Signalling via ADP-ribosylation factor 6 lies downstream of phosphatidylinositide 3-kinase

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ADP-ribosylation factor (ARF) 6 regulates plasma membrane trafficking and cortical actin formation by cycling between inactive GDP and active GTP-bound conformations. Here we show that agonist stimulation of phosphatidylinositide 3-kinase (PI 3-kinase) activates a pathway that leads to ARF6 activation. We also describe experiments that propose a central role in this pathway for the PI 3-kinase-dependent plasma membrane recruitment of the cytohesin-1 family of PtdIns(3,4,5)P_3-binding ARF-exchange factors.

Key words: ARNO, cytohesin-1, general receptor for phosphoinositides-1, phosphatidylinositol 3-kinase.

INTRODUCTION

Receptor-mediated activation of phosphoinositide 3-kinase (PI 3-kinase) represents a major pathway by which biological signalling systems operate to regulate diverse cellular functions, including vesicle transport, membrane ruffling and motility [1]. Of the 3′-polyphosphoinositides generated by PI 3-kinase activation, PtdIns(3,4,5)P_3 has aroused considerable interest as a second messenger. A number of proteins have been identified based on their ability to specifically bind PtdIns(3,4,5)P_3, including the cytohesin-1 family of ADP-ribosylation factor (ARF) guanine-nucleotide exchange factors. This family, which comprises cytohesin-1 [2], ARF nucleotide-binding site opener (ARNO) [3] and general receptor for phosphoinositides-1 (GRP1) [4], is characterized by an N-terminal coiled-coil domain, a PtdIns(3,4,5)P_3-binding C-terminal pleckstrin homology (PH) domain, and a central Sec7 domain, which stimulates the exchange of GDP with GTP on members of the ARF family of GTPases.

ARFs play critical roles in a number of different eukaryotic vesicle-trafficking pathways [5]. ARF6, the least conserved of the six mammalian ARF proteins, is associated with and controls the integrity of peripheral membranes and appears to cycle between the plasma membrane and a recycling endosomal compartment depending on its nucleotides status [6–9]. For instance, ARF6–GTP is localized to the plasma membrane where it induces membrane invaginations, decreases transferrin uptake and triggers a redistribution of transferrin receptors to the plasma membrane [10]. This contrasts with ARF6–GDP, which accumulates on a pericentriolar, tubulovesicular compartment that is morphologically similar to the recycling endosomal compartment [6,8–10]. In addition, once accumulated at the plasma membrane, ARF6–GTP has also been implicated in stimulating cortical actin cytoskeletal rearrangements [8,11–12].

On agonist stimulation of PI 3-kinase, members of the cytohesin-1 family undergo rapid plasma membrane recruitment as a result of their PH domains binding PtdIns(3,4,5)P_3 [13–18]. Since ARF6 appears to reside at the plasma membrane [6–9], we, and others, have proposed that ARF6 signalling may be regulated following agonist stimulation in a PI 3-kinase-dependent manner [13,19–20]. In the present work, we show that ARF6 is indeed activated *in vivo* following agonist stimulation of PI 3-kinase. Furthermore, we describe experiments designed to establish the molecular entities and interactions required to link agonist stimulation to ARF6 activation. This has allowed us to propose a basic pathway whereby agonist stimulation enhances the catalytic activity of the cytohesin-1 family of ARF6 exchange factors by inducing their PtdIns(3,4,5)P_3-dependent plasma membrane recruitment.

MATERIALS AND METHODS

Molecular cloning

Site-directed mutants were generated using specific mutagenic primers and pEGFP-ARNO as template according to [27]. pEGFP-ARNO and the corresponding mutants were membrane targeted by attaching a C-terminal CAAX motif using PCR and a 3′-sequence-specific primer containing the coding sequence for the CAAX motif from K-Ras (KDGKKKKKKSKTKCVIM).

