to PtdIns(4,5)P_2 and PtdIns(3,4)P_2, and an N-terminal 200-amino acid region with high similarity to a domain within Sec7, a yeast protein that is required for transport of polypeptides from the endoplasmic reticulum to and through the Golgi membranes [10]. Functionally, this Sec7 domain has been reported to catalyse guanine-nucleotide exchange on the ARF family of small GTP-binding proteins [6,11–13].

Members of the ARF family are key regulators of vesicle trafficking in eukaryotic cells [14]. Of the six mammalian ARF genes, ARF1, the most extensively studied, functions to recruit coat proteins to membranes of the Golgi apparatus [14,15], whereas ARF6, the least-well characterized, appears to be localized to the plasma membrane and endosomes where it may regulate some aspect of endocytosis [16–19]. Indeed a potential interaction between PI 3-kinase and ARF mutants has been suggested based on the characteristic changes of endosome morphology elicited by the specific PI 3-kinase inhibitor wortmannin [20]. The ability of ARFs to regulate such membrane-fusion events may, in part, be due to their ability to stimulate the activity of certain isoforms of phospholipase D (PLD) [21,22].

Although cytohesin-1, ARNO and GRP1 all function as exchange factors for ARF1 *in vitro* [6–9,23,24], they do have distinct specificities with respect to other ARF isoforms. For

Abbreviations used: NGF, nerve growth factor; EGF, epidermal growth factor; ARF, ADP-ribosylation factor; PI 3-kinase, phosphatidylinositol 3-kinase; PH, pleckstrin homology; GFP, green fluorescent protein; PtdIns, phosphatidylinositol; GST, glutathione S-transferase; PLD, phospholipase D; ARNO, ARF nucleotide-binding-site opener.

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The nucleotide sequence data referred to in this paper have been submitted to the EMBL/GenBank/DDBJ Nucleotide Sequence Databases under accession no. AJ005197.

instance, cytohesin-1 catalyses guanine-nucleotide exchange on ARF3 but not ARF5 [11], whereas ARNO functions on ARF6 [23] and GRP1 on ARF5 [13]. Of particular importance has been the recent demonstration that PtdIns(3,4,5)P_3 can specifically increase the GRP1-catalysed ARF1 guanine-nucleotide-exchange activity *in vitro* [13].

Here we have addressed whether GRP1 does in fact bind PtdIns(3,4,5)P_3 *in vivo*. To do this we have cloned the human equivalent of GRP1 from a blood cDNA library. Recombinant GRP1 specifically binds, via its PH domain, the inositol head group of PtdIns(3,4,5)P_3, with a pharmacology consistent with its role as a putative PtdIns(3,4,5)P_3 receptor. Importantly we demonstrate that upon stimulation of PC12 cells with either nerve growth factor (NGF) or epidermal growth factor (EGF), GRP1 translocates rapidly to the plasma membrane in response to the activation of PI 3-kinase. This translocation is dependent upon the PH domain and occurs with a time course that parallels the production of PtdIns(3,4,5)P_3.

MATERIALS AND METHODS

Cloning of human blood GRP1

EST01394 (accession no. M79246) was isolated by PCR using sequence-specific primers (sense, 5′-CTTATTGATGACATTG-AGAGGCTGAAATAT-3′, Primer 1; antisense, 5′-CAGGTA-GTCCCCAATGACGGTCTTATTTAG-3′, Primer 2) and a human blood cDNA library as template [Strategene no. 938202; oligo(dT) and randomly primed]. The PCR was carried out for 30 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C using High Fidelity *Taq* DNA polymerase (Boehringer-Mannheim). The PCR product, after sequencing, was radio-labelled with [α-^{32}P]dCTP by using a random priming kit (Boehringer-Mannheim). The labelled probe was used to screen 1×10^6 p.f.u. of the above-mentioned cDNA library. Hybridization was performed in QuickHyb (Strategene) containing 1×10^6 d.p.m. per ml of ^{32}P-labelled probe for 12 h at 68 °C. The hybridized filters were washed 3 times with $2 \times$ SSC (0.15 M NaCl/0.015 M sodium citrate)/0.1 % SDS followed by 3 times with $0.2 \times$ SSC/0.1 % SDS, each wash being performed for 15 mins at 65 °C. The washed blots were exposed to X-ray film overnight at −80 °C to pick positive clones. Following secondary and tertiary secreening under identical conditions, one positive plaque was obtained that was subsequently excised and sequenced. This revealed a partial clone lacking the 5′ region, which was obtained as a 550 bp fragment by PCR (parameters as described above, cDNA library as template) using Primer 2 against a T7 primer specific for the cDNA library-containing phagemid. After sequencing, this PCR product was attached to the 5′ end of the truncated GRP1 cDNA to obtain the full-length clone.

Sub-cloning of GRP1 into pGEX and pEGFP-C1

Primers (sense, 5′-CGCAGATCTATGGATGAAGACGGCG-GCGGCGAG-3′ [Primer 3] and antisense, 5′-CGCGTCGACC-TATTTTTTATTGGCAATCCTTCG-3′ [Primer 4], containing the underlined *Bgl*II and *Sal*I restriction sites) were designed to amplify the entire coding sequence of human GRP1 by PCR using High Fidelity *Taq* Polymerase. The PCR product was digested with *Bgl*II/*Sal*I, gel-purified and cloned into the corresponding restriction sites of pEGFP-C1 (Clontech). The resultant pEGFP-GRP1 construct was fully sequenced prior to use. GRP1 was cloned into pGEX by digestion of pEGFP-GRP1 using *Bam*H1/*Sal*I with the resultant DNA being cloned into the corresponding restriction sites within pGEX4T-3 (Pharmacia).

