# A role for ADP-ribosylation factor 1, but not COP I, in secretory vesicle biogenesis from the trans-Golgi network

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**Abstract** **A synthetic *N*-myristoylated peptide corresponding to the amino-terminal domain of ADP-ribosylation factor 1 (ARF1) markedly increases, in a cell-free system using post-nuclear supernatant from PC12 cells, the biogenesis of constitutive secretory vesicles and immature secretory granules from the trans-Golgi network (TGN). The related *N*-myristoylated ARF4 peptide only weakly stimulates, and the non-myristoylated ARF1 and ARF4 peptides inhibit, the biogenesis of these secretory vesicles. In a modified cell-free system using TGN membranes, coatomer-depleted cytosol supports the biogenesis of TGN-derived secretory vesicles to the same extent as control cytosol. These results suggest a role for ARF1, but not the COP I coat, in secretory vesicle biogenesis from the TGN, possibly via the activation of phospholipase D.**

*Key words:* Budding; Coatomer; Fission; Phospholipase D

## 1. Introduction

Over the past few years, considerable progress has been made in understanding the molecular mechanisms that underlie the biogenesis of vesicles mediating membrane traffic in the secretory pathways of eukaryotic cells [1,2]. In particular, the biogenesis of transport vesicles derived from the *cis*-Golgi of mammalian cells [2] has served as a conceptual paradigm for the study of vesicle biogenesis from other donor compartments. Two classes of cytosolic proteins have emerged as key components in the biogenesis of these vesicles, (i) the small GTP-binding protein ADP-ribosylation factor (ARF) and (ii) the coatomer complex forming the COP I coat [2]. ARF binds to Golgi membranes in the GTP state via its amino-terminal myristate [3], and mediates the membrane recruitment of coatomer [4,5]. The GDP-GTP exchange on ARF is regulated by a membrane-associated guanine nucleotide exchange factor that is inhibited by the drug brefeldin A [6,7] which blocks membrane recruitment of coatomer and vesicular transport [8].

We have previously reconstituted, in a cell-free system derived from the neuroendocrine cell line PC12, the biogenesis of constitutive secretory vesicles (CSVs) and immature secretory granules (ISGs) from the trans-Golgi network (TGN) [9]. Interestingly, in this cell-free system [10] and in intact cells [10,11], the biogenesis of both CSVs and ISGs, collectively referred to as TGN-derived secretory vesicles, is inhibited by brefeldin A. If one extrapolates from the observations on the formation of *cis*-Golgi-derived vesicles to the biogenesis of TGN-derived secretory vesicles, the inhibition by brefeldin A would be indicative of an involvement of ARF in the latter process [12]. In the present study, we have examined a possible role of ARF in the biogenesis of TGN-derived secretory vesicles. In addition, we have addressed the related question of whether or not coatomer is required for the biogenesis of these vesicles.

## 2. Materials and methods

### 2.1. Peptides and antibodies

All peptides were synthesised on a 25 μmol scale with an Abimed AMS 422 Multiple Peptide Synthesiser using a PyBOP activation and Fmoc/tBu/Boc protection scheme. For *N*-myristoylation, the Fmoc group on the N-terminal glycine was removed, and 25 μmol of the resin-attached peptide was myristoylated for twice 3 h with a 10-fold molar excess of myristoyl chloride in 450 μl *N*,*N*-dimethylformamide and 50 μl 50% 4-methylmorpholine/*N*,*N*-dimethylformamide. The myristoylated peptides were cleaved from the resin (*p*-hydroxymethyl-phenoxymethyl polystyrene), and deprotected, with 95% trifluoroacetic acid/5% water for 3 h, precipitated with ice-cold diethyl ether, dissolved in 70% acetonitrile/water, and lyophilized overnight. All peptides were analyzed and purified by reverse-phase HPLC, and their predicted composition verified by matrix-assisted laser desorption ionization time of flight mass spectrometry, and amino acid analysis. Peptides were stored at −20°C as 10 mM stocks; those with a free amino terminus were dissolved in $dH_2O$, while *N*-myristoylated peptides were dissolved in dimethyl sulphoxide (DMSO). Monoclonal antibody CM1A10 was a kind gift of Dr. Jim Rothman.

