Regulation and recruitment of phosphatidylinositol 4-kinase on immature secretory granules is independent of ADP-ribosylation factor 1

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Heterotrimeric G-proteins, as well as small GTPases of the Rho and ADP-ribosylation factor (ARF) family, are implicated in the regulation of lipid kinases, including PtdIns 4-kinases and PtdIns(4)*P* 5-kinases. Here, we describe a PtdIns 4-kinase activity on immature secretory granules (ISGs), regulated secretory organelles formed from the *trans*-Golgi network (TGN), and investigate the regulation of PtdIns4*P* levels on these membranes. Over 50 % of the PtdIns 4-kinase activity on ISGs is inhibited by both a low concentration of adenosine and the monoclonal antibody 4C5G, a specific inhibitor of the type II PtdIns 4-kinase. Treatment of ISGs with mastoparan 7 (M7) stimulates the type II PtdIns 4-kinase via pertussis-toxin-sensitive G_i/G_o proteins, which, in contrast with previous results obtained with chromaffin granules [Gasman, Chasserot-Golaz, Hubert, Aunis and Bader (1998) *J. Biol. Chem.* **273**, 16913–16920], does not require Rho A, B or C. M7 treatment also leads to an inhibition

in the recruitment of ARF to ISG membranes: this inhibition is not dependent on G_i/G_o activation, and is not linked to the stimulation of PtdIns 4-kinase observed with M7. PtdIns 4-kinase activity on ISGs is not regulated by myristoylated ARF1–GTP, in contrast with results obtained with Golgi membranes [Godi, Pertile, Meyers, Marra, Di Tullio, Iurisci, Luini, Corda and De Matteis (1999) *Nat. Cell Biol.* **1**, 280–287; Jones, Morris, Morgan, Kondo, Irvine and Cockcroft (2000) *J. Biol. Chem.* **275**, 13962–13170], whereas ARF1–GTP does regulate the production of PtdIns(4,5)*P*₂. Our results suggest that the regulation of PtdIns 4-kinase on the ISGs differs in comparison with that on the TGN, and might be related to a specific requirement of ISG maturation.

Key words: G-proteins, mastoparan, secretory granule.

INTRODUCTION

Phosphatidylinositol 4-phosphate (PtdIns4*P*) and phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)*P*₂] are involved in many post-Golgi vesicle trafficking steps, in particular those resulting in regulated secretion of hormones and neurotransmitters [1–3]. Regulated secretion of hormones and neurotransmitters occurs from secretory granules (SGs), which, after secretagogue stimulation, undergo exocytosis with the plasma membrane and release their contents into the extracellular space. Exocytosis of SGs requires the sequential action of a PtdIns 4-kinase and PtdIns(4)*P* 5-kinase, which generate PtdIns4*P* and PtdIns(4,5)*P*₂ respectively [4]. SGs are formed in the *trans*-Golgi network (TGN) and undergo maturation, a process that alters their size and composition. Immature SGs (ISGs) are an intermediate in the formation of SGs. A key step in the maturation of ISGs to mature SGs (MSGs) is the remodelling of the membrane by adaptor protein-1 (AP-1)-containing clathrin coats. AP-1 is recruited to ISGs via interaction with ADP-ribosylation factor-1 (ARF1)–GTP [5]. The clathrin coat recruits and removes proteins not required for MSG function, such as furin and the mannose-6-phosphate receptors. In contrast, MSGs neither bind ARF1–GTP nor have AP-1-containing clathrin coats, in support of data showing that proteins are lost or inactivated from the maturing SG. The initiation of coat recruitment to membranes is

also modulated by phosphorylated lipids originating from the PtdIns 4-kinase pathway [1–3].

Two families of PtdIns 4-kinases have been characterized: type II [6,7] and type III PtdIns 4-kinase [8]. The type II PtdIns 4-kinase activity has been identified as the basal activity on a variety of membranes, including Golgi membranes, whereas the recruitment and activity of the type III PtdIns 4-kinase to Golgi membranes is thought to be regulated by ARF1 [9,10]. PtdIns 4-kinases have been identified on isolated chromaffin granules [6,11], which would be MSGs and most likely unable to recruit ARF1–GTP.