Construction of the GRP1 deletion mutants

This was achieved using PCR by the following primer combinations, which contained either underlined *Bgl*II (sense) or *Sal*I (antisense) restriction sites: for GRP1PH, sense 5′-CGCAGATCTATGCGCGAGGGCTGGCTCCTGAAG-3′ and antisense Primer 4; for GRP1ΔPH, sense Primer 3 and antisense 5′-CGCGTCGACCTAGTCGTTCCCGCTGTCCT-CCGG-3′. PCR cycles were as described above, with the resultant products being digested with the *Bgl*II/*Sal*I prior to cloning into the corresponding sites within pEGFP-C1. The resultant plasmids were fully sequenced prior to use. In a similar manner to that described above, pEGFP-GRP1PH and pEGFP-GRP1ΔPH were digested with *BamH*I/*Sal*I and the resultant insert was cloned into the corresponding restriction sites in pGEX4T-3.

Expression and purification of glutathione S-transferase (GST)-fusion proteins

The pGEX plasmids containing the required GRP1 clone were transformed into the *Escherichia coli* strain *BL*21 (*DE*3) to express and purify the corresponding GST fusion protein as follows. Luria–Bertani broth (5 ml) containing ampicillin (100 μg/ml) was inoculated with a single colony of the transformed strain and grown overnight at 37 °C. This was then diluted 1:100 in fresh Luria–Bertani broth containing ampicillin and grown, with shaking, at 37 °C until the cell density had reached a D_{600} of 0.5 (approx. 3 h). Protein expression was induced with 0.2 mM isopropyl β-D-thiogalactoside for 3 h at 37 °C, after which cells were collected by centrifugation. The resultant pellet was resuspended in 50 ml ice-cold buffer A (PBS containing 1 mM EDTA, 1 mM EGTA, 0.2 mM PMSF and 1 mM β-mercaptoethanol). Cells were lysed by sonication (3×20 s pulses, with 1 min at 4 °C between each sonication) and incubated at 4 °C with 1 % Triton-X 100 for 1 h with gentle mixing prior to the removal of cell debris by centrifugation. A 50 % slurry (1 ml) of glutathione–Sepharose 4B resin (Pharmacia), prewashed with buffer A, was added to the resultant supernatant and incubated at 4 °C overnight with constant shaking. The resin was washed with 3×10 ml of ice-cold buffer A prior to elution of the bound GST-fusion protein with 50 mM Tris/HCl containing 10 mM glutathione (pH 8.0) by incubating with constant mixing for 10 min at room temperature. Protein content of the eluate was estimated by the Bradford method using BSA as a standard.

[^{32}P]Ins(1,3,4,5)P_4-binding assays

These assays were performed as described previously [25]. An individual binding assay contained 100 mM KCl, 20 mM NaCl, 10 mM Hepes/NaOH (pH 7.0), 1 mM EDTA, 30000 d.p.m. [^{32}P]Ins(1,3,4,5)P_4 (prepared as in [26]), 0.5–1.0 μg of GST–GRP1 and various concentrations of competing unlabelled inositol phosphates in a final volume of 0.5 ml. Equilibrium binding was reached by a 15 min incubation at 4 °C after which the receptor–ligand complex was precipitated by addition of 100 μl of 5 mg/ml γ-globulin and 1 ml of 25 % (w/v) polyetheylene glycol. The samples were spun for 10 min prior to removal of the supernatant with the resultant pellet being washed briefly before counting.

Cell culture and transfection

PC12 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum/5% horse serum/100 μg/ml streptomycin/100 units of penicillin/ml (Gibco)/10 mM Hepes (pH 7.0). For transfection, 3.5×10^5 cells, cultured on 22 mm diameter cover slips, were transfected for 2 h with 1 μg of pEGFP-GRP1 in serum-free medium using Tfx-50 (Promega). In all experiments, cells were cultured on collagen-coated dishes, and isolated by trypsin treatment.

Confocal microscopy of transfected PC12 cells

After transfection (18–24 h), cells were serum starved for 2 h before fluorescence analysis at 37 °C in Krebs–Ringer phosphate buffer (136 mM NaCl/4.7 mM KCl/1.25 mM $MgSO_4$/1.25 mM $CaCl_2$/5 mM sodium phosphate) containing 2 mM $NaHCO_3$ and 25 mM Hepes (pH 7.4). Fluorescence imaging was performed with a Leica DM IRBE inverted confocal microscope controlled with TCS-NT4 software (Leica). In some experiments, cells were fixed with 4.5% paraformaldehyde and mounted on to glass slides with Mowiol containing 2.5% diazabicyclo[2.2.2]octane (Sigma). Where inhibitors were used, cells were incubated with wortmannin (100 nM) or LY294002 (50 μM) for 10 min prior to agonist stimulation.