### 2.2. Cell-free system using PNS

Cell-free biogenesis of TGN-derived secretory vesicles using PNS from [$^{35}$S]sulphate-labelled PC12 cells was carried out for 60 min at 37°C as described previously [9,13], with ARF peptides being added as indicated in the legend to Fig. 1.

### 2.3. Modified cell-free system using TGN membranes supplemented with cytosol

*2.3.1. Preparation of TGN membranes.* All the following manipulations were performed at 4°C. A [$^{35}$S]sulphate-labelled PNS (3 ml), prepared from six 15 cm dishes of PC12 cells as described previously [9,13], was layered over a 5 ml step of 0.8 M sucrose on top of a 5 ml cushion of 1.2 M sucrose in an 'Ultra-clear' tube for the Beckman SW40 rotor, all sucrose solutions being buffered with 10 mM HEPES-KOH to pH 7.2. Centrifugation was carried out at 25000 rpm ($110000 \times g_{av}$) for 30 min in a Beckman SW40 rotor. The 0.8–1.2 M sucrose interface was then collected using a 1 ml syringe and 22 gauge needle. This interface, containing TGN membranes, typically had a volume of 500–600 μl, a sucrose concentration of 1.0 M, a total $^{35}$S radioactivity of $2 \times 10^3$ cpm/μl, and a protein concentration of 1.6–1.7 mg/ml. For cell-free biogenesis of TGN-derived secretory vesicles, 80 μl aliquots of this membrane suspension, each corresponding to approximately one labelled 15 cm dish of PC12 cells, were used immediately.

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*Abbreviations:* ARF, ADP-ribosylation factor; CSVs, constitutive secretory vesicles; ISGs, immature secretory granules; PNS, post-nuclear supernatant; TGN, trans-Golgi network.