Transient transfection and indirect immunofluorescence

HeLa cells were plated on to glass coverslips and transfected with vector DNA at 50–60 % confluency by lipofection using Lipofectamine (Gibco BRL) at a ratio of 0.15 μg DNA/μl cationic lipid. At 48 h post-transfection, cells were fixed in paraformaldehyde and indirect immunofluorescence was performed using an anti-haemagglutinin (HA) monoclonal antibody (BAbCO, Richmond, CA, U.S.A.) and either anti-mouse IgG FITC- or tetramethyl Rhodamine β-isothiocyanate ('TRITC')-conjugated secondary antibody (Boehringer). In some instances, actin was visualized using Rhodamine-conjugated phallodin. Immunofluorescence staining was visualized with a confocal microscope (Leica TCS-NT) equipped with a Kr/Ar laser or an UltraView (E. G. & G. Wallac).

Abbreviations used: ARF, ADP-ribosylation factor; ARNO, ARF nucleotide-binding site opener; BFA, brefeldin-A; EGF, epidermal growth factor; GRP1, general receptor for phosphoinositides-1; GFP, green fluorescent protein; HA, haemagglutinin; PH, pleckstrin homology; PI 3-kinase, phosphatidylinositide 3-kinase.

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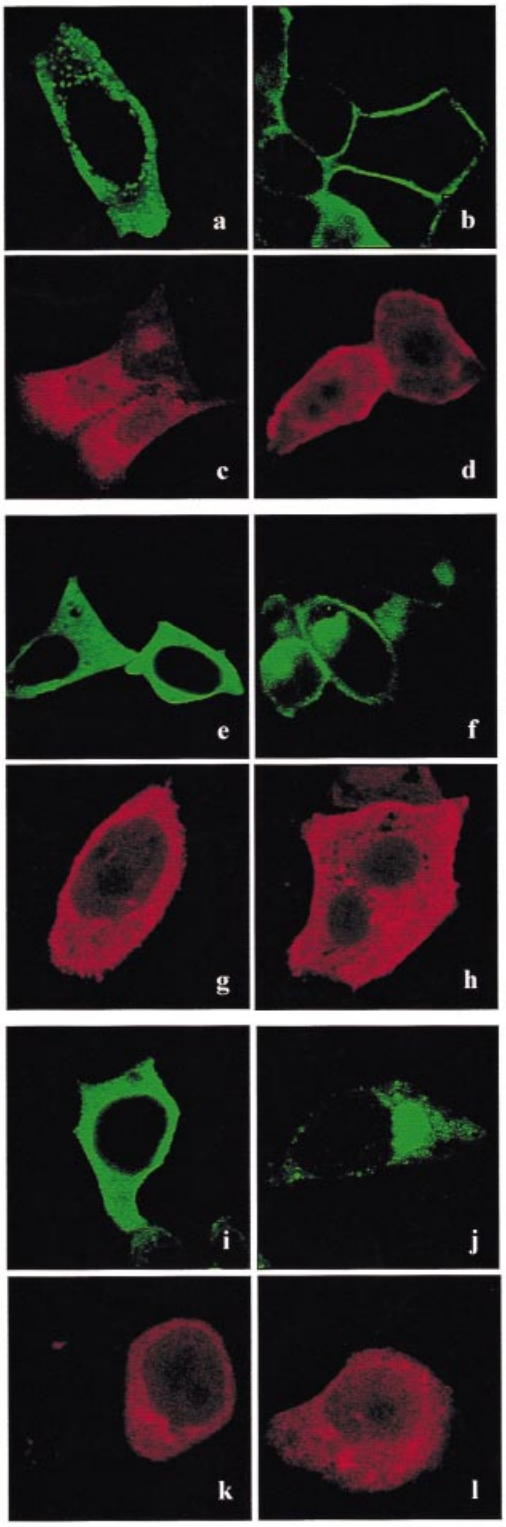

