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Effect of Protein Kinase A Activity on the Association of ADP-ribosylation Factor 1 to Golgi Membranes*

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The small GTP-binding protein ADP-ribosylation factor 1 (ARF1) is an essential component of the molecular machinery that catalyzes the formation of membrane-bound transport intermediates. By using an *in vitro* assay that reproduces recruitment of cytosolic proteins onto purified, high salt-washed Golgi membranes, we have analyzed the role of cAMP-dependent protein kinase A (PKA) on ARF1 incorporation. Addition to this assay of either pure catalytic subunits of PKA (C-PKA) or cAMP increased ARF1 binding. By contrast, ARF1 association was inhibited following C-PKA inactivation with either PKA inhibitory peptide or RIIα as well as after cytosol depletion of C-PKA. C-PKA also stimulated recruitment and activation of a recombinant form of human ARF1 in the absence of additional cytosolic components. The binding step could be dissociated from the activation reaction and found to be independent of guanine nucleotides and saturable. This step was stimulated by C-PKA in an ATP-dependent manner. Dephosphorylated Golgi membranes exhibited a decreased ability to recruit ARF1, and this effect was reverted by addition of C-PKA. Following an increase in the intracellular level of cAMP, ARF proteins redistributed from cytosol to the perinuclear Golgi region of intact cells. Collectively, the results show that PKA exerts a key regulatory role in the recruitment of ARF1 onto Golgi membranes. In contrast, PKA modulators did not affect recruitment of β-COP onto Golgi membranes containing prebound ARF1.

The small GTP-binding protein known as ADP-ribosylation factor 1 (ARF1)[1] is required for the recruitment of both COPI and adaptor coat proteins from cytosol and therefore plays an essential regulatory role in membrane trafficking processes along the endocytic and biosynthetic pathways (1–3). Like other Ras-related GTPases, ARF1 cycles between inactive GDP-bound and active GTP-bound conformations. Posttranslational *N*-myristoylation allows ARF1 to become inserted into membranes in a way that is coupled to the GDP-GTP conformational switch (4). Thus, inactive ARF-GDP is found in cytosol, whereas active ARF-GTP binds to intracellular membranes in a myristate-dependent manner. Activation depends on guanine nucleotide-exchange factors (GEFs) that promote the exchange of GDP for GTP. Several GEFs specific for ARF have been identified (5, 6). They all share a 200-amino acid region, referred to as the Sec7 domain, that is responsible for the exchange activity. However, the different ARF-specific GEFs differ in structural organization and sensitivity to brefeldin A, a drug that inhibits ARF1 activation and association to Golgi membranes (7–9). It is well established that once bound to a membrane ARF1-GTP promotes the recruitment of cytosolic coat components, but the exact mechanism is controversial at present. According to one view, activated ARF1 interacts directly with coat proteins serving as a membrane anchor for them (10, 11). This model is supported by the finding that ARF1 binds β-COP (12), a component of the coatomer complex which along with ARF1 itself form the COPI coat (13–15). Alternatively, the role of ARF1 in coat assembly has been proposed to be indirect. ARF1-GTP has been shown to activate phospholipase D, which catalyzes the hydrolysis of phosphatidylcholine to phosphatidic acid (16). Ktistakis *et al.* (17) reported conditions in which, in the absence of ARF1, coatomer could still bind to Golgi membranes containing a high level of phosphatidic acid. Additionally to its role on coat recruitment and phospholipase D activation, ARF1 has been described very recently to control both sorting of cargo molecules into transport vesicles (18) and the phospholipid composition of the Golgi membranes (19).

Golgi membranes contain sites where ARF1-GTP insertion preferentially occurs with high affinity. Although how these docking sites are recognized is unknown at present it possibly involves ARF1 interaction with membrane molecules (20–22). Aluminum fluoride, an activator of heterotrimeric G proteins, promotes stable association of both ARF1 and coatomer to a subset of binding sites in the Golgi membranes (23). The opposite effect applies to G protein $\beta\gamma$ subunits that inhibit ARF1 binding (24). Direct interaction of ARF1 with G$\beta\gamma$ and Gα_s has also been reported (25, 26). The aluminum fluoride effect may be mediated by protein kinase C (27). ARF1 has also been described to interact with phosphoinositides (28) and to be involved in several cell-signaling cascades (29–33). Collectively, these studies suggest that ARF1 recruitment and/or activation could be regulated by signal transduction molecules. We show in this report that cAMP-dependent protein kinase A (PKA) influences the interaction of ARF1 with the Golgi membranes. Addition of exogenous PKA catalytic subunits (C-PKA) increased binding of both cytosolic and recombinant ARF1 to purified Golgi membranes, whereas either depletion or inacti-

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[1] The abbreviations used are: ARF1, ADP-ribosylation factor 1; GEF, guanine nucleotide-exchange factor; PKA, cAMP-dependent protein kinase A; C-PKA, PKA catalytic subunit; PKI, PKA inhibitory peptide; TGN, trans-Golgi network; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; DTT, dithiothreitol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; NRK, normal rat kidney.