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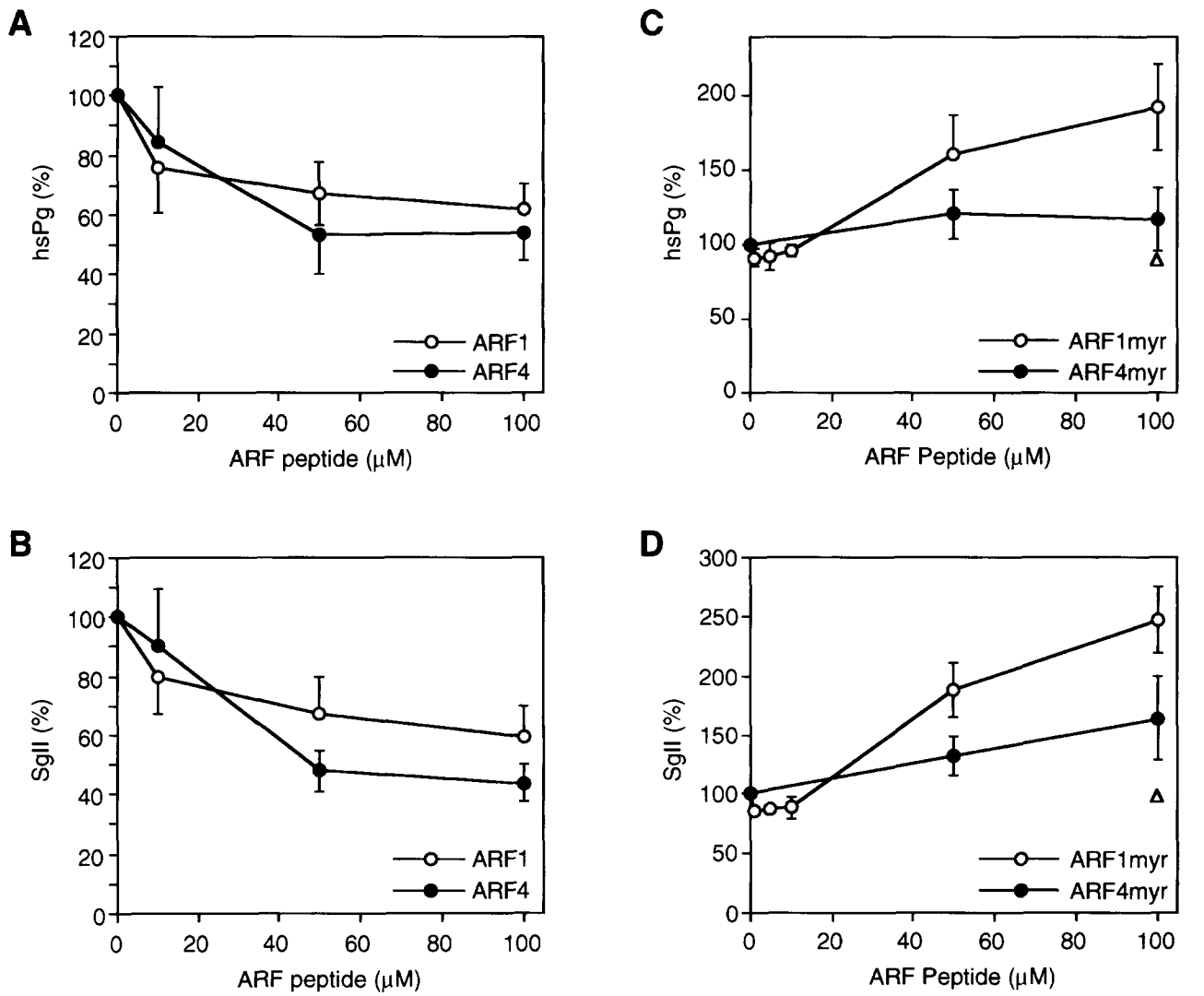


Fig. 1. Effects on the cell-free biogenesis of TGN-derived secretory vesicles of non-myristoylated and *N*-myristoylated peptides corresponding to the amino-terminal domain of ARF1 and ARF4. Cell-free reactions using PNS were carried out in the presence of the indicated concentrations of either unmodified (A and B) or *N*-myristoylated (C and D) peptides corresponding to the amino-terminal 15 amino acid residues of ARF1 (open circles) and ARF4 (closed circles). The unmodified peptides (A and B) were added from a 10 mM stock in $dH_2O$, while the *N*-myristoylated peptides (C and D) were added from a 10 mM stock in DMSO. Two control reactions received DMSO only, at an amount corresponding to the solvent present at the highest peptide concentration used (open triangles in C and D). The biogenesis of CSVs (hsPg, A and C) and ISGs (SgII, B and D) was determined as described in section 2. The budding efficiencies are expressed as a percentage of the control (no ARF peptide) and are the means of three independent experiments. Bars indicate the standard deviation. The variation for the two control reactions with DMSO was ~5% and is not shown for clarity.

*2.3.2. Preparation of control cytosol.* A PNS was prepared from unlabelled PC12 cells following the previously published procedure [9,13] but made to be twice the standard concentration. This PNS was centrifuged at 4°C for 20 min at 45000 rpm ($170000 \times g_{av}$) in a Beckman TLS-55 rotor. For cytosol made from rat brain the same proceedure was followed except that a $12500 \times g_{av}$ supernatant derived from rat brain homogenate [14] was used instead of the PC12 cell PNS. The typical protein concentration of the PC12 cell and rat brain cytosol was 6 mg/ml and 3 mg/ml, respectively. Cytosols were snap frozen in liquid nitrogen and stored at −80°C.