Figure 1 Partial redistribution of ARF6 to the plasma membrane induced by EGF

HeLa cells were grown and transiently transfected with either (**a**) pXS-HA-ARF6(T27N); (**b**) pXS-HA-ARF6(Q67L); (**c**, **d**) pXS-HA-ARF6; (**e**) pXS-HA-ARF1(T31N); (**f**) pXS-HA-ARF1(Q71L); (**g**, **h**) pXS-HA-ARF1; (**i**) pXS-HA-ARF5(T31N); (**j**) pXS-HA-ARF5(Q71L) or (**k**, **l**) pXS-HA-ARF5. After 48 h, transfected cells were starved of serum for 4 h in serum-free Dulbecco's modified Eagle's medium and incubated either in the absence of stimulation (**a**–**c**, **e**–**g**, **i**–**k**) or for 5 min at 37 °C with 100 ng/ml EGF (**d**, **h**, **l**). Cells were fixed in paraformaldehyde and indirect immunofluorescence was performed using an anti-HA monoclonal antibody and either anti-mouse IgG FITC- or TRITC-conjugated secondary antibodies. Similar data were obtained in at least a further 100 cells.

Table 1 Inhibition of PI 3-kinase blocks the EGF-induced redistribution of ARF6

HeLa cells were transiently transfected with pXS-HA-ARF6 and cultured as described in the legend to Figure 1. After 48 h, transfected cells were starved of serum for 4 h before incubation with either wortmannin or LY294002 for 10 min. Cells were immediately stimulated for 5 min with 100 ng/ml EGF, fixed and mounted as described in Figure 1. In the case of dominant negative p85 (Δp85), cells were co-transfected with Δp85 and pXS-HA-ARF6 in a ratio of 4:1, fixed and imaged as above. The percentage of cells showing HA-ARF6 at the plasma membrane was determined. The data are from one experiment, similar results were obtained in two further experiments. Numbers in parentheses are the number of cells examined.

Conditions	Cells with HA-ARF6 localized at the plasma membrane (%)
− EGF	4.3 (278)
+ EGF	70.5 (254)
+ EGF + Δp85	10.9 (92)
+ EGF + wortmannin (100 nM)	9.7 (237)
+ EGF + LY294002 (50 μM)	7.4 (284)

RESULTS

To address the activation state of ARF6 *in vivo*, we initially made use of the observation that the subcellular distribution of ARF6 is dependent upon its bound guanine nucleotide. As can be seen in Figure 1(a), in HeLa cells expressing a constitutively ARF6–GDP-bound mutant, ARF6(T27N), a clear intracellular vesicular staining pattern was observed. Conversely, in cells expressing the constitutively ARF6–GTP-bound mutant, ARF6(Q67L), a predominant plasma membrane localization was visualized (Figure 1b). These observations were entirely consistent with those reported previously for endogenous ARF6 and these, and other, ARF6 mutants [9,11,12,21]. Thus, by analysing the subcellular distribution of ARF6, an assay of its activation state *in vivo* can be achieved.

In serum-starved HeLa cells transiently transfected with a plasmid expressing HA-tagged wild-type ARF6 (HA-ARF6), an intracellular vesicular staining pattern was observed (Figure 1c). However, on stimulation with epidermal growth factor (EGF) (100 ng/ml), a partial brefeldin-A (BFA)-insensitive (5 μg/ml; results not shown) redistribution of HA-ARF6 to the plasma membrane was detected (Figure 1d). This redistribution was specific for HA-ARF6, since no detectable alteration in the subcellular distribution of HA-ARF1 or HA-ARF5 was found on EGF stimulation, although clear differences were seen between their dominant negative GDP-bound [ARF1(T31N) and ARF5(T31N)] and constitutively active GTP-bound mutants [ARF1(Q71L) and ARF5(Q71L)] (Figures 1e–l). Thus EGF can induce the specific activation of ARF6, possibly via the stimulation of a BFA-insensitive ARF6 exchange factor.