Therefore, to clarify the relationship between ARF1 recruitment and PtdIns4*P* in the regulation of coat recruitment to ISGs, we sought to determine whether ISGs have PtdIns 4-kinase activity. We have identified and characterized a type II PtdIns 4-kinase activity on purified ISG membranes. This activity on ISGs is regulated by G_i/G_o, but not Rho A, Rho B or Rho C. Using cell-free assays to study the recruitment of type II PtdIns 4-kinase and ARF1–GTP to ISGs, we have shown that the recruitment of the type II PtdIns 4-kinase activity does not require ARF1–GTP. In addition, no change in the level of PtdIns4*P* was observed during recruitment assays, under conditions which promote recruitment of ARF1–GTP and AP-1. Thus coat formation on ISG membranes is not linked to the production of PtdIns4*P* on ISG membranes.

Abbreviations used: AP-1, adaptor protein-1; ARF, ADP-ribosylation factor; BAMC, bovine adrenal medulla cytosol; GDP[β S], guanosine [β -thio]diphosphate; GTP[γ S], guanosine [γ -thio]triphosphate; HB, homogenization buffer; (I/M)SGs, (immature/mature) secretory granules; mAb, monoclonal antibody; M7/M17, mastoparan 7 or 17 respectively; mARF-1, myristoylated ARF-1; PT, pertussis toxin; SCRM7, scrambled M7; TGN, *trans*-Golgi network.

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Figure 1 Characterization of a type II PtdIns 4-kinase activity on ISG membranes which is stimulated by M7

(a) PtdIns kinase assays were performed on ISGs (60 μ g) essentially as described by Whitman et al. [15] without or with 0.1% Triton X-100 (Tx100), 50 μ M adenosine (Adeno) or 30 μ g/ml 4C5G mAb. Labelled phospholipids were extracted, separated using TLC, visualized using autoradiography (top panels), and quantified by PhosphorImager and expressed as percentages of the maximum signal (bottom panels). (b and c) ISGs were pre-incubated for 10 min on ice in the presence or absence of (b) 40 mg/ml 4C5G, 50 mM SCRM7, 50 mM M7 or both 4C5G and M7, or (c) 100 mM LY294002 (Ly) or 50 mM M7, as indicated. The PtdIns kinase reaction and quantification were performed as detailed above for (a). All results are the averages of duplicates from representative experiments repeated independently at least three times.

EXPERIMENTAL

Materials

Mastoparan 7 (M7; sequence INLKALAALAKALL), scrambled M7 (SCRM7; INLLAKAALAKALL) and mastoparan 17 (M17; INLKAKAALAKKLL) [12] were synthesized by solid-phase synthesis and purified by HPLC. Pertussis toxin (PT) was provided by Professor R. Rappuoli (Siena, Italy), and *Clostridium botulinum* C3 transferase was purchased from Cytoskeleton (Denver, CO, U.S.A.).

Preparation of ISGs, recombinant myristoylated ARF1 (mARF1), bovine adrenal medulla cytosol (BAMC) and binding assays

ISGs were prepared from PC12 cells as described previously [13]. Briefly, a post-nuclear supernatant was prepared from PC12 cells by homogenization of the cells in homogenization buffer (HB; 250 mM sucrose/10 mM Hepes (pH 7.2)/1 mM magnesium acetate, 1 mM EDTA), followed by centrifugation at 1000 g for 10 min to remove the nuclei and unbroken cells. Of this post-nuclear supernatant, 1.3 ml was loaded on to a linear sucrose gradient of 0.3–1.2 M sucrose in 10 mM Hepes/KOH, pH 7.2, and subjected to velocity-controlled centrifugation. Fractions (4 ml from the top of the velocity gradient) containing ISGs were pooled, and subjected to equilibrium sucrose-gradient centrifugation. Fractions of 1 ml were collected from the top and fractions 7–9 were pooled. Aliquots were stored in liquid nitrogen and used within 6 months. All gradient preparations were routinely checked using [35 S]sulphate labelling to ensure that the preparations were consistent, and that correct sorting of the soluble proteins was achieved [14]. Recombinant mARF1 was prepared as described previously [5], and was found to be > 95% pure and > 95% myristoylated. BAMC was prepared from freshly dissected glands homogenized in HB with 1 mM dithiothreitol, and subjected to two centrifugation steps (30 min at 30000 g in a Ti70 rotor, followed by a spin at 100000 g in