*2.3.3. Preparation of coatomer-depleted cytosol.* The mouse monoclonal IgG CM1A10, raised against the native coatomer, was coupled to protein G-agarose as described [15]. The CM1A10 protein G-agarose was stored at 4°C as a 50% suspension in PBS with 0.5% azide. Rat brain cytosol was immunodepleted of coatomer by sequential incubations with CM1A10 protein G-agarose (100 μg/mg of cytosolic protein) for 6 h, 6 h and 12 h at 4°C with mixing. The extent of immunodepletion was assessed by immunoblot analysis of untreated and immunodepleted cytosol using a rabbit polyclonal antibody to the 36 kDa subunit of the mammalian coatomer. Immunoblots were quantitated using laser-scanning densitometry.

*2.3.4. Cell-free reactions.* 80 μl of [$^{35}$S]sulphate-labelled TGN membranes were supplemented on ice with 50 μl of 10 mM HEPES-KOH pH 7.2, 200 μl homogenisation buffer [9,13], 800 μl of cytosol as indicated in the figure legends, 50 μl of 10 mM $Mg(OAc)_2$ and then 50 μl of an ATP regenerating system [9,13]. In some experiments, the conditions were modified by use of an ATP depleting system [9,13] instead of the ATP regenerating system, or by the addition of GTPγS, as indicated in the figure legends. The samples were mixed by inverting twice. Cell-free reactions were then carried out at 4°C and 37°C for 60 min, and samples placed on ice thereafter.

*2.4. Analysis of cell-free biogenesis of TGN-derived secretory vesicles*

TGN-derived secretory vesicles were separated from the TGN by means of velocity-controlled centrifugation on linear, 0.3 M to 1.2 M, sucrose gradients as described previously [9,13]. In some experiments,

Fig. 2. The modified cell-free system. (A and B) Modified cell-free reactions using TGN membranes supplemented with PC12 cell cytosol to a final protein concentration of 2 mg/ml were performed as follows: 4°C in the presence of ATP and a regenerating system (4°C +ATP); 37°C in the presence of an ATP depleting system (37°C −ATP); 37°C in the presence of ATP and a regenerating system (37°C +ATP); 37°C in the presence of ATP, a regenerating system, and 20 μM GTPγS (37°C +ATP+GTPγS). The biogenesis of CSVs (hsPg, A) and ISGs (SgII, B) was determined as described in section 2. The budding efficiencies are expressed as a percentage of the control (37°C +ATP) and are the means of two independent experiments. Bars represent the deviation of the individual values from the mean. The mean budding efficiency for the control condition was 22% for the hsPg and 28% for SgII. (C) Modified cell-free reactions were carried out using TGN membranes supplemented with PC12 cell cytosol to the indicated final protein concentrations. The biogenesis of CSVs (hsPg, closed circles) and ISGs (SgII, open circles) was determined and is expressed as budding efficiency as described in section 2.

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TGN-derived secretory vesicles were further fractionated by equilibrium centrifugation [9,13] using a modified linear, 0.5 M to 1.6 M, sucrose gradient [16]. Aliquots of velocity and equilibrium sucrose gradient fractions were analysed by SDS-PAGE and fluorography [9,13]. To quantitate the cell-free biogenesis of CSVs and ISGs, the 'budding efficiencies' were determined; these were defined, calculated and expressed as described previously [17].

## 3. Results

### 3.1. *Effects on cell-free secretory vesicle biogenesis from the TGN of unmodified and* N*-myristoylated peptides corresponding to the amino-terminal domains of ARF1 and ARF4*

To investigate a possible role of ARF in the biogenesis of TGN-derived secretory vesicles, we examined, in the cell-free system using PC12 cell PNS, the effects of synthetic peptides corresponding in sequence to the N-terminal 15 residues of ARF1 (GNIFANLFKGLFGKK) and ARF4 (GLTISSLFSRLFGKK), which are two representative members of the ARF family [3]. The amino-terminal region of ARF was chosen because it has been implicated as an effector domain [3,18]. Panels A and B of Fig. 1 show that with 10 μM to 100 μM ARF1 (open circles) and ARF4 (closed circles) peptide, the biogenesis of both CSVs (hsPg, Fig. 1A) and ISGs (SgII, Fig. 1B) was progressively inhibited. At 50 μM and 100 μM, the ARF4 peptide was a more potent inhibitor than the ARF1 peptide.