To address whether the HA-ARF6 redistribution required the ability of EGF to induce PI 3-kinase activation, we initially preincubated transiently transfected HeLa cells with the chemically unrelated PI 3-kinase inhibitors, wortmannin and LY294002. In the presence of either 100 nM wortmannin or 50 μM LY294002 a dramatic reduction in EGF-induced HA-ARF6 redistribution occurred (Table 1). To confirm this and to negate any non-specific effects of these drugs, we co-expressed a dominant-negative p85 regulatory subunit of PI 3-kinase (Δp85) which blocks the elevation in PtdIns(3,4,5)P_3 by uncoupling the ability of activated tyrosine-kinase-linked receptors to stimulate PI 3-kinase. Again, a clear inhibition in EGF-induced HA-ARF6 redistribution was observed (Table 1). Therefore EGF can induce

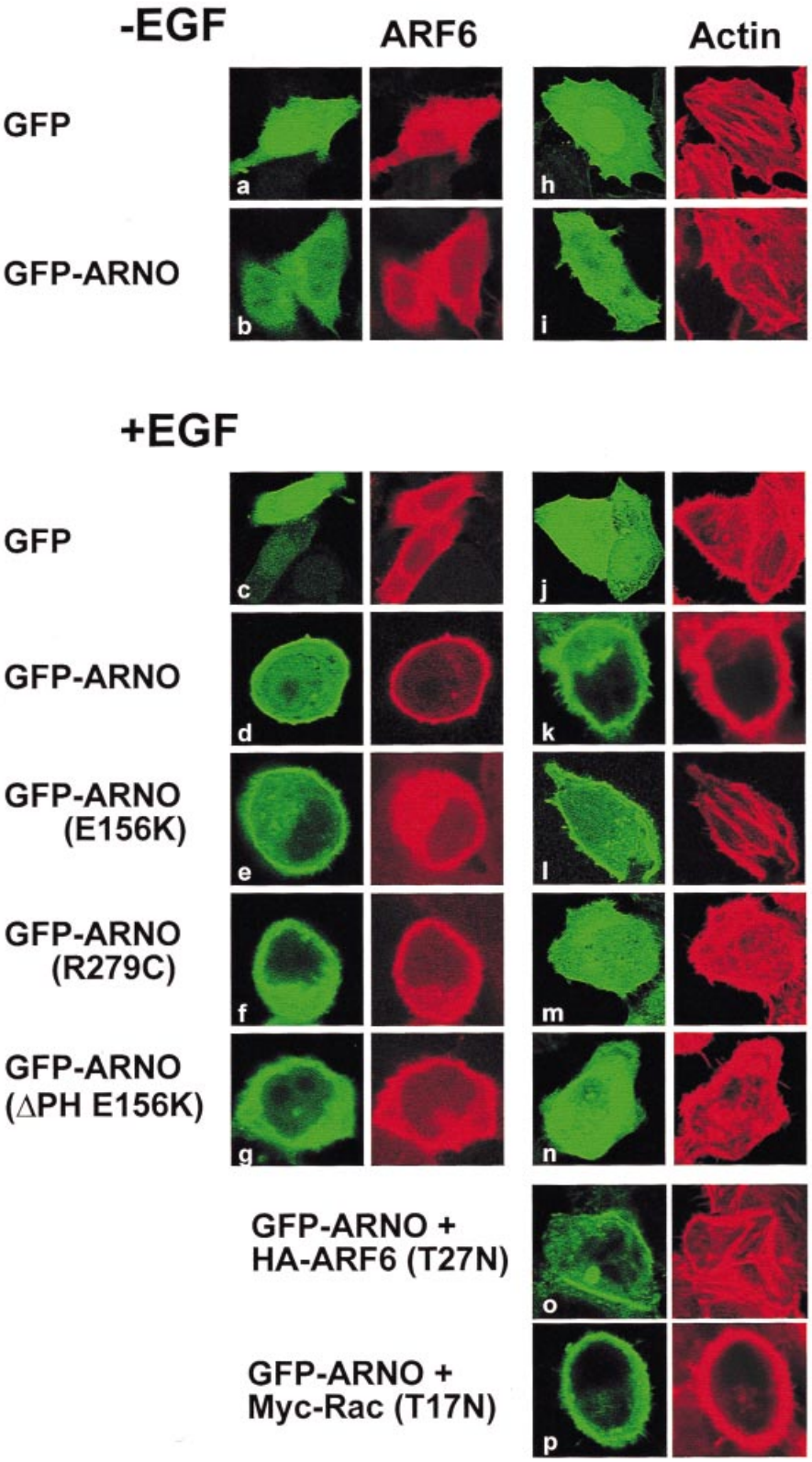

Figure 2 Enhancement of the ability of EGF to induce ARF6 redistribution and cortical actin formation by transient transfection with GFP-ARNO