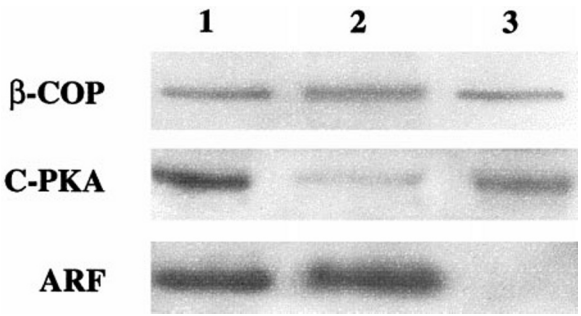

FIG. 1. **Analysis of different cytosol preparations.** Three different cytosol preparations were used as follows: crude bovine brain cytosol (*lane 1*), C-PKA-depleted cytosol (*lane 2*), and ARF-depleted cytosol (*lane 3*). 10 μg of each preparation were analyzed by SDS-PAGE and immunoblotting with anti-β-COP (M3A5), anti-C-PKA, and anti-ARF1 (1D9) antibodies.

vation of endogenous C-PKA decreased ARF1 binding. In addition, PKA activation caused ARF redistribution in intact cells. These data indicate that PKA exerts a key regulatory role in ARF1 recruitment from cytosol to intracellular membranes. Such effect explains the influence of PKA activity on protein transport processes along the exocytic (34–39) and endocytic (34, 39–42) pathways and is discussed in the context of evagination of transport intermediates from donor membranes. In particular, we propose the existence of protein targets in the Golgi membranes that when phosphorylated by PKA act as high affinity sites for ARF1 binding.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Mouse monoclonal antibody 1D9 recognizing ARF1, -3, -5, and -6 was a generous gift of Dr. R. A. Kahn (Emory University). M2 anti-FLAG and M3A5 anti-β-COP mouse monoclonal antibodies were from Sigma. A polyclonal antibody against bacterially expressed His-tagged Cα subunit of murine PKA (anti-C-PKA) was raised in rabbits. Antisera were subjected to ammonium sulfate precipitation and affinity-purified on recombinant protein coupled to activated Sepharose 4 (Amersham Pharmacia Biotech). Rabbit polyclonal antibody against Golgi mannosidase II has been previously described (43). Goat anti-mouse or anti-rabbit IgG secondary antibodies conjugated to fluorescein or rhodamine were from Tago (Burlingame, CA), and secondary antibodies conjugated to peroxidase were from BIOSOURCE International (Camarillo, CA). C-PKA and calphostin C were purchased from Calbiochem; PKI, forskolin, nucleotides, okadaic acid, and apyrase were from Sigma, and proteinase K and calf intestinal alkaline phosphatase were from Roche Molecular Biochemicals. Purified, recombinant RIIα was prepared as described previously (44).

Cytosol Preparations—Bovine brain cytosol was prepared according to Taylor *et al.* (45). Briefly, brains were homogenized at 4 °C in 25 mM Tris-HCl, pH 8.0, 500 mM KCl, 250 mM sucrose, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and centrifuged at 100,000 × *g* for 1 h. Supernatant was dialyzed extensively against 25 mM Hepes-KOH (pH 7.2), 25 mM KCl, 2.5 mM $MgCl_2$, and centrifuged at 30,000 × *g* for 30 min to remove precipitates. To immunodeplete C-PKA, 6 ml of crude cytosol (9 mg/ml) were incubated overnight at 4 °C with 3.5 mg of anti-C-PKA IgG and passed twice over a 4-ml protein A-Sepharose CL-4B column (Sigma). C-PKA-depleted cytosol was then concentrated to the original volume using an Ultrafree-10K (Millipore, Bedford, MA). For ARF depletion, 2 ml of concentrated cytosol (60 mg/ml) was fractionated at 24 ml/h on a Sephacryl S-200 column (60 × 2.6 cm, Amersham Pharmacia Biotech) equilibrated in cytosol buffer. Fractions that were shown by Western blotting to be depleted of ARF1 were pooled and concentrated at 7.5 mg/ml. A comparative immunoblotting analysis of the different cytosol preparations used is shown in Fig. 1.

Golgi Membrane Preparations—Intact Golgi stacks were prepared from rat liver as described (46). Membranes were incubated on ice with 3 M KCl for 10 min, recovered by centrifugation (12,000 × *g*, 20 min) on a 2 M sucrose cushion, resuspended in 25 mM Hepes-KOH, pH 7.2, 25 mM KCl, 2.5 mM $MgCl_2$, at 1 mg/ml, frozen in liquid nitrogen, and stored at −80 °C.

Preparation of Recombinant ARF1-FLAG—Recombinant baculovirus encoding human ARF1 tagged at the carboxyl terminus with the FLAG epitope was generated as described previously (47). Sf9 insect cells (2×10^8) were infected with 0.1 plaque-forming unit/cell for 1 h. They were harvested 48 h after infection and lysed in 4 ml of 50 mM Tris-HCl, pH 8.0, containing 400 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 100 μM GDP, and protease inhibitors (5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 100 μg/ml soybean trypsin inhibitor, 20 μg/ml aprotinin, and 10 μg/ml of each leupeptin, antipain, and pepstatin A). Cell lysate was clarified by centrifugation (20,000 × *g*, 20 min), and the supernatant was incubated overnight at 4 °C with 1 ml of anti-FLAG M2 affinity gel (Sigma). Beads were rinsed three times with lysis buffer, and bound ARF1-FLAG was eluted with acid. Protein was dialyzed against 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 5 mM $MgCl_2$, 10% (v/v) glycerol, concentrated at 0.2 mg/ml, and stored at −80 °C. Similar results to those reported in the present study with ARF1-FLAG were obtained with a myristoylated, untagged form of ARF1 expressed in bacteria and purified according to Liang and Kornfeld (48).

ARF1/β-COP Binding Assay—Binding of either ARF1 or β-COP to Golgi membranes was assayed in 1.5-ml siliconized microcentrifuge tubes at 37 °C for 15 min according to a previously described method (48–50). 10 μg of high salt-washed Golgi membranes were incubated with 25 μM GTPγS, ATP-regenerating system (1 mM ATP, 5 mM creatine phosphate, and 10 units/ml creatine kinase), and either 3 mg/ml cytosol or 2 μg of recombinant ARF1-FLAG in 25 mM Hepes-KOH (pH 7.2), 25 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT. The final volume of the assay was 50 μl. The reaction was stopped by transferring the tubes to ice and adding 1 ml of ice-cold assay buffer to each tube. Samples were transferred to new siliconized microcentrifuge tubes and centrifuged at 12,000 × *g* for 20 min. Membranes were recovered on a 35-μl 2 M sucrose cushion and transferred to new tubes. They were washed twice with assay buffer followed by solubilization in electrophoresis sample buffer. Proteins were reduced with 10 mM DTT and analyzed by 12.5% SDS-PAGE and immunoblotting. Immunoblots were revealed by enhanced chemiluminescence (SuperSignal Ultra Chemiluminescent Substrate, Pierce).