RESULTS

Molecular cloning of human GRP1

The expressed-sequence-tagged database (dbEST) was searched using BLAST to identify human DNA sequences similar to mouse brain GRP1. This identified one expressed sequence tag sequence (accession no. M79246) that showed substantial similarity to mouse GRP1. Using this information we designed two oligonucleotide primers, which were used in the PCR to obtain a 261 bp fragment from a human blood cDNA library. This PCR product, after sequencing to confirm its authenticity, was used to probe the human blood cDNA library. One partial cDNA clone was obtained, which lacked 12 bases of the 5′ coding sequence; these were obtained using a PCR-based method (see Materials and methods). The entire nucleotide sequence of this clone has been deposited in the EMBL database (accession no. AJ005197).

Table 1 Mapping of the Ins(1,3,4,5)P_4 binding site to the PH domain of GRP1

Ins(1,3,4,5)P_4 binding to the various recombinant GRP1 deletion mutants was determined as described in Material and methods. Data are the means ± S.E.M. for 3 independent determinations.

Deletion mutant	[^{32}P]Ins(1,3,4,5)P_4 binding (d.p.m./μg of protein)
GST–GRP1	573 ± 32
GST–GRP1ΔPH	25 ± 11
GST–GRP1PH	538 ± 18

Sequence analysis of human GRP1

Human GRP1 contains a 75 nucleotide 5′-untranslated region that includes a possible Kozak's consensus sequence upstream of the first ATG codon. The open reading frame of 1200 bp encodes for a protein of 399 amino acids with a calculated molecular mass of 46.3 kDa. Sequence analysis revealed the predicted protein to be highly homologous with proteins of the brefeldin A-insensitive ARF guanine-nucleotide-exchange factor family, having 98.8, 82.7 and 79.5% identity with mouse GRP1, human cytohesin-1 and ARNO. The deduced amino acid sequence reveals the presence of three domains, an N-terminal coiled-coil domain (residues 21–59), a central Sec7 domain (residues 76–247) and a C-terminal PH domain (residues 263–380).

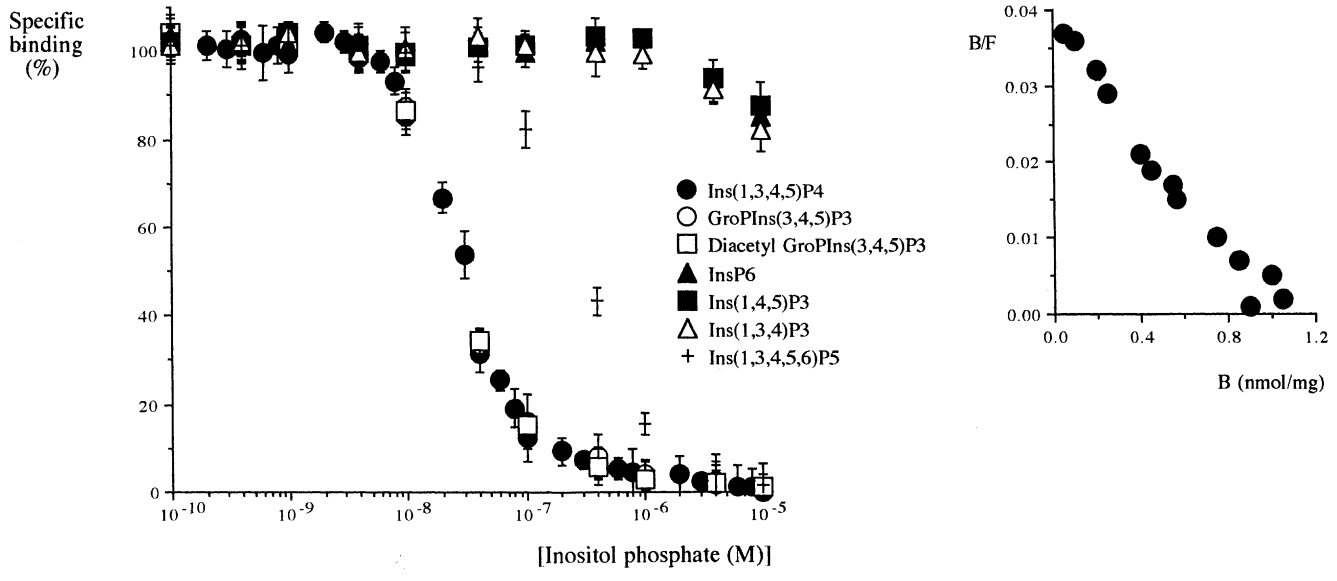

Figure 1 Characterization of [^{32}P]Ins(1,3,4,5)P_4 binding to recombinant full-length human GRP1

Displacement curve for Ins(1,3,4,5)P_4 and other competing inositol phosphates to a fusion protein of GST–GRP1. Each point is from 3 independent determinations and represents mean ± S.E.M. The inset (right) shows Scatchard transformation of the Ins(1,3,4,5)P_4 competition curve using the EBDA-LIGAND computer program. Binding was performed as described in Materials and methods. B/F, bound/free.