HeLa cells were transiently transfected with either pEGFP (**a**, **c**, **h**, **j**) or various pEGFP-ARNO constructs together with either pXS-HA-ARF6 (**a**–**g**) or pXS (**h**–**n**). In the case of (**o**) and (**p**), cells were co-transfected with pEGFP-ARNO and either pXS-HA-ARF6(T27N) (**o**) or pCDNA3-Myc-Rac1(T17N) (**p**). After 48 h, transfected cells were starved of serum and fixed either in the absence of stimulation (− EGF) or 5 min after the addition of 100 ng/ml EGF (+ EGF). Cells were stained for either ARF6 (**a**–**g**) or actin (**h**–**p**) using an anti-HA monoclonal antibody and a TRITC-conjugated secondary antibody (see Figure 1) or Rhodamine-conjugated phallodin respectively. In the case of (**o**) and (**p**) cells were stained for either ARF6(T27N) (**o**) or Rac1(T17N) (**p**) using anti-HA or anti-Myc antibodies respectively and a Cy5-conjugated secondary anti-mouse antibody (Jackson ImmunoResearch; results not shown). The images are representative of at least 50 cells for each condition.

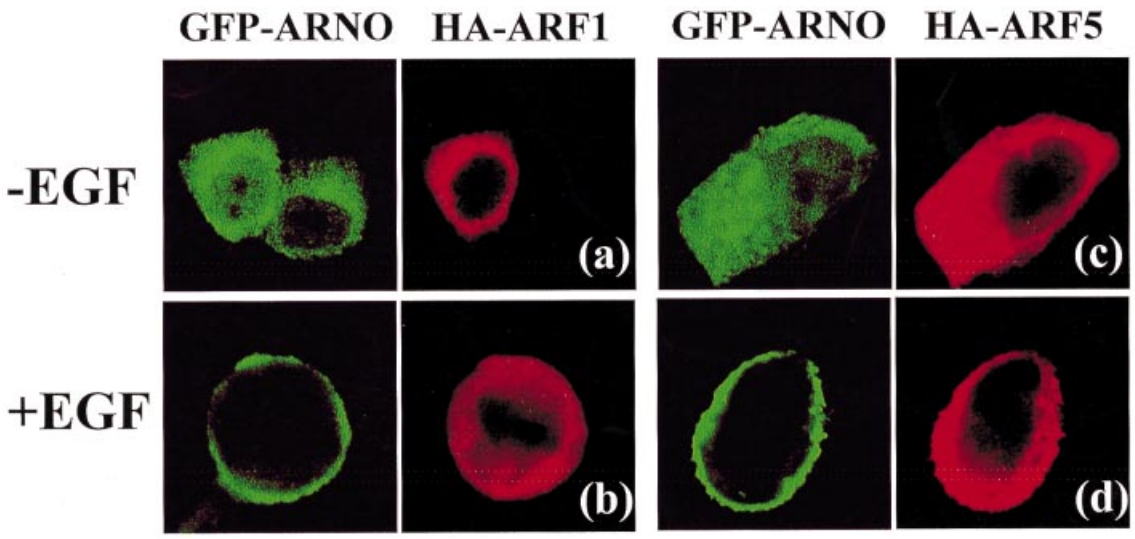

Figure 3 Transient transfection of GFP-ARNO does not induce redistribution of ARF1 or ARF5 even after EGF stimulation