a Ti60 rotor for 90 min). The cytosol was then dialysed overnight against binding buffer (25 mM Hepes/KOH/25 mM KCl/2.5 mM magnesium acetate). Before use, the cytosol was incubated for 30 min at 37 $^{\circ}$ C, and clarified by centrifugation at 100000 g in a TLA 45 rotor for 60 min. ARF and AP-1 recruitment assays were performed as described elsewhere [13]. After incubation at 37 $^{\circ}$ C, the samples were diluted 4-fold in binding buffer, centrifuged (100000 g for 1 h), re-suspended in SDS/PAGE sample buffer and analysed as detailed in Figure legends.

PtdIns kinase assays

PtdIns kinase assays were performed essentially as described by Whitman et al. [15] in PtdIns kinase assay buffer [20 mM Hepes (pH 7.5)/100 mM NaCl], in a volume of 35 μ l without or with additions, as indicated in the Figure legends. The reaction was started by the addition of ATP solution containing 40 μ M ATP, 12 mM $MgCl_2$ and 5 μ Ci of [γ - 32 P]ATP, allowed to proceed for 20 min at 37 $^{\circ}$ C in a final volume of 50 μ l, and stopped by the addition of 100 μ l of 1 M HCl and 200 μ l of chloroform/methanol (1:1, v/v). Phospholipids were separated by TLC oxalate-coated silica gel-60 plates in chloroform/acetone/methanol/acetic acid/distilled deionized water (40:15:13:12:5, by vol.). Labelled phospholipids [compared with standard PtdIns(4)P lipid stained with iodine vapour] were visualized using autoradiography, and quantified using PhosphorImager analysis.

PT and *C. botulinum* C3 transferase treatments

ISGs were incubated in PT ADP-ribosylation buffer [50 mM Hepes (pH 7.5)/100 μ M GTP/10 mM thymidine/100 μ M EDTA] containing 0.5 μ Ci of [32 P]NAD $^{+}$ in the presence or absence of 0.5 μ g of PT in a final volume of 50 μ l for 15 min at 37 $^{\circ}$ C. Alternatively, ISGs were incubated in C3 ADP-ribosyl-

ation buffer [50 mM Hepes (pH 7.5)/200 μ M GDP/1 mM dithiothreitol/1 mM MgCl₂] containing 0.5 μ Ci of [³²P]NAD⁺, in the presence or absence of C3 transferase (0.1–1 μ g) in a final volume of 35 μ l for 15 min at 37 °C. The reactions were terminated by the addition of SDS sample buffer, and labelled proteins were resolved by SDS/PAGE (12.5% gels) before detection by autoradiography.

RESULTS

Identification and characterization of type II PtdIns 4-kinase on ISGs

Figures 1(a) and 1(b) shows the PtdIns kinase activity detected on ISG membranes using endogenous PtdIns as substrate. PtdInsP was the only ³²P-labelled derivative of PtdIns found in lipid extracts of the ISG membranes labelled with [γ -³²P]ATP. PtdIns kinase assays performed in the presence of LY294002, an inhibitor of PtdIns 3-kinase, did not inhibit the formation of

Figure 2 Type II PtdIns 4-kinase on ISG membranes is inhibited by PT, but not C3 transferase