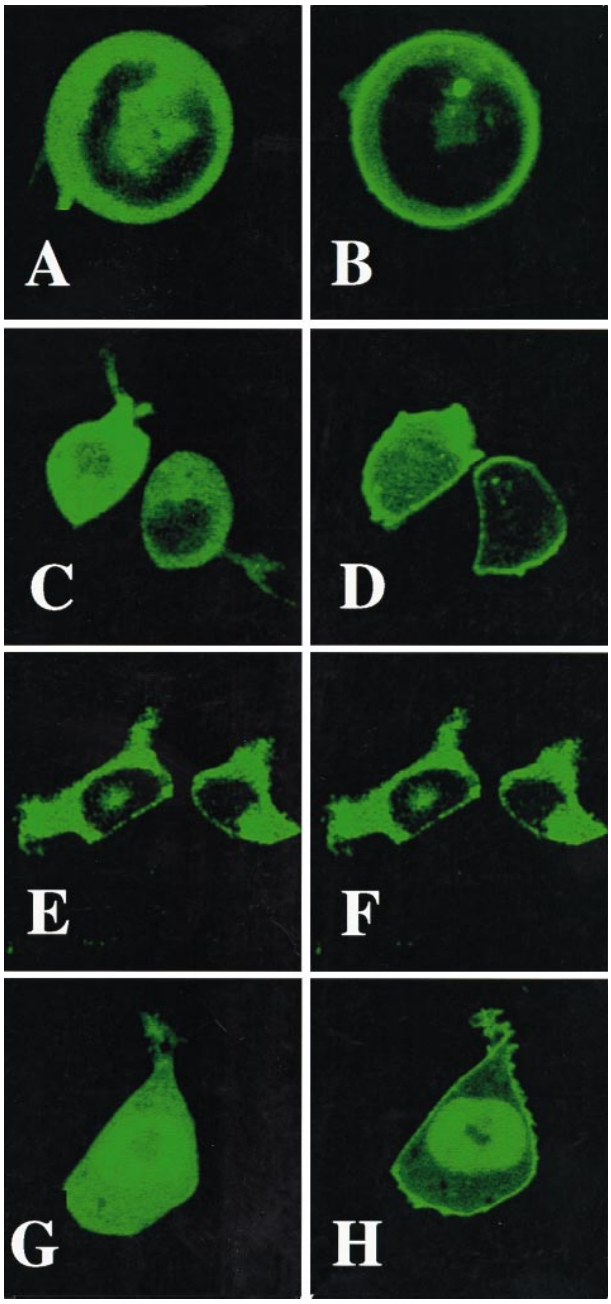

Figure 2 EGF- and NGF-stimulated translocation of GFP–GRP1 is dependent on a functional PH domain

PC12 cells were transfected with (**A**–**D**) pEGFP-GRP1, (**E**,**F**) pEGFP-GRP1ΔPH and (**G**,**H**) pEGFP-GRP1PH. After 24 h, cells were serum starved and imaged by laser-scanning confocal microscopy in the absence of agonist (**A**,**C**,**E**,**G**) or following addition of (**B**) NGF (100 ng/ml) or (**D**,**F**,**H**) EGF (100 ng/ml). In the case of NGF, images were captured 2.5 min after addition, whereas with EGF images were obtained 1 min after addition. The nuclear localization of GFP–GRP1PH in **G** and **H** is due to the small size, which allows free access through the nuclear pore; this is restricted for the larger 74 kDa GFP–GRP1.

[^{32}P]Ins(1,3,4,5)P_4-binding to recombinant GRP1

Full-length GRP1 was expressed as a GST-fusion protein in *E. coli* and purified using glutathione–Sepharose 4B as described in the Materials and methods. Recombinant GRP1 bound Ins(1,3,4,5)P_4 with nanomolar affinity and a high degree of inositol phosphate specificity (Figure 1). Detailed Scatchard analysis revealed a single class of binding sites with a K_d for Ins(1,3,4,5)P_4 of 32.3 ± 5.2 nM and a B_{max} of 0.9 nmol/mg of protein (Figure 1, inset), which, assuming a stoichiometry of one Ins(1,3,4,5)P_4-binding site per GRP1 molecule, is close to the predicted maximum binding for a 46 kDa protein of 2.2 nmol/mg of protein. The inositol phosphate specificity of this binding activity was determined using Ins(1,3,4,5,6)P_5, InsP_6, Ins(1,4,5)P_3 and Ins(1,3,4)P_3, which had K_d values of 283 ± 32, >10000, >10000 and >10000 nM respectively.

Of particular interest with respect to the nature of GRP1 as a putative PtdIns(3,4,5)P_3 receptor is that esterification of the 1-phosphate of Ins(1,3,4,5)P_4, with either glycerol or diacetylglycerol [to form GroPIns(3,4,5)P_3 and diacetyl GroPIns(3,4,5)P_3, respectively], does not dramatically alter their ability to interact with GRP1 (K_d values of 38.1 ± 4.2 and 40.3 ± 6.3 nM, respectively). This is consistent with GRP1 functioning as a PtdIns(3,4,5)P_3 receptor since we have predicted previously that a PtdIns(3,4,5)P_3 receptor may in fact bind Ins(1,3,4,5)P_4 *in vitro* [9], because of the fact that PtdIns(3,4,5)P_3 has Ins(1,3,4,5)P_4 as its inositol head group [with the distinction that the 1-phosphate is not free, as in Ins(1,3,4,5)P_4, but is attached via glycerol to the fatty acids]. It would be expected however, that esterification of the 1-phosphate would result in a decrease in binding affinity to an Ins(1,3,4,5)P_4 receptor, but would not affect, or may even increase, the binding affinity to a PtdIns(3,4,5)P_3 receptor. The data described above are consistent with this view, since GRP1 specifically recognizes the 1,3,4,5-phosphate configuration on the inositol ring in preference to the 1,3,4 and 1,4,5 configurations of PtdIns(3,4)P_2 and PtdIns(4,5)P_2, and furthermore it can accommodate a glycerol substitution on the 1-phosphate.