Guanine Nucleotide Exchange Assay—10-μl beads containing immunoabsorbed ARF1-FLAG were added to a reaction mixture consisting of 5 μg of Golgi membranes, 1 mM ATP, 1 mg/ml BSA, 250 pmol of [^{35}S]GTPγS (1000 Ci/mmol) in a final volume of 50 μl of 25 mM Hepes, pH 7.0, 50 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT. Incubation was carried out at 37 °C for 15 min in 1.5-ml siliconized microcentrifuge tubes. Samples were cooled on ice, and then 1 ml of ice-cold assay buffer was added to each tube, and the complete mixture was transferred to a new presiliconized microcentrifuge tube. Beads were washed twice with assay buffer before ^{35}S quantitation by liquid scintillation counting. Samples lacking either Golgi membranes or ARF1-FLAG were similarly processed and used to determine background binding of [^{35}S]GTPγS.

Experiments with Intact Cells—COS-7 and NRK cells were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. In experiments involving treatment with forskolin, they were first preincubated in serum-free medium for 2 h at 37 °C. For subcellular fractionation, cells were pelleted, rinsed with cold PBS, and resuspended in 3 ml of PBS containing protease inhibitors. They were homogenized in a ball-bearing homogenizer. The postnuclear supernatant was centrifuged at high speed (100,000 × *g*, 1 h at 4 °C) to obtain a total microsomal pellet. Membranes were rinsed, lysed in 1% (v/v) Triton X-100, and the amount of protein determined before processing for SDS-PAGE and immunoblotting. Cells grown on round glass coverslips were used for immunofluorescence. Microinjection was performed in complete culture medium containing 25 mM Hepes using an Eppendorf microinjection system (Hamburg, Germany). Cascade blue-conjugated bovine serum albumin (Molecular Probes, Eugene, OR) was used as a coinjection marker. Cells to be processed for immunofluorescence were fixed in 3% (w/v) formaldehyde, prepared from paraformaldehyde, in PBS, rinsed with plain PBS first, and then with 0.5% (w/v) bovine serum albumin, 0.05% (w/v) saponin in PBS (PBS/BSA/saponin). Incubation with antibodies, diluted in PBS/BSA/saponin, was performed in a moist chamber at 37 °C for 30 min. Coverslips were rinsed with PBS and mounted in 10% PBS, 90% glycerol.

RESULTS

Effect of PKA on Binding of Cytosolic ARF1 to Golgi Membranes—An *in vitro* binding assay was used to study recruitment of endogenous ARF1 from cytosol to the Golgi (48–50). Golgi membranes, deprived of peripheral proteins by high salt wash, were incubated at 37 °C with cytosol in the presence of ATP-generating system and GTPγS, which makes ARF binding

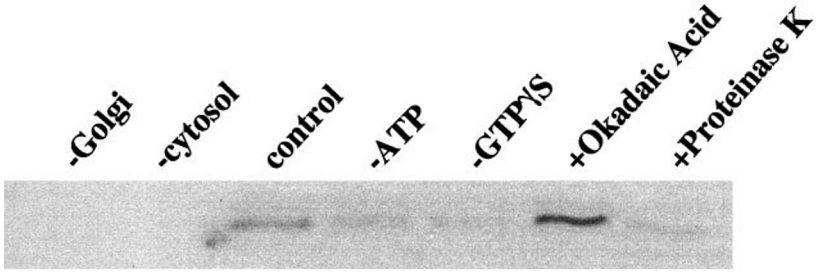

FIG. 2. **Requirements for ARF1 binding to Golgi membranes.** The standard binding assay consisted of high salt-washed Golgi membranes (10 μg) incubated at 37 °C for 15 min with cytosol in the presence of ATP-regenerating system and GTPγS (control). Membranes were reisolated, rinsed with buffer, and bound ARF1 detected by immunoblotting with 1D9 monoclonal antibody. Samples lacking either Golgi membranes, cytosol, or GTPγS were also similarly processed. Additionally, other samples contained cytosol preincubated for 30 min at 37 °C with either 0.6 units/ml apyrase to deplete ATP or 20 μM okadaic acid. Results derived from pretreatment on ice of Golgi membranes with 0.5 mg/ml proteinase K for 30 min prior to incubation in the complete binding assay are also shown.