ISGs were pre-incubated in a final volume of 35 μ l (a) in the presence or absence of 1 μ g of C3 transferase (C3) in C3 ADP-ribosylation buffer containing 100 μ M NAD⁺ for 15 min at 37 °C. Samples were then incubated in the presence or absence of 50 μ M M7, and PtdIns kinase activity was assayed and quantified as described in the legend to Figure 1. The results shown in the top panel are from a representative experiment. In the lower panel the results are expressed as the means $\pm$ S.E.M. (b) ISGs were incubated in 1 μ g of C3 transferase in the C3 ADP-ribosylation buffer containing 0.5 μ Ci of [³²P]NAD⁺. The labelled proteins were resolved by SDS/PAGE and detected by autoradiography. ³²P-labelled Rho protein (approx. 23 kDa) is indicated with an arrow. (c) ISGs were pre-incubated in a final volume of 35 μ l in the presence or absence of 0.35 μ g of PT in PT ADP-ribosylation buffer containing 100 μ M NAD⁺ for 15 min at 37 °C. Samples were then placed on ice and incubated in the presence or absence of 50 μ M M7. PtdIns kinase activity was assayed and quantified as described in (a). The results shown in the top panel are from a representative experiment. In the lower panel, the results shown are the average of duplicates from representative experiments repeated independently. (d) ISGs were incubated in PT ADP-ribosylation buffer containing 0.5 μ Ci of [³²P]NAD⁺, in the presence or absence of 0.5 μ g of PT for 15 min at 37 °C. The labelled proteins were resolved by SDS/PAGE and detected by autoradiography. ³²P-labelled α_i/α_o subunits (approx. 42 kDa) are indicated with an arrow.

Figure 3 M7 reduces ARF and AP-1 recruitment to ISG membranes independent of PT inactivation of G_i/G_o

(a and b) ISGs (125 μ l) were incubated in binding buffer with 2 mg/ml BAMC and ATP in the presence or absence of 100 μ M guanosine [β -thio]diphosphate (GDP[β S]) (GDP[β S] or GTP[γ S]) (GTP[γ S]) without or with 50 μ M M7, SCRM7 or M17. Recruitment of ARF (a) and AP-1 (b) was analysed by immunoblotting with anti-ARF (1A9) mAb or anti-(γ -adaplin), 100/3 (Sigma) [5], followed by ¹²⁵I-labelled Protein A. ARF (c) or AP-1 γ -adaplin subunit (d) that was bound to ISGs was quantified using PhosphorImager analysis. The results shown in (a) and (b) are from a representative experiment. In (c) and (d) the results are expressed as the means $\pm$ S.E.M. (e and f) ISGs (60 μ g) were pre-incubated in a final volume of 50 μ l in the presence or absence of 0.5 μ g of PT in PT ADP-ribosylation buffer for 15 min at 37 °C. Samples were then incubated as above in binding buffer with 2 mg/ml BAMC and ATP, in the presence or absence of 50 μ M M7 and 100 μ M GDP[β S] (GDP[β S] or GTP[γ S]) (GTP[γ S]), and analysed by immunoblotting. The inserts show a representative experiment of the results using the antibodies raised against ARF (e) and AP-1 γ -adaplin (f). The lower panels show the ARF and AP-1 immunoreactivity bound to ISGs, quantified using PhosphorImager analysis. The results shown in (e) and (f) are the averages of duplicates from representative experiments repeated independently.

PtdInsP on ISG membranes (Figure 1c). The PtdIns 4-kinase activity on ISG membranes was stimulated by Triton X-100, and inhibited both by low concentrations of adenosine and by 4C5G, a monoclonal antibody (mAb) that specifically inhibits type II PtdIns 4-kinase activity [16]. M7, a cationic amphiphilic peptide that activates heterotrimeric G_i and G_o proteins by stimulating the exchange of GDP for GTP, thereby activating α_i and α_o subunits [17], strongly stimulated the formation of radiolabelled PtdInsP on ISG membranes (Figures 1b and 1c). A peptide corresponding to an SCRM7 sequence caused only a slight stimulation. A mastoparan-induced activation of PtdIns 4-kinase activity has also been observed in chromaffin-granule membranes [18]. In addition, the M7-induced stimulation of PtdInsP is inhibited by 4C5G (Figure 1b). These results suggest that a type