The ARF proteins are *N*-myristoylated at their amino-terminal glycine residue, a modification shown to be important for their membrane association and, hence, their biological activity [3]. Membrane association of ARF proteins has also been shown to be regulated by their nucleotide state. When in the GTP form, they become membrane-associated, and this membrane association is greatly enhanced by their *N*-myristoylation [3]. Given the importance of *N*-myristoylation for the membrane association and biological activity of ARF, the effects of the synthetic *N*-myristoylated peptides ARF1myr and ARF4myr, corresponding to the N-terminal 15 residues of *N*-myristoylated ARF1 and ARF4, respectively, were investigated. In contrast to the unmodified peptides ARF1 and ARF4 (Fig. 1A,B), the *N*-myristoylated peptides did not inhibit the biogenesis of TGN-derived secretory vesicles (Fig. 1C,D). Addition of the ARF4myr peptide (closed circles) caused a small stimulation of the biogenesis of CSVs (hsPg,

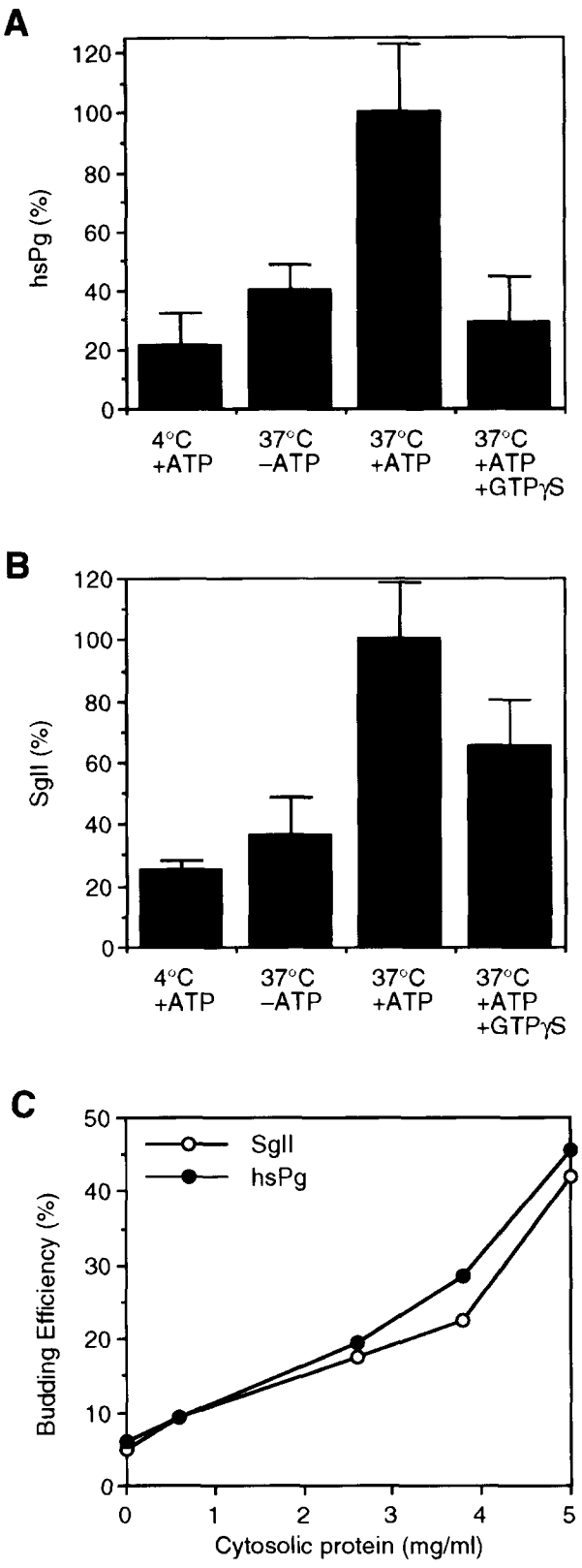


Fig. 1C) and ISGs (SgII, Fig. 1D). Addition of the ARF1myr peptide (Fig. 1C,D, open circles) resulted in a much greater stimulation of the biogenesis of TGN-derived secretory vesicles, which was 2-fold for CSVs (Fig. 1C) and 2.5-fold for ISGs (Fig. 1D) at 100 μM of the peptide. In conclusion, the

data obtained with the unmodified and the *N*-myristoylated peptides indicate a role for ARF proteins, specifically ARF1, in the biogenesis of CSVs and ISGs from the TGN.

### 3.2. *Biogenesis of TGN-derived secretory vesicles in a modified cell-free system*

One of the functions of ARF in vesicular transport is thought to be in the recruitment of the COP I coat [2,4,5]. To investigate whether or not the COP I coat is involved in the biogenesis of secretory vesicles from the TGN, we modified the previously established cell-free system [9,13], which reconstitutes the biogenesis of ISGs and CSVs in a PNS, such that TGN membranes could be supplemented with cytosol.