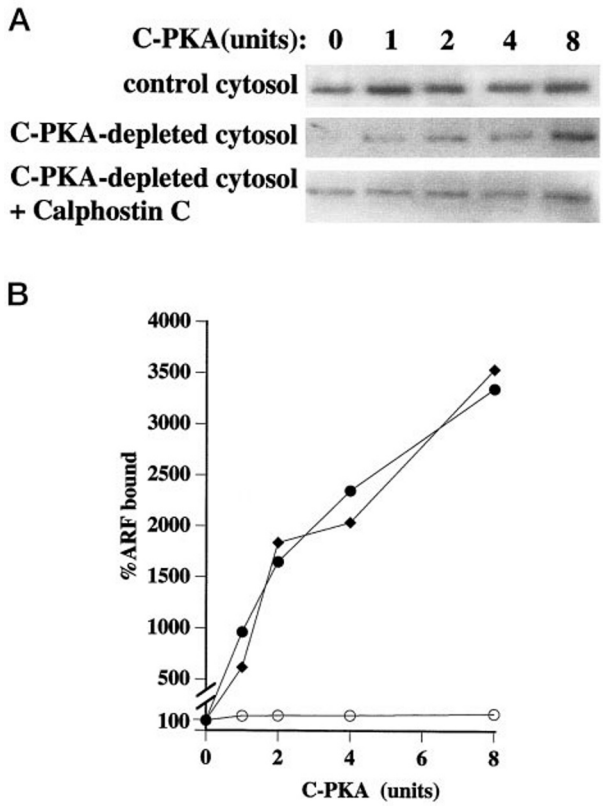

FIG. 3. **Effect of C-PKA addition on ARF1 recruitment from cytosol.** The binding assay contained either crude cytosol or C-PKA-depleted cytosol, and it was supplemented with the indicated amounts of pure C-PKA. Where indicated C-PKA-depleted cytosol preincubated for 15 min at 37 °C with 2 μM light-activated calphostin C was used. *A,* representative immunoblots of ARF1 incorporated from either crude (*control*) or C-PKA-depleted cytosol are shown. *B,* quantitation by scanning densitometry of ARF1 incorporated from both crude (○) and C-PKA-depleted cytosol in the presence (♦) or absence (●) of calphostin C. Values are average of three different experiments and expressed as percentage of the amount of ARF1 bound in the absence of C-PKA added.

relatively irreversible. Membranes were then reisolated, rinsed with buffer, and bound ARF1 detected by immunoblotting with 1D9 monoclonal antibody. While efficient recruitment of cytosolic ARF1 occurred during incubation of Golgi membranes with the other components, no apparent signal was detected when either membranes or cytosol were omitted from the incubation medium (Fig. 2). ARF1 binding was drastically reduced although not completely abolished (see below) in the absence of either ATP or GTPγS. The role of ATP was further investigated. ARF1 incorporation was increased 3–4-fold following preincubation for 30 min at 37 °C of crude cytosol with 10–20 μM okadaic acid, a specific serine/threonine phosphatase inhibitor (Fig. 2). This suggested the involvement of protein kinase activities in ARF1 recruitment from cytosol.

Accordingly, addition to this assay of exogenous, pure C-PKA slightly increased ARF1 binding. Thus, 8 units of C-PKA with a specific activity of 750 units/μg gave rise to a 1.5–2-fold increase (Fig. 3*A*). By contrast, addition of PKA regulatory (RIIα) subunits instead of catalytic subunits decreased ARF1 incorporation (Fig. 4*B*). C-PKA also increased 1.2–1.6-fold ARF1 binding to native, untreated Golgi membranes (not shown), but in order to evaluate the PKA effect high salt-washed membranes with little if any of prebound ARF1 were routinely used. Additionally, to assess further this effect cytosol was immunodepleted of C-PKA. As judged by immunoblotting, this cytosol preparation still contained ~30% of the original C-PKA content with no apparent decrease in the presence of both ARF1 and β-COP (Fig. 1, *lanes 1* and *2*). In comparison with crude cytosol ARF1 incorporation was reduced to less than 10% of the original value when C-PKA depleted cytosol was used as a source of ARF1 in the binding assay (Fig. 3*A*). Addition of exogenous C-PKA to this cytosol preparation, however, reestablished ARF1 recruitment. Thus, the level of ARF1 binding was comparable with both crude cytosol and C-PKA-depleted cytosol supplemented with 8 units of C-PKA. This implied a 33-fold increase in the case of C-PKA-depleted cytosol (Fig. 3*B*). To exclude the possibility that the stimulatory effect observed with C-PKA was due to a different serine/threonine protein kinase such as PKC the experiment was carried out in the presence of light-activated calphostin C which at the dose used, 2 μM, inhibits most PKC isoforms. As shown in Fig. 3 C-PKA stimulated ARF1 incorporation regardless of the presence or not of calphostin C in the incubation medium.

To reveal further the PKA effect, we used a peptide, PKI, containing the inhibitory sequence of the thermostable PKA inhibitor, a protein that functions as a natural, highly specific inhibitor of PKA activity (51). When added to the crude assay this 20-amino acid sequence caused significant inhibition of ARF1 binding (Fig. 4*A*). Complete inhibition required a high (10–20 μM) concentration of PKI although a 56 ± 3% decrease was already observed at 1 nM (Fig. 4*B*). Although it is possible that at high concentrations PKI has some nonspecific effects, the fact that considerable inhibition still occurred in the nanomolar range supported the involvement of PKA activity in ARF1 recruitment. In addition, PKI inhibition was comparable to that obtained with RIIα (Fig. 4*C*) consistent with the competitive interaction of both agents with the substrate-binding site of the kinase (52).

Effect of cAMP on ARF Redistribution—The above data suggested that ARF1 recruitment could be regulated by PKA ac-