Figure 4 Recruitment of mARF1-GTP elevates PtdIns(4,5)P₂ but not PtdIns(4)P levels on ISG membranes

ISGs (125 μl) were incubated (step 1) without or with 1 μM mARF1 in the presence of 100 μM GDP[βS] (GDPβS) or GTP[γS] (GTPγS) for 25 min at 37 °C. The ISGs were then (Step 2) diluted 4-fold, subjected to centrifugation at 33 000 rev./min in a Ti50.2 rotor for 30 min, rinsed and re-suspended in 2 mg/ml BAMC in binding buffer, ATP and an ATP-regenerating system, incubated for 25 min at 37 °C, pelleted and then rinsed and resuspended (step 3) in PtdIns kinase buffer with [γ-³²P]ATP for 15 min at 37 °C in the (a) absence or (b) presence of 4C5G. Labelled [³²P]PtdInsP (a and b) and PtdInsP₂ (c) were quantified as described in the legend to Figure 1. The data shown in (a) and (c) represent three independent experiments performed in duplicate; those shown in (b) are a representative experiment performed in duplicate.

II PtdIns 4-kinase [6,7] is present on the ISG membrane, and that the increase in PtdInsP observed from M7 treatment is generated via the activation of this kinase.

M7 stimulation of type II PtdIns 4-kinase is not dependent on Rho A, B or C, but is regulated by G_i/G_o

Experiments by Gasman et al. [18] have shown that activation of G_i/G_o proteins by M7 on chromaffin-granule membranes results in the stimulation of PtdIns 4-kinase, which is mediated by the small GTPase Rho. To investigate whether Rho is also involved in the M7-induced stimulation of PtdIns 4-kinase on ISG membranes, we made use of the specific ADP-ribosylation of Rho A, B and C with *C. botulinum* C3 ADP-ribosyltransferase to inactivate Rho A–C [19]. As shown in Figure 2(a), pre-treatment of ISG membranes with C3 transferase before M7 addition had no effect on the M7-induced activation of PtdIns 4-kinase activity. This suggests that, in contrast with the results observed with chromaffin-granule membranes [18], Rho A–C is not involved in the pathway downstream of M7-activated G_i/G_o proteins, which results in the stimulation of PtdIns 4-kinase on ISG membranes. The assay conditions used in the pre-treatment of ISG membranes using C3 transferase could support ADP-ribosylation. ISG membranes, incubated in parallel with C3 transferase and [³²P]NAD⁺, were analysed by gel electrophoresis and autoradiography for the presence of substrates for C3 transferase. Figure 2(b) shows that C3 transferase labelled a 21 kDa protein, which corresponds to the apparent molecular mass for Rho protein. Taken together, these data indicate that the C3 transferase used is functional, and that a Rho protein exists on ISG membranes, but has no role in the M7-induced activation of PtdIns 4-kinase.

We next asked whether the ability of M7 to stimulate PtdIns 4-kinase activity was via activation of G-proteins. PT-catalysed

ADP-ribosylation of α_i and α_o subunits blocks their ability to interact functionally with receptor proteins and, as a result, pre-treatment of G_i/G_o proteins with PT will abolish the ability of M7 to activate these G-proteins. To confirm that PT treatment results in ADP-ribosylation of α_i/α_o subunits, ISG membranes were incubated with PT and [³²P]NAD⁺. Figure 2(d) shows that PT labelled a 42 kDa protein, which corresponds to the apparent molecular mass for α_i/α_o subunits, previously shown to be present on TGN membranes [20]. Pre-treatment of ISGs with PT reduced the M7-stimulated PtdIns 4-kinase activity by approx. 50 % (Figure 2c), indicating that activation of a PtdIns 4-kinase by M7 is via G_i/G_o proteins.