HeLa cells were transiently co-transfected with pEGFP-ARNO and either pXS-HA-ARF1 (**a**, **b**) or pXS-HA-ARF5 (**c**, **d**). After 48 h, transfected cells were starved of serum and fixed either in the absence of stimulation (−EGF) or 5 min after the addition of 100 ng/ml EGF (+EGF). Cells were stained for either ARF1 (**a**, **b**) or ARF5 (**c**, **d**) using an anti-HA monoclonal antibody and a TRITC-conjugated secondary antibody (see Figure 1). The images are representative of at least 50 cells under each condition.

the subcellular redistribution of ARF6 via a pathway that requires PI 3-kinase activation.

In the light of the observation that cytohesin-1 family members undergo rapid plasma membrane recruitment following agonist activation of PI 3-kinase [13–16], we investigated whether these proteins formed the link between PI 3-kinase and ARF6 activation. In serum-starved HeLa cells co-transfected with HA-ARF6 and green fluorescent protein (GFP)-ARNO, a clear intracellular vesicular staining pattern was observed for HA-ARF6 (Figures 2a and 2b), suggesting that cytosolic overexpression of ARNO does not lead to activation of ARF6 signalling. Furthermore, GFP-ARNO overexpression did not affect the integrity of the Golgi complex, which was consistent with ARNO not functioning as an ARF1-exchange factor *in vivo* ([22] and results not shown).

In contrast, stimulation of GFP-ARNO-transfected cells with EGF resulted in the plasma membrane recruitment of ARNO and a marked enhancement in the redistribution of HA-ARF6 to the plasma membrane (Figures 2c and 2d). This effect was dependent on both the catalytic activity and plasma membrane association of ARNO since upon membrane recruitment of the PtdIns(3,4,5)P_3-binding, catalytically inactive GFP-ARNO (E156K) mutant (Figure 2e), and the catalytically active GFP-ARNO(R279C) mutant, which is unable to bind PtdIns(3,4,5)P_3 and hence remains cytosolic (Figure 2f), were both incapable of increasing EGF-induced HA-ARF6 redistribution. Furthermore, in experiments in which cells were co-transfected with GFP-ARNO and either HA-ARF1 or HA-ARF5, no effect was observed on the subcellular distribution of these ARF isoforms either before or after EGF stimulation (Figure 3).

All of the assays so far described rely on examination of the redistribution of overexpressed HA-ARF6. As an independent assay of the activation of endogenous ARF6, we analysed the effect of GFP-ARNO overexpression on the actin cytoskeleton. Clearly, overexpression of cytosolic GFP-ARNO had no effect on the actin cytoskeletal network (compare Figures 2h and 2i). However, a clear increase in cortical actin formation was apparent in GFP-ARNO-transfected cells following EGF stimulation (Figures 2j and 2k). Again this effect was dependent on both the plasma membrane association and catalytic activity of ARNO (Figures 2l and 2m). We also confirmed that this increase in cortical actin formation occurred via activation of endogenous ARF6, since co-transfection with dominant-negative ARF6, ARF6(T27N), but not dominant-negative Rac1, Rac1(T17N), inhibited actin rearrangements (Figures 2o and 2p).