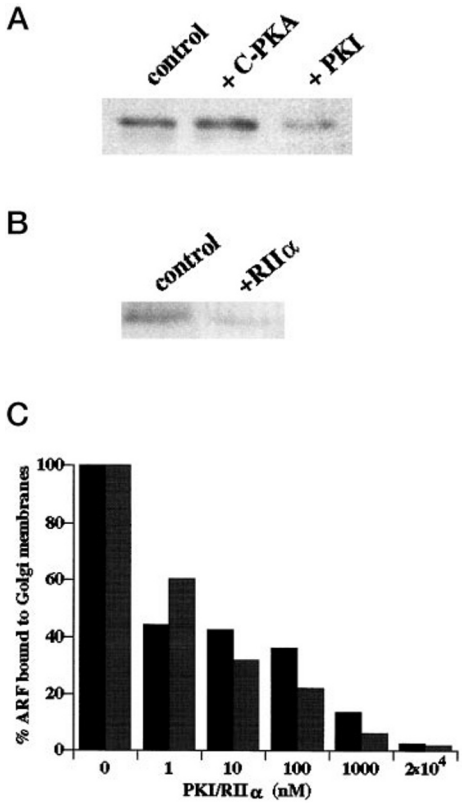

FIG. 4. **Effect of C-PKA inactivation on ARF1 recruitment from cytosol.** The assay that reproduces ARF1 binding from crude cytosol was carried out. *A* and *B,* immunoblots showing ARF1 recruited from cytosol supplemented or not (control) with either 8 units of C-PKA, 1 μM PKI, or 1 μM RIIα. *C,* quantitation of the amount of ARF1 incorporated in incubations containing the indicated concentrations of either PKI (*black bars*) or RIIα (*gray bars*). Data represent average of three different experiments. They are expressed as percentage of the amount of ARF1 bound in the absence of any inhibitor.

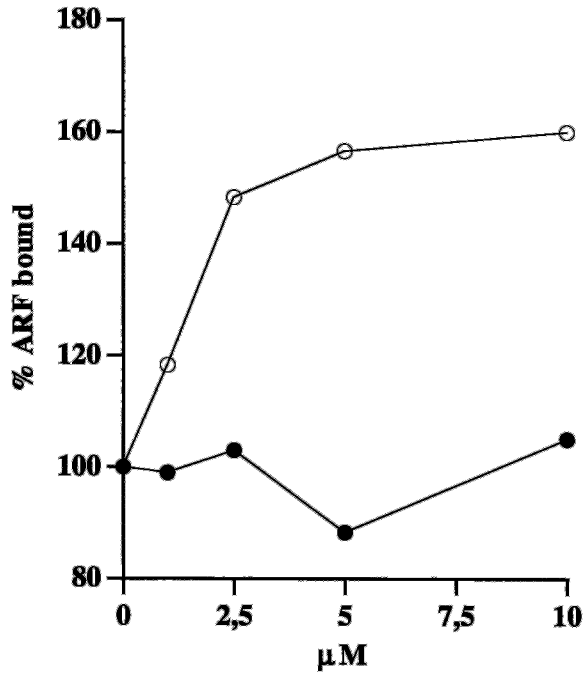

FIG. 5. **Effect of cAMP addition on ARF1 recruitment from cytosol.** The complete binding assay containing crude cytosol was supplemented with the indicated concentrations of either cAMP (○) or AMP (●). Data are average of two different experiments. They represent the amount of ARF1 bound to Golgi membranes and are expressed as percentage of the amount bound in the absence of cAMP/AMP added.

tivity, and in that case, modulators of this kinase should modify its intracellular distribution. As a first indication we studied the effect of cAMP on the recruitment of endogenous ARF1 from crude cytosol. Addition of 5–10 μM cAMP increased ARF1 binding by 1.5-fold. By contrast, addition of AMP either had no effect or even slightly decreased ARF1 incorporation (Fig. 5). We next analyzed the *in vivo* relevance of this observation. COS-7 cells were microinjected with either cAMP to activate PKA or, alternatively, with AMP as a negative control. Cells were fixed and processed for immunofluorescence to determine the intracellular distribution of ARF proteins (Fig. 6*A*). Both noninjected and AMP-injected cells showed a typical diffuse, cytoplasmic staining pattern. In cAMP-injected cells, however, a significant amount of ARF was concentrated in the perinuclear region and showed extensive colocalization with the Golgi marker galactosyltransferase (not shown). Similarly, noninjected NRK cells treated with forskolin which activates adenylate cyclase showed ARF redistribution from cytosol to the Golgi where it showed colocalization with mannosidase II (Fig. 6*B*). ARF1 redistribution induced by forskolin was quantitated by immunoblotting analysis of total microsomal membranes. The amount of ARF1 associated to intracellular membranes in cells treated with 250 μM forskolin for 30 min doubled that in control cells (Fig. 6*C*). Taken together these data support a regulatory role of PKA activity in ARF recruitment.

Effect of PKA on ARF1-FLAG Binding—In principle, PKA may affect ARF1 incorporation by inducing the phosphorylation of particular protein substrates either in the cytosol and/or in the Golgi membranes. To clarify this point, we examined recruitment of a recombinant form of ARF1 containing the FLAG epitope at the carboxyl terminus. Bound ARF1-FLAG was detected with an antibody against the tag. As shown in Fig. 7 efficient recruitment of ARF1-FLAG occurred in the absence of any other soluble protein. However, this incorporation was stimulated by addition of pure C-PKA. This was particularly evident at a low dose of both ARF1-FLAG and C-PKA. Thus, a 3.4-fold increase was observed with 1 unit of C-PKA and 2 μg of ARF1-FLAG (Fig. 7*B*). Maximal stimulation, *i.e.* ~5-fold increase, required 4 units of C-PKA, and a further increase up to 20 units of C-PKA did not cause further stimulation. Importantly, no stimulatory effect was induced in the absence of ATP or when heat-inactivated C-PKA was used (Fig. 7*B*) indicating that PKA enzymatic activity, rather than just the protein, was necessary in order to stimulate ARF1 recruitment.