M7 inhibits ARF1 recruitment to ISGs

While mastoparan has been shown to increase binding of the coat protein β-COP to Golgi membranes [21], it also has been shown to inhibit secretory-vesicle formation from the TGN, a process which requires heterotrimeric G-proteins and ARF1 [20,22]. To investigate the effect of M7 on the recruitment of ARF and the clathrin-coat adaptor AP-1 to ISG membranes, we used a cell-free assay that reproduces ARF-dependent AP-1 recruitment to ISGs [13]. Using BAMC as a source of ARF and AP-1, Figures 3(a)–3(d) confirm that there is a guanosine [γ-thio]triphosphate (GTP[γS])-dependent recruitment of ARF and AP-1 to ISG membranes respectively. Addition of M7 appeared to decrease recruitment of ARF and AP-1 to ISG membranes, while SCRM7 or the peptide M17 (INLKAKAALAKKLL; an analogue of M7 that is unable to activate G_i/G_o proteins [12]) had little effect on the recruitment of ARF or AP-1 to ISG membranes (Figures 3a and 3b respectively). This demonstrates that the observed inhibition of ARF and AP-1 binding to ISG membranes in reconstitution assays containing M7 is specific to the M7 peptide.

To investigate whether M7 is inhibiting recruitment of ARF to ISG membranes via a mechanism involving G_i/G_o proteins, we examined the affect of PT on ARF and AP-1 binding to ISG membranes. When ISG membranes are pre-treated with PT before the addition of M7, a decrease in ARF and AP-1 recruitment to ISG membranes is still observed (Figures 3e and 3f respectively). In contrast with the observed activation of PtdIns 4-kinase, these findings suggest that M7 is not acting via G_i/G_o proteins to inhibit the binding of ARF to ISG membranes.

It has been suggested that mastoparan can cause membrane damage, and thereby has the potential for introducing artefacts [23]. It is possible that the decrease in the binding of ARF and AP-1 to ISG membranes observed upon the addition of M7 might be due to disruption of the membrane, resulting in a decreased amount of ISG membrane recovered. To preclude this possibility, syntaxin 6, a type I membrane protein present on ISGs [24], was used as a marker for membrane stability. ARF/AP-1 recruitment assays *in vitro* were performed in the absence or presence of M7, and the amount of syntaxin 6 present in the membranes was determined by immunoblotting (see Figure 6d, lanes 2 and 4). The presence of M7 made no difference to the amount of syntaxin 6 compared with samples incubated in the absence of peptide, indicating that incubation with M7 does not result in a loss of ISG membranes.

Recruitment of ARF1-GTP to ISGs does not influence PtdIns 4-kinase activity

The synthesis of PtdIns4P and PtdIns(4,5) P_2 on the Golgi complex can be stimulated by ARF1. In particular, the stimulation of PtdIns4P synthesis was shown to be due to the recruitment of the type III PtdIns 4-kinase β to Golgi membranes [9,10]. Our results suggest that over 50 % of the endogenous PtdIns 4-kinases on ISG membranes is the type II enzyme, and M7 stimulates this activity. In contrast, M7 inhibits ARF1 recruitment to ISGs. To investigate the role of mARF1 in the recruitment of PtdIns 4-kinases to ISG membranes, and the regulation of PtdIns4P synthesis on ISGs, a two-step assay was performed, as described previously [9]. Purified recombinant mARF1 was first recruited to ISG membranes in the presence of GTP[γ S], the membranes were re-isolated and then incubated in BAMC. Finally, after re-isolation of the membranes the production of PtdIns4P and PtdIns(4,5) P_2 was assayed (Figure 4). While incubation of the ISG membranes in BAMC increased the level of PtdIns4P on the ISGs at least 3-fold, prior recruitment of mARF1-GTP had no effect on the production of PtdIns4P over the levels obtained in the presence of cytosol alone (Figure 4a). The increase in the PtdIns4P levels on the ISG membranes was inhibited by approx. 50 % by 4C5G (Figure 4c). In agreement with previous reports using Golgi membranes [9,10], recruitment of mARF1-GTP had a pronounced stimulatory effect on the levels of PtdIns(4,5) P_2 over that observed with cytosol alone, or with cytosol and mARF1-GDP (Figure 4c). Addition of M7 with BAMC had the same stimulatory effect on the PtdIns 4-kinase activity as shown in Figure 1, as well as stimulating the PtdIns(4,5) P_2 levels (results not shown).