To establish and characterize the modified cell-free system, TGN membranes were prepared from [$^{35}$S]sulphate-pulse-labelled PC12 cells, supplemented with PC12 cell cytosol to a final concentration of 2 mg/ml, and used in cell-free reactions with an ATP regenerating or depleting system and 20 μM GTPγS as shown in Fig. 2A,B. At 37°C in the presence of an ATP depleting system (37°C −ATP), no significant increase in the biogenesis of either hsPg- (Fig. 2A) or SgII- (Fig. 2B) containing vesicles over the background value obtained at 4°C in the presence of ATP (4°C +ATP) was observed. When the temperature was raised to 37°C in the presence of an ATP regenerating system, a marked increase in the biogenesis of these vesicles was observed (Fig. 2A,B, 37°C +ATP). Analysis by equilibrium sucrose gradient centrifugation of the hsPg- and SgII-containing vesicles formed in this condition showed that, similar to previous observations using the PNS-based system [9,16,17], the SgII-containing vesicles peaked at higher buoyant density than the hsPg-containing vesicles (data not shown), as would be expected for ISGs and CSVs, respectively [9]. Therefore the modified cell-free system, as is the case for the PNS-based system [9], reconstitutes the biogenesis of CSVs (hsPg) and ISGs (SgII) from the TGN in a temperature and ATP dependent manner.

Another criterion for the reconstitution of CSV and ISG biogenesis in a cell-free system is the inhibition of vesicle budding by non-hydrolysable GTP analogues [16,19]. When modified cell-free assays were performed in the presence of 20 μM GTPγS, the biogenesis of TGN-derived secretory vesicles was inhibited (Fig. 2A,B, 37°C +ATP+GTPγS). These results suggest that in the modified cell-free system (see also ref. [20]), as in the PNS-based system [16,17,19], the regulation of secretory vesicle biogenesis by GTP-binding proteins is also reconstituted.

To further characterise the biogenesis of TGN-derived secretory vesicles in the modified cell-free system, the requirement for cytosol was investigated. Modified cell-free reactions were carried out using TGN membranes supplemented with PC12 cell cytosol to the final protein concentrations (between 0.5 and 5.0 mg/ml) indicated in Fig. 2C. Addition of increasing concentrations of cytosol resulted in a progressive increase in the biogenesis of TGN-derived secretory vesicles, indicating that cytosolic components were limiting in the cell-free system.