Influence of the Phosphorylation State of Golgi Membranes on ARF1 Binding—The above data indicated that although ARF1 can bind Golgi membranes in the absence of additional cytosolic factors, pure C-PKA stimulates such a recruitment. Since ARF1 itself does not contain a consensus PKA phosphorylation site, it was postulated that the relevant PKA substrates would be Golgi integral membrane proteins that once phosphorylated would function as high affinity binding sites for ARF1. To test this possibility we evaluated the importance of the phosphorylation state of the Golgi membranes for ARF1 binding. Golgi membranes were subjected to dephosphorylation by preincubation with alkaline phosphatase and then tested for their ability to bind either recombinant ARF1-FLAG (Fig. 8) or cytosolic ARF1 (not shown). In both cases, a significant decrease in ARF1 binding was observed following pretreatment of Golgi membranes with alkaline phosphatase. This reduction ranged from 40 to 30% of the control value depending of the amount of enzyme (50–250 units). The preparation of alkaline phosphatase was tested for activity and shown to cause the loss of almost 50% (47 ± 5%) of ^{32}P counts incorporated into microsomal membranes during overnight radiolabel-

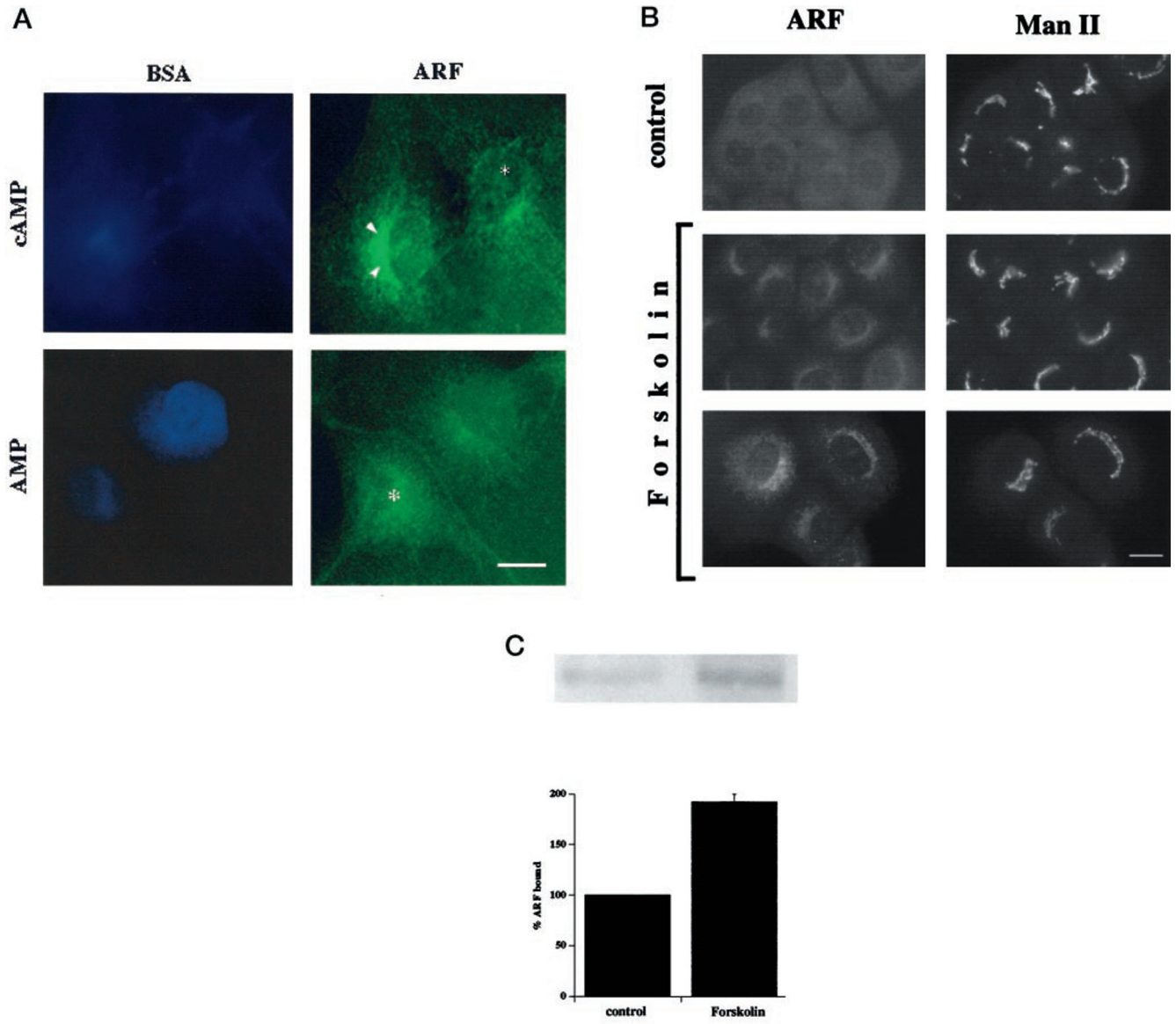

FIG. 6. **Effect of PKA activation *in vivo* on ARF intracellular distribution.** *A,* COS-7 cells were microinjected with 10 μM of either cAMP or AMP mixed with 2 mg/ml cascade blue-conjugated BSA (*BSA*) used as co-injection marker. Cells were fixed immediately and processed for indirect immunofluorescence with 1D9 monoclonal antibody. ARF concentration in the perinuclear region is indicated (*arrowheads*). *Asterisks* indicate noninjected cells. *Bar*, 25 μm. *B* and *C,* NRK cells were preincubated at 37 °C for 2 h in serum-free medium and then incubated or not (control) with 250 μM forskolin for 30 min. *B,* cells were fixed and processed for immunofluorescence with 1D9 monoclonal antibody against ARF and polyclonal antibody against Golgi mannosidase II (*Man II*). *Bar,* 25 μm. *C,* total microsomal membranes were prepared from cell homogenates. 7 μg of each membrane preparation were resolved by SDS-PAGE and analyzed by immunoblotting with 1D9 monoclonal antibody against ARF. Quantitative data (mean ± S.D. of three different determinations) are expressed as percentage of the amount of ARF1 bound to membranes in untreated, control cells.

ing of COS-7 cells with 1 mCi of [^{32}P]orthophosphate. Importantly, incubation of previously dephosphorylated Golgi membranes with C-PKA reestablished ARF1 incorporation (Fig. 8). Therefore, these data indicated that the phosphorylation state of the Golgi membranes affects ARF1 recruitment and pointed to PKA as one of the kinases involved in this process.