To map this Ins(1,3,4,5)P_4-binding site, we constructed GST-fusion proteins comprising either GRP1 lacking the C-terminal PH domain (GST-GRP1ΔPH) or just the PH domain itself (GST-GRP1PH). Clearly (see Table 1), only GST-GRP1PH bound Ins(1,3,4,5)P_4 and, importantly, it did so with an affinity and inositol phosphate specificity similar to that of full-length GRP1 (results not shown).

Table 2 Inhibition of EGF-stimulated GFP–GRP1 translocation by specific PI 3-kinase inhibitors

Cells were transfected with the pEGFP–GRP1 and cultured for 24 h as described in Figure 2. In the case of wortmannin (100 nM) and LY294002 (50 μM), these were added 10 min prior to the addition of EGF (100 ng/ml); 1 min after EGF addition cells were fixed and mounted as described in Materials and methods. Cells were imaged and the percentage showing GFP–GRP1 at the plasma membrane determined. In the case of dominant negative p85 (Δp85), cells were co-transfected with Δp85 and pEGFP–GRP1 in a ratio of 4:1, fixed and imaged as above. In all cases the number of cells imaged are as shown.

Condition	Cells in which GFP–GRP1 is at the plasma membrane (%)
Control	1.2 ($n = 85$)
+ EGF	89.6 ($n = 69$)
+ EGF + wortmannin	7.4 ($n = 54$)
+ EGF + LY294002	8.6 ($n = 70$)
+ EGF + Δp85 (1:4)	5.7 ($n = 35$)

EGF and NGF stimulate a rapid PH domain-dependent translocation of GRP1 to the plasma membrane of PC12 cells

These binding studies *in vitro* suggest that GRP1 may function *in vivo* as a PtdIns(3,4,5)P_3 receptor and, to address this more