### 3.3. *Lack of effect of coatomer immunodepletion on the cell-free biogenesis of TGN-derived secretory vesicles*

The dependence of the biogenesis of TGN-derived secretory vesicles on cytosol allowed us to use the modified cell-free system to investigate a possible role of coatomer in this process. Rat brain cytosol, which like PC12 cell cytosol supported

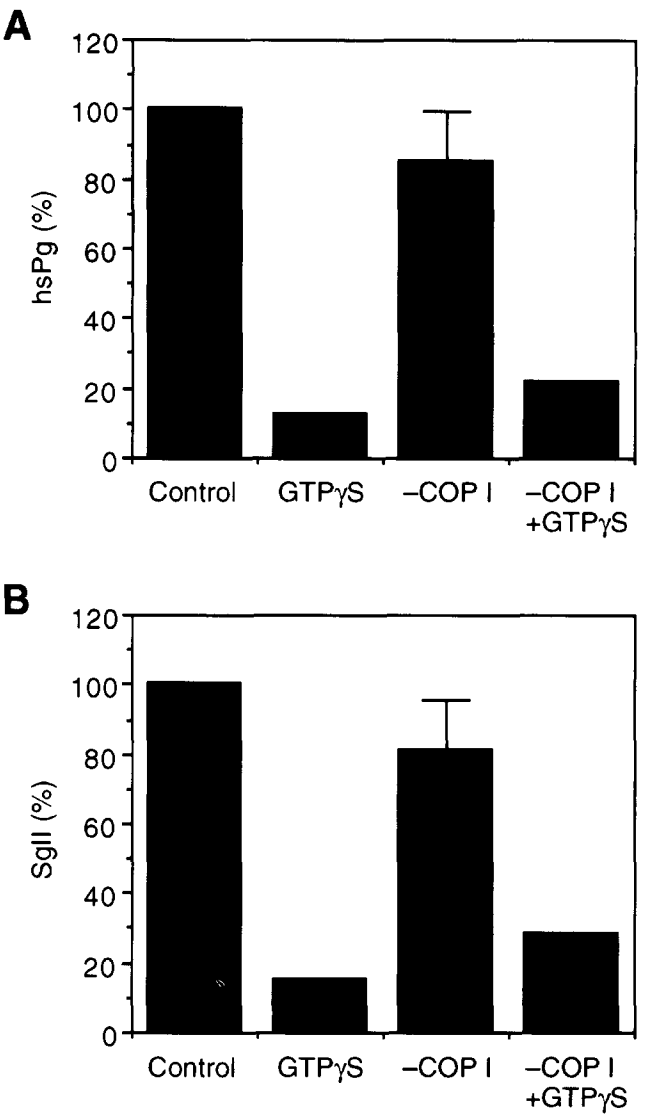


Fig. 3. Coatomer-depleted cytosol supports the cell-free biogenesis of TGN-derived secretory vesicles. Modified cell-free reactions were carried out using TGN membranes supplemented with either control (Control, GTPγS) or coatomer-depleted (−COP I, −COP I +GTPγS) rat brain cytosol to a final protein concentration of 2 mg/ml, in the absence (Control, −COP I) or presence (GTPγS, −COP I+GTPγS) of 20 μM GTPγS. The biogenesis of CSVs (hsPg, A) and ISGs (SgII, B) was determined as described in section 2. The budding efficiencies are expressed as a percentage of the control, and are the means of two independent experiments (Control, −COP I) or from a single experiment (GTPγS, −COP I+GTPγS). Bars indicate the variation of the individual values from the mean.

the biogenesis of TGN-derived secretory vesicles (data not shown), was depleted of coatomer by immunoadsorption. The immunodepleted cytosol contained less than 15% of the amount of coatomer found in untreated cytosol as determined by immunoblotting (data not shown). This level of coatomer depletion has been shown by morphological criteria [15] to reduce the formation of coated *cis*-Golgi-derived vesicles by 80%. We then compared the biogenesis of TGN-derived secretory vesicles in the modified cell-free system using either control or coatomer-depleted cytosol. This comparison should reveal a requirement for the COP I coat in the biogenesis of