Effect of PKA on ARF1 Activation by Golgi Membranes—It is well documented that in the Golgi membranes resides a GEF activity that catalyzes the exchange of GDP for GTP on ARF1 causing its activation (7, 8, 53). Since PKA exerts its stimulatory role on ARF1 binding by inducing the phosphorylation of selected Golgi proteins, we next determined whether ARF1 activation by Golgi membranes would also be affected by PKA activity. Purified ARF1-FLAG loaded with GDP and bound to an affinity gel was incubated at 37 °C with high salt-washed Golgi membranes in the presence of ATP and [^{35}S]GTPγS. Beads were rinsed, and the radioactivity associated was measured and taken as an indication of the GDP/[^{35}S]GTPγS exchange catalyzed by the Golgi membranes. Under standard incubation conditions 2.14 ± 0.6 pmol of [^{35}S]GTPγS were incorporated to each nmol of ARF1-FLAG. This value was increased 1.4–1.7-fold in incubations containing 8–10 units of C-PKA (Fig. 9). Interestingly, no stimulation occurred when native, untreated Golgi membranes were used. In this case membranes exhibited a strong exchange activity (higher than 15 pmol of [^{35}S]GTPγS/nmol ARF1-FLAG) that presumably masked a possible stimulatory effect caused by exogenous C-PKA. Background radioactivity present in samples lacking either Golgi membranes or ARF1-FLAG-beads was, in any case, less than 6% of control value. Similar to what happened with the ARF1-FLAG binding assay, the stimulatory effect

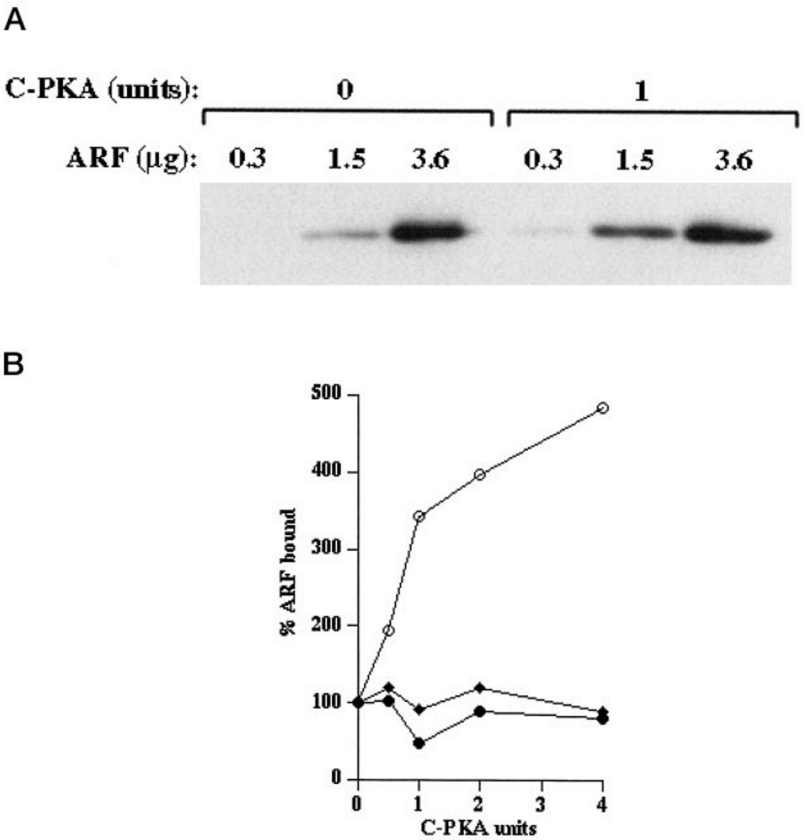

FIG. 7. **Effect of C-PKA addition on ARF1-FLAG recruitment.** A recombinant, tagged form of human ARF1, ARF1-FLAG, was used instead of cytosol. Protein bound to the Golgi membranes was detected with anti-FLAG antibody. *A,* immunoblot showing ARF1-FLAG incorporated to the Golgi membranes in incubations containing or not C-PKA and different amounts of ARF1-FLAG. *B,* the binding assay contained 2 μg of ARF1-FLAG and the indicated amounts of either native (○) or heat-inactivated (90 °C, 30 min) (●) C-PKA. Results from incubations containing native C-PKA and apyrase (0.6 units/ml) (♦) are also shown.