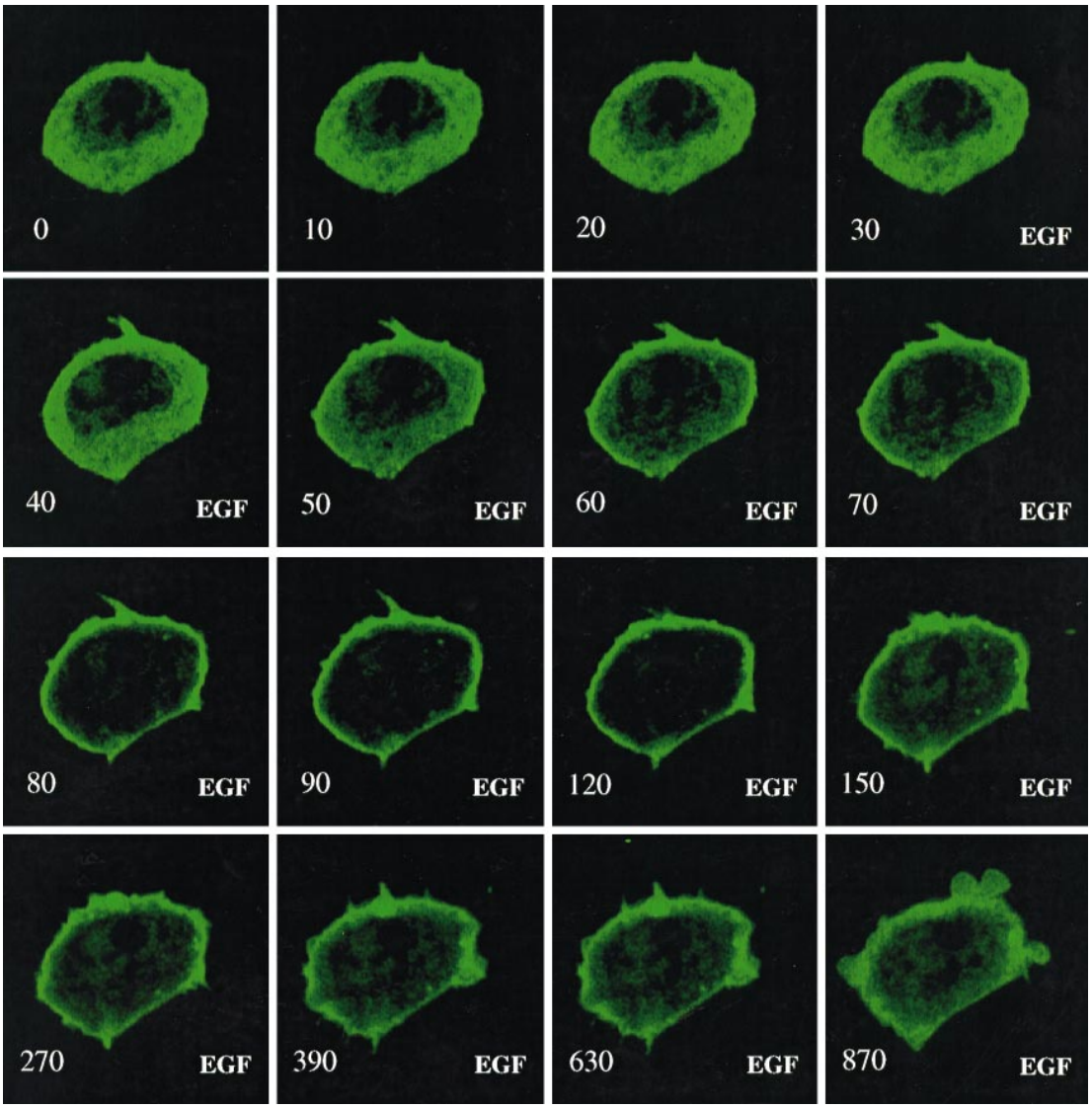

Figure 3 Time-lapse confocal imaging of the effect of EGF stimulation on the subcellular localization of GFP–GRP1 in PC12 cells

pEGFP–GRP1 was transfected into PC12 cells and a selected cell was imaged 24 h later; the number in each panel refers to time (s) at which images were collected. EGF (100 ng/ml) was added immediately after collection of the 20 s image. A similar time course of translocation was seen following stimulation with 50 ng/ml EGF (results not shown). Culturing and manipulation of transfected cells was as described in Figure 2.

directly, we have transfected PC12 cells with a chimaera of green fluorescent protein (GFP) fused to the N-terminus of GRP1. Using laser-scanning confocal microscopy we have studied the resultant localization of GFP–GRP1 in single living cells prior to, and during, stimulation with either EGF or NGF. In unstimulated cells, GFP–GRP1 resides entirely within the cytosol (Figure 2A), whereas stimulation with either EGF or NGF (both at 100 ng/ml) resulted in a complete translocation of GFP–GRP1 to the plasma membrane (Figures 2B–2D). This agonist-dependent translocation required the PH domain of GRP1 as (i) its deletion prevented translocation (Figures 2E and 2F) and (ii) a fusion protein between GFP and just the PH domain of GRP1 (amino acids 267–399; GFP–GRP1PH) translocated in response to agonist stimulation in a similar manner to full-length GRP1 (Figures 2G and 2H).

EGF- and NGF-dependent translocation of GFP–GRP1 requires activation of PI 3-kinase

To examine whether this translocation requires the agonist-dependent activation of PI 3-kinase, we preincubated transfected PC12 cells with the chemically unrelated PI 3-kinase inhibitors wortmannin (100 nM) and LY294002 (50 μM). In the presence of either inhibitor, EGF-stimulated translocation of GFP–GRP1 was almost completely inhibited (see Table 2). This inhibition also occurred when we co-expressed a dominant negative p85 (Δp85), which blocks the elevation in PtdIns(3,4,5)P_3 by uncoupling the ability of activated tyrosine kinase receptors to stimulate PI 3-kinase [27] (Table 2). Similar data were also obtained with NGF-stimulated GFP–GRP1 translocation (results not shown).