Interestingly the GFP-ARNO(E156K), but not the GFP-ARNO(R279C), mutant was capable of inhibiting both the EGF-stimulated HA-ARF6 redistribution and the EGF-stimulated, endogenous ARF6-dependent, cortical actin formation (compare the right hand panels of Figure 2c with Figure 2e, and Figure 2j with Figure 2l). This was dependent on the PH domain, since the GFP-ARNOΔPH(E156K) mutant was ineffective (Figures 2g and 2n). Thus GFP-ARNO(E156K) uncouples agonist stimulation from ARF6 activation. Mechanistically this may result from the PH domain of GFP-ARNO(E156K) competing with endogenous cytohesin-1 family members for binding to PtdIns(3,4,5)P_3, which is generated on EGF stimulation.

In view of the evidence suggesting a requirement for plasma membrane association in the activation of ARNO, we generated a membrane-targeted version by incorporation of a C-terminal CAAX motif (GFP-ARNO$_{CAAX}$). Transfection of HeLa cells with GFP-ARNO$_{CAAX}$ resulted in ARNO being expressed predominantly at the plasma membrane (Figure 4a). Intriguingly, this alteration in the subcellular distribution resulted both in the redistribution of HA-ARF6 to the plasma membrane (Figure 4a) and in increased cortical actin formation (Figure 4d).

The ability of GFP-ARNO$_{CAAX}$ to induce HA-ARF6 redistribution and cortical actin formation was dependent on the ARF-exchange activity, since the GFP-ARNO$_{CAAX}$(E156K) mutant was ineffective (Figures 4b and 4e). However, it was independent of a functional PtdIns(3,4,5)P_3-binding PH domain, since GFP-ARNO$_{CAAX}$(R279C) was equally effective as the wild