TGN-derived secretory vesicles if coatomer, in analogy to the formation of *cis*-Golgi-derived vesicles [2,15,21], were indeed a limiting component in our cell-free system. However, as shown in Fig. 3A,B, coatomer-depleted cytosol (−COP I) supported the biogenesis of TGN-derived secretory vesicles to the same extent as control cytosol. In either condition, the biogenesis of TGN-derived secretory vesicles was sensitive to inhibition by GTPγS (Fig. 3A,B, GTPγS, −COP+GTPγS). The hsPg- and SgII-containing vesicles formed with coatomer-depleted cytosol showed the same differential distribution on equilibrium sucrose gradients (indicative of the biogenesis of CSVs and ISGs, respectively) as those formed with control cytosol (data not shown).

## 4. Discussion

Our results implicate ARF proteins, specifically ARF1, but not the COP I coat, in the biogenesis of secretory vesicles from the TGN. Our findings are consistent with the observation that ARF, but not β-COP, is associated with CSVs of hepatocytes [22]. The lack of effect of coatomer depletion in the present modified cell-free system is in agreement with the lack of stimulation of secretory vesicle biogenesis by a coatomer-enriched cytosol fraction in a similar cytosol-dependent cell-free system [23]. Our observations are also in line with the results of a previous study addressing the role of coatomer in vivo, which shows that β-COP antibodies block membrane transport from the endoplasmic reticulum to the Golgi complex but not from the TGN to the plasma membrane [24]. Hence, it appears that the biogenesis of TGN-derived secretory vesicles is a COP I-independent process. Perhaps the biogenesis of TGN-derived secretory vesicles involves coats other than COP I, such as the 'lace-like' coat in the case of CSVs [25], or no coat, as has been discussed for ISGs [26].

The extent of stimulation and inhibition of TGN-derived secretory vesicle biogenesis by the *N*-myristoylated and the non-myristoylated ARF peptides, respectively, was different for ARF1 and ARF4. The *N*-myristoylated ARF1 peptide was a much more potent stimulator than the *N*-myristoylated ARF4 peptide, whereas the non-myristoylated ARF4 peptide was slightly more inhibitory than the non-myristoylated ARF1 peptide. This may reflect differences in the mechanisms underlying the stimulatory and inhibitory effects of the ARF peptides. The inhibitory effect of the non-myristoylated ARF1 and ARF4 peptides on the biogenesis of TGN-derived secretory vesicles may be interpreted in the same way as the inhibition by such peptides of vesicle formation from more proximal compartments of the secretory pathway [27,28], i.e. as a competitive action on endogenous ARF (presumably ARF1). If so, our data would imply that both the non-myristoylated ARF1 and ARF4 peptides can act as such competitive inhibitors [27]. However, the ability of the *N*-myristoylated ARF peptides, especially the ARF1 peptide, to *stimulate* secretory vesicle formation is remarkable, in particular if the only role of ARF in the biogenesis of TGN-derived secretory vesicles were the recruitment of, as yet poorly characterized, coat proteins. In this case, one would have to assume that this peptide, which is only 15 amino acid residues long, suffices to mediate the recruitment of these coat proteins to the TGN membrane. An alternative interpretation, which we favour, is that the stimulation of biogenesis of TGN-derived secretory vesicles by the *N*-myristoylated ARF1 peptide reflected the activation of phospholipase D. Phospholipase D appears to be present on Golgi membranes [29], is stimulated by ARF via ARF's myristoylated amino-terminal domain [30–32], and this stimulation is inhibited by brefeldin A [29]. Perhaps the *N*-myristoylated ARF1 peptide was more potent in eliciting a phospholipase D activation relevant to TGN-derived secretory vesicle biogenesis than the *N*-myristoylated ARF4 peptide [33]. A role of phospholipase D in the biogenesis of TGN-derived secretory vesicles would be consistent with the growing evidence implicating lipid modifications in the course of vesicle budding and fission [23,34–37].

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