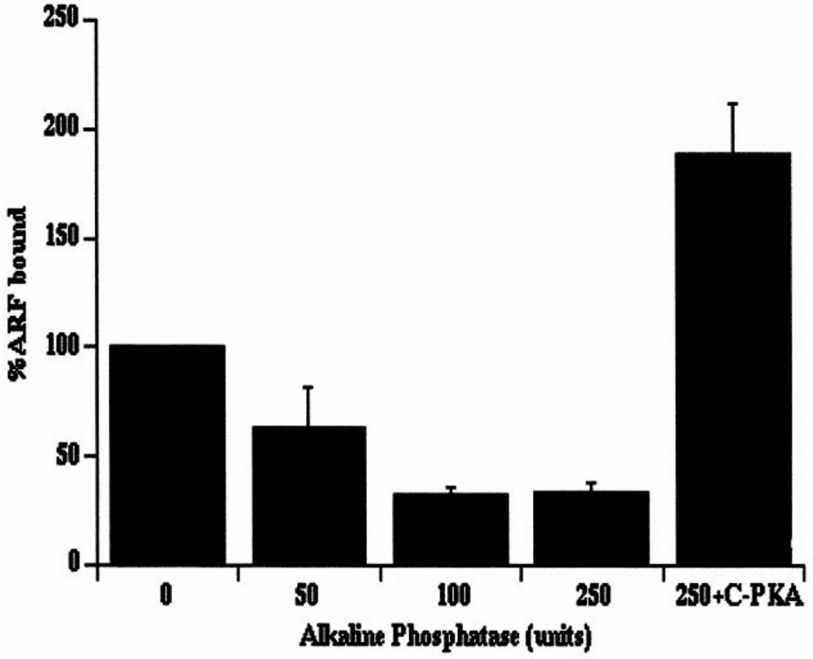

FIG. 8. **Effect of pretreatment of Golgi membranes with alkaline phosphatase on ARF1 recruitment from cytosol.** High salt-washed Golgi membranes (10 μg) were preincubated at 37 °C for 30 min with the indicated units of calf intestinal alkaline phosphatase in 50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 1 mM $MgCl_2$, and protease inhibitors. Membranes were rinsed twice with cold 25 mM Hepes-KOH, pH 7.2, 25 mM KCl, 2.5 mM $MgCl_2$, 10 mM sodium pyrophosphate prior to incubation in the ARF1-FLAG binding assay. Where indicated 8 units of C-PKA were added to the assay. Data (mean ± S.D. of three different experiments) represent the amount of ARF1 bound to Golgi membranes and are expressed as percentage of the amount bound to membranes preincubated in the absence of alkaline phosphatase.

induced by C-PKA on ARF1-FLAG activation depended on the simultaneous presence of ATP in the incubation medium (not shown) supporting a catalytic role for PKA in this stimulation.

C-PKA Effect on ARF1 Binding Is Independent on Guanine Nucleotides—As mentioned above for the crude binding assay some ARF1 recruitment took place in the absence of GTPγS

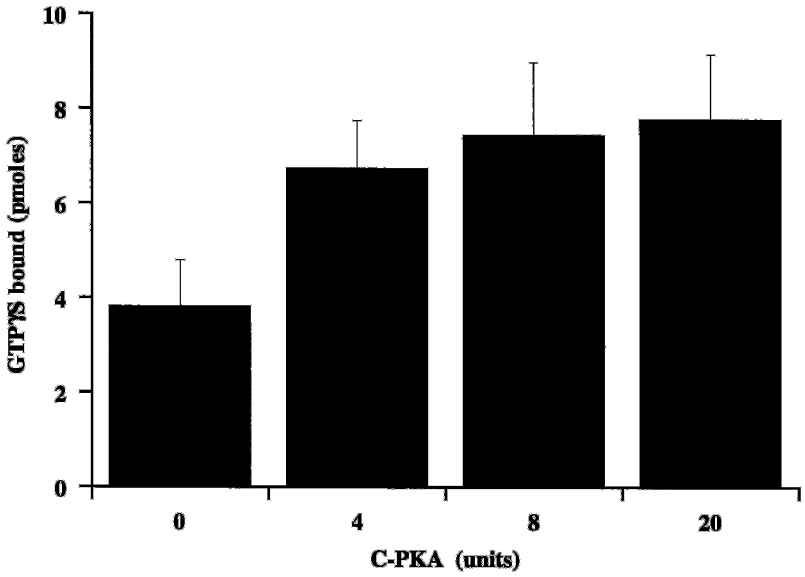

FIG. 9. **Effect of C-PKA addition on ARF1-FLAG activation.** Beads with GDP-ARF1-FLAG bound were incubated at 37 °C for 15 min with Golgi membranes, ATP, [^{35}S]GTPγS, and the indicated amounts of pure C-PKA. They were rinsed and processed for liquid scintillation counting. Data (mean ± S.D.) are average of three different determinations.

(Fig. 2). This was also evident with recombinant ARF1-FLAG in assays that were scaled up 2-fold. Binding of ARF1-FLAG to Golgi membranes in the presence of either GDP, GTP (Fig. 10*A*), or in the absence of guanine nucleotides (Fig. 10*B*) was strongly decreased in comparison to binding in the presence of GTPγS. Nevertheless, the amount of ARF1-FLAG incorporated under these conditions was sufficient to allow us to evaluate recruitment independently of ARF activation. Therefore, we monitored the time course of ARF1-FLAG incorporation to high salt-washed Golgi membranes in the absence of guanine nucleotides in the incubation medium (Fig. 10*B*). Either in the presence or in the absence of C-PKA binding increased linearly during the initial 5 min of incubation and reached saturation over 10–15 min of incubation. At later time points, 20–30 min, the amount of ARF1-FLAG remaining bound to the membranes started to decrease possibly as consequence of dissociation (not shown). At all time points examined C-PKA increased ARF1-FLAG binding. However, the kinetic of recruitment was similar in both cases since $t_{1/2}$ was 0.8 min in the absence of C-PKA *versus* 1.2 min in the presence of C-PKA (Fig. 10*B*). This suggests a role for C-PKA in the generation of ARF1 high affinity binding sites.

Effect of PKA on β-COP Recruitment—Some components of the coatomer complex including β-COP and δ-COP are serine-phosphorylated *in vivo* (54) and contain consensus PKA phosphorylation sequences (3 sites in the case of rat β-COP and 2 sites in the case of human δ-COP). We wondered whether coatomer recruitment would also be affected by PKA activity regardless of the PKA effects on ARF1 binding. We therefore examined β-COP incorporation using the above binding assay (Fig. 11). Addition of pure C-PKA to the incubation medium containing crude cytosol resulted in a marginal (~10–20%) increase on β-COP association to the Golgi membranes (Fig. 11*A*). This stimulation, however, became evident when C-PKA-depleted cytosol was used instead. In this case, a significant 2.5-fold increase was observed following addition of 8 units of C-PKA (Fig. 11*A*). It is important to note that this increase on β-COP binding includes the stimulatory effect of