Our inability to demonstrate any role for mARF1 in the recruitment of PtdIns 4 kinases led us to investigate further the opposing effects of M7 on PtdIns4P levels and ARF recruitment. First, using the same approach employed in Figure 4, i.e. recruiting mARF1 to membranes before the addition of BAMC, we examined whether M7 interferes with ARF recruitment, and thereby also inhibits binding of AP-1 to ISG membranes. When mARF1-GTP is first pre-bound to ISG membranes before incubation with BAMC, there is little effect of M7 on the

Figure 5 mARF1-GTP and AP-1 bound to ISG membranes are resistant to the effect of M7

AP-1 and ARF binding assays were performed on ISG membranes in two stages, as described in the legend to Figure 3, except that 50 μ M M7 was added to step 2. Samples were incubated and then centrifuged as above and analysed by immunoblotting, as described in the legend to Figure 3. The radioactivity bound to ISGs corresponding to (a) mARF1 and (b) AP-1 was quantified using PhosphorImager analysis. All results are the averages of duplicates from representative experiments repeated independently at least three times.

mARF1-GTP levels on the ISG membranes (Figure 5a) or AP-1 (Figure 5b). As expected, addition of M7 also had no effect on mARF1 or AP-1 levels (Figures 3a and 3b). These results suggest that M7 acts by interfering with the recruitment of mARF1-GTP to ISG membranes. In addition, the decrease in the binding of AP-1 to ISG membranes in the presence of M7 most probably results from a decrease in mARF1 recruitment.

An increase in PtdIns4P levels does not influence ARF recruitment

Since M7 appears to induce an increase in PtdIns 4-kinase activity, and yet concurrently inhibits the recruitment of ARF, we asked whether PtdIns4P levels had any effect on the binding of ARF to ISG membranes. To investigate this, PtdIns 4-kinase assays were performed on ISG membranes in parallel with ARF and AP-1 recruitment assays in the presence of BAMC, and the presence or absence of mAb 4C5G, M7 and SCRM7 peptide (Figure 6). Recruitment of ARF and AP-1 was largely unaffected by the addition of 4C5G (Figures 6b and 6c, lanes 2 and 3, and Figures 6f and 6g), although, as expected, there was a decrease in

Figure 6 Effects of M7-induced PtdIns kinase activity on the binding of AP-1 and ARF to ISG membranes

PtdIns kinase assays were performed on ISGs under conditions that would support AP-1 and ARF recruitment. ISGs (60 μ g) were incubated in binding buffer in the presence or absence of 2 mg/ml BAMC and, as indicated, 40 μ g/ml 4C5G, 50 μ M M7, 50 μ M SCRM7 and 100 μ M GDP[β S] (GDP β S) or GTP[γ S] (GTP γ S) in a volume of 45 μ l. (a and e) The PtdIns kinase reaction was then initiated by the addition of [γ - 32 P]ATP and allowed to proceed for 20 min at 37 $^{\circ}$ C in a final volume of 60 μ l. Labelled PtdInsP was extracted, separated using TLC and quantified using PhosphorImager analysis. The recruitment of AP-1 and ARF to ISGs was analysed in parallel samples. ISGs (125 μ l) were incubated in binding buffer with 8 μ l of ATP-regenerating system, in the presence or absence of 2 mg/ml BAMC, 50 μ M M7, 50 μ M SCRM7, 40 μ g/ml 4C5G mAb and 100 μ M GDP[β S] (GDP β S) or GTP[γ S] (GTP γ S) in a final volume of 250 μ l, and analysed by immunoblotting as described in the legend to Figure 3. The radioactivity bound to ISGs corresponding to ARF (b and f) and AP-1 (c and g) was quantified using PhosphorImager analysis. (d) Syntaxin 6 immunoreactivity on the ISGs incubated as above was detected on the ISGs membranes using anti-syntaxin 6 antibody [24] and a horseradish-peroxidase-conjugated anti-rabbit antibody. All results, except for (a), are the averages of duplicates from representative experiments repeated independently at least three times.