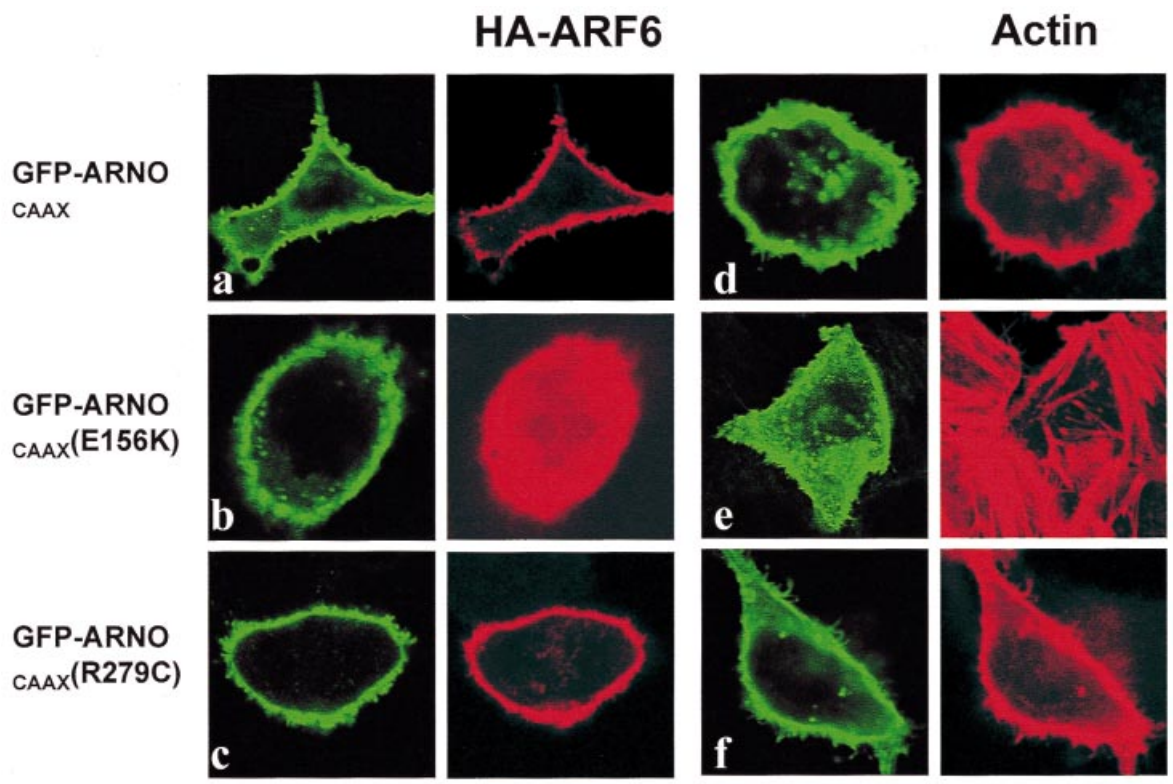

Figure 4 Plasma membrane targeting of ARNO results in constitutive activation of ARF6 signalling

HeLa cells were transiently co-transfected with the various membrane-targeted versions of pEGFP-ARNO and either pXS-HA-ARF6 (**a**–**c**) or pXS (**d**–**f**). Cells were starved of serum, fixed and stained for either ARF6 (**a**–**c**) or actin (**d**–**f**) before imaging.

type (Figures 4c and 4f). Thus, by targeting ARNO to the plasma membrane, downstream ARF6 signalling could be activated in the absence of agonist stimulation.

DISCUSSION

Here we have shown that on agonist stimulation of PI 3-kinase, HA-ARF6 undergoes a BFA-insensitive redistribution from intracellular vesicles to the plasma membrane; a process indicative of an increase in ARF6–GTP. We have demonstrated that, although ARNO appears to be inactive when cytosolic, its plasma membrane recruitment results in an increase in ARF6 exchange activity, as assayed by HA-ARF6 redistribution or endogenous ARF6-dependent, Rac1-independent rearrangement of the actin cytoskeleton [11]. These data, when taken with similar results obtained for GRP1 and cytohesin-1 (not shown), suggest that these proteins do function as ARF6 exchange factors [20,22] and that they may indeed form a link between PI 3-kinase and ARF6 activation.

As alluded to above, the plasma membrane association of ARNO leads to an increase in its ability to stimulate HA-ARF6 redistribution and endogenous ARF6-induced actin rearrangements. Interestingly, this increase in exchange activity can be achieved simply by artificially targeting ARNO to the plasma membrane, conditions in which a functional PtdIns(3,4,5)P_3-binding PH domain does not appear to be required. This suggests that the role of PtdIns(3,4,5)P_3 might be simply to recruit ARNO to the plasma membrane in order to allow efficient interaction with ARF6.

The activation of ARNO upon plasma membrane association implies that GDP/GTP exchange takes place when the recycling endosomal compartment comes into juxtaposition with the plasma-membrane-bound ARF6 exchange factor. This is distinct from previous suggestions that nucleotide exchange occurs on the recycling endosome [9]. ARF6 activation close to the plasma membrane may therefore act as a signal to drive, first, phospholipase-D-regulated fusion of the recycling endosome with the plasma membrane [23,24] and, secondly, actin rearrangements via a Rac1-independent pathway [11]. ARF6 may therefore co-ordinate the increase in plasma membrane phospholipid/protein content and cytoskeletal rearrangements, which together are prerequisites for enhanced cell motility [25].

In conclusion, we have experimentally defined a pathway whereby agonist stimulation of PI 3-kinase increases the catalytic activity of the cytohesin-1 family of ARF6 exchange factors by inducing their PtdIns(3,4,5)P_3-dependent plasma membrane association. The rapid plasma membrane dissociation of these exchange factors following agonist removal, coupled with the PtdIns(3,4,5)P_3-dependent plasma membrane recruitment of potential ARF6 GTPase-activating proteins [26], would effectively switch off ARF6 activation. Therefore ARF6 signalling may be dynamically regulated by the degree of plasma membrane association and, hence, activation of the cytohesin-1 family of ARF6 exchange factors.

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