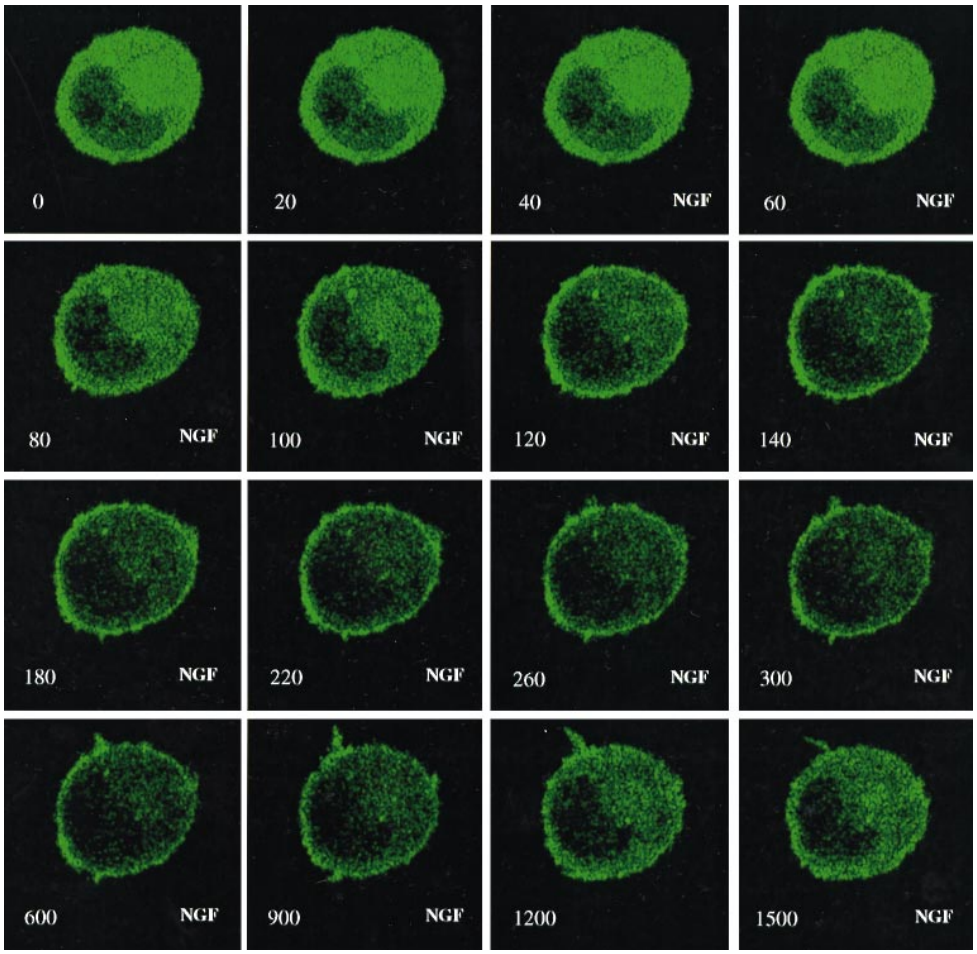

Figure 4 Time-lapse imaging of the effect of NGF stimulation on the subcellular distribution of GFP–GRP1 in PC12 cells

Cells were transfected with pEGFP–GRP1 as described in Figure 3. The number in each panel refers to the time (s) at which images were collected. NGF (100 ng/ml) was added immediately after collection of the 20 s image. Culturing and manipulation of transfected cells were as described in Figure 2. Note the distinct time points at which images were captured compared with Figure 3.

NGF and EGF stimulate GFP–GRP1 translocation with distinct time courses

Stimulation of PC12 cells with either NGF or EGF results in a distinct rate at which these two agonists generate PtdIns(3,4,5)P_3 [28]. EGF stimulation causes a rapid transient peak of PtdIns(3,4,5)P_3, which is maximal after approx. 1 min, and decays such that after 15 min its level is slightly elevated above basal [28]. When stimulated with NGF, however, the peak of PtdIns(3,4,5)P_3 is not reached until approx. 5 min and, although still transient in nature, remains significantly above basal even after 30 min [28]. We have therefore examined the kinetics of the translocation of GFP–GRP1 using time-lapse confocal microscopy. An initial appearance of GFP–GRP1 at the plasma membrane occurred within 20 s of EGF addition, and was complete within 60 s, at which time most of the cytosolic GFP–GRP1 had translocated (Figure 3). The EGF-stimulated translocation of GFP–GRP1 was transient in nature, having almost completely returned to the cytosol after approx. 15 min of stimulation. Interestingly, the time course by which NGF stimulated the translocation of GFP–GRP1 was somewhat different (Figure 4). Following NGF addition, GFP–GRP1 did not appear at the plasma membrane until 1 min after stimulation, and the translocation was complete after approx. 2.5 min, at which time almost all of the cytosolic GFP–GRP1 had translocated. As with EGF, the response to NGF was transient in nature with most of the GFP–GRP1 having returned to the cytosol after approx. 25 min.

Retention of GFP–GRP1 at the plasma membrane is dependent upon the continual activation of PI 3-kinase

The transient nature of the agonist-generated GFP–GRP1 translocation suggests that a continual activation of PI 3-kinase is required to maintain the protein at the plasma membrane. Indeed, time-lapse confocal imaging of cells treated previously for 60 s with EGF demonstrated that addition of wortmannin (100 nM) promoted the rapid displacement of GFP–GRP1 from the plasma membrane back into the cytosol, a process that was initiated after 20 s and was complete within 60 s (Figure 5).

DISCUSSION

Here we have described the cloning and characterization of a human cDNA encoding for a protein that shows a high degree of

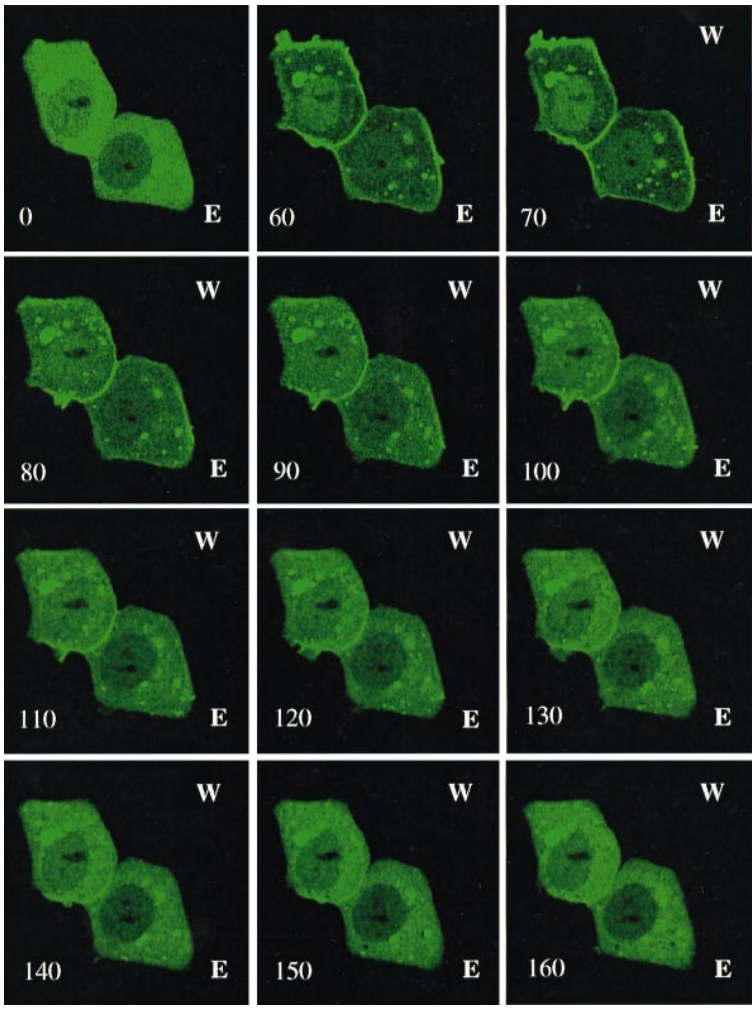

Figure 5 Time-lapse sequence of the return of GFP–GRP1 to the cytosol following the addition of wortmannin