the PtdInsP levels (Figure 6a, lanes 2 and 3, and Figure 6e). Furthermore, while addition of 4C5G reduced the M7-induced stimulation of PtdIns 4-kinase (Figure 6a, lanes 4 and 5, and Figure 6e), 4C5G had no effect on the inhibition of ARF and AP-1 recruitment by M7 (Figures 6b and c, lanes 4 and 5, and Figures 6f and 6g). This suggests that the M7-induced increase in PtdIns4P levels on ISG membranes does not influence the M7-induced decrease in ARF recruitment.

DISCUSSION

Our data show that M7 induces an increase in PtdIns 4-kinase activity on ISG membranes via a pathway that involves G_i/G_o proteins, but, unlike chromaffin-granule membranes [18], is

independent of Rho A, B and C. Inhibition of the endogenous and the M7-induced increase in PtdInsP production by mAb 4C5G suggests that the type II PtdIns 4-kinase recently identified [6,7] is present on ISG membranes. However, we could find no evidence for a role of ARF1-GTP in recruitment of either type II or type III PtdIns 4-kinases to ISG membranes. In addition, our results suggest that while PtdIns 4-kinase activity is regulated by heterotrimeric G-proteins G_i/G_o , the recruitment of ARF (and AP-1) is not. Our results also show that M7 inhibits ARF1-GTP recruitment, whereas the association of ARF1-GTP pre-bound to ISG membranes was not affected by M7. The inhibition of ARF1-GTP recruitment to ISGs is independent of PT inactivation of heterotrimeric proteins G_i/G_o . It has been demonstrated that the M7 peptide can bind to the lipid bilayer of

membranes [25]. It is possible, therefore, that M7 could be binding to ISG membranes and blocking the access of mARF to the membrane. Alternatively, M7 may be involved in regulating effector molecules, such as guanine-nucleotide exchange factors [26], which function in activating and/or recruiting ARF to ISG membranes.

While we found no link between recruitment of ARF1 and recruitment of the type II PtdIns 4-kinase on ISG membranes, previous experiments using Golgi membranes have demonstrated that ARF can regulate recruitment of the type III PtdIns 4-kinase β . However, on ISGs we did not observe an overall increase in the PtdIns4P levels after recruitment of ARF1-GTP. This may be due to the inability of ISG membranes to recruit the type III PtdIns 4-kinase β . Preliminary evidence using antibodies specific for the type III PtdIns 4-kinase β suggest that there is no specific recruitment to ISG membranes (C. Panaretou, unpublished work). Although ISGs are derived from the TGN, they do not contain resident TGN markers such as tyrosyl protein sulphotransferase, a resident TGN membrane protein [14,27]. It is possible that the proteins required for recruitment of the type III PtdIns 4-kinase β are also not present on ISG membranes.

In addition to clathrin-coat-driven membrane remodelling, ISG maturation involves homotypic fusion and changes in the size of the dense core secretory vesicle [28]. PtdIns4P on the ISG may serve as a precursor for the production of PtdIns(4,5) P_2 . A wide variety of intracellular activities require PtdIns(4,5) P_2 ; in particular, regulated exocytosis and endocytosis in mammalian cells [1–3]. Moreover, an ongoing synthesis of PtdIns(4,5) P_2 is required to maintain the structure of the Golgi apparatus in mammalian cells [9]. The precise role of PtdIns(4,5) P_2 in vesicle formation is not yet clear, however. While we could not detect an ARF-dependent increase in PtdIns levels, we could detect an ARF-dependent increase in PtdIns(4,5) P_2 levels. Intriguingly, PtdIns(4,5) P_2 has been shown to be required for homotypic vacuole fusion in yeast [29]. The presence of a type II PtdIns 4-kinase, and possibly other isoforms of PtdIns 4-kinase, on ISG membranes suggests that PtdIns4P and PtdIns(4,5) P_2 production may have a physiological significance on events leading to ISG maturation.

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