pEGFP–GRP1-transfected cells were stimulated for 60 s with 100 ng/ml EGF (E), and wortmannin (100 nM; W) was added immediately after capture of the 60 s image. Images were subsequently captured every 10 s. These images are presented as time-lapse movies that can be accessed through the World Wide Web by connection to URL: http://www.BiochemJ.org/bj/335/bj3350139add.htm.

similarity (98.8 % identity) to the mouse brain protein GRP1 [8]. *In vitro*, recombinant human GRP1 specifically binds, via its PH domain, Ins(1,3,4,5)P_4 with a nanomolar affinity and a high degree of specificity for the 1,3,4,5-phosphate configuration on the inositol ring. Furthermore, the addition of glycerol or diacetylglycerol to the 1-phosphate of Ins(1,3,4,5)P_4, in the form of GroPIns(3,4,5)P_3 or diacetyl GroPIns(3,4,5)P_3, respectively, does not dramatically alter their ability to compete with Ins(1,3,4,5)P_4 for binding to GRP1. These results suggest that GRP1 constitutes a putative PtdIns(3,4,5)P_3 receptor, in that, *in vitro*, it (i) specifically recognizes the 1,3,4,5-phosphate configuration on the inositol ring in preference to the 1,4,5 or 1,3,4 configurations of PtdIns(4,5)P_2 and PtdIns(3,4)P_2, respectively, and (ii) can accomodate glycerol substitution on the 1-phosphate. This interpretation is further strengthened by the recent direct demonstration *in vitro* of specific PtdIns(3,4,5)P_3 binding to mouse GRP1 [13].

The results presented here using live-cell imaging clearly demonstrate that upon stimulation of PC12 cells with either NGF or EGF, GFP–GRP1 specifically and rapidly translocates to the plasma membrane, a recruitment that is dependent upon the presence of a functional GRP1 PH domain and the ability of the agonist to stimulate PI 3-kinase. Furthermore, we have shown that the time course by which NGF and EGF induce this translocation parallels the rate, and transient nature, by which these two agonists stimulate the production of PtdIns(3,4,5)P_3 [28]. These data, taken together with the pharmacology of Ins(1,3,4,5)P_4 binding to GRP1 *in vitro*, suggest that GRP1 preferentially binds PtdIns(3,4,5)P_3 over PtdIns(3,4)P_2 [and for that matter PtdIns(4,5)P_2] *in vivo*, a binding that is visualized as the translocation of GFP–GRP1 to the plasma membrane of PC12 cells. Clearly, therefore, this suggests strongly that GRP1 constitutes a true PtdIns(3,4,5)P_3 receptor *in vivo*.

At present the physiological function of GRP1 remains unclear. The study of Klarlund et al. [13] has clearly demonstrated that GRP1 functions *in vitro* as an exchange factor for ARF1 and ARF5, but apparently not for ARF6. Interestingly, the ARF-exchange activity on ARF1 is greatly enhanced by the presence of PtdIns(3,4,5)P_3 [13], data which suggest that not only does PtdIns(3,4,5)P_3 function to recruit GRP1 to the plasma membrane but that once there it may also stimulate the catalytic activity. It should be stressed however that a detailed description

of the exchange activity of GRP1 on the other isoforms of ARF is lacking, and so the identification of the exact ARF isoform to which GRP1 interacts *in vivo* is at present unknown.

ARF proteins have been implicated in a wide range of vesicle-transport and fusion steps along the secretory pathway [14,15]. These include budding, transport and fusion steps in the Golgi complex, in the endoplasmic reticulum and in the endocytotic and exocytotic pathways [15,29]. The ability to regulate such membrane-fusion events may in part be a result of ARFs functioning as effective activators of PLD [21,30–32]. PLD hydrolyses the non-fusogenic phospholipid phosphatidylcholine to generate choline and phosphatidic acid, the latter of which is a fusogenic lipid [33]. Plasma-membrane-localized PLD activity has been described [34–36] and it is therefore interesting to speculate that the PtdIns(3,4,5)P_3-induced recruitment and activation of GRP1 may result in a stimulation of PLD via an activation of ARF, resulting in a stimulation of fusion events at the plasma membrane. It is noteworthy that several targets of receptor-activated PI 3-kinase signalling involve plasma-membrane-trafficking systems, including insulin-senstive GLUT4 translocation to the plasma membrane in muscle and adipocytes [5,37]. In this respect it is therefore tempting to suggest a role for GRP1 in regulating such an event in response to an increase in PtdIns(3,4,5)P_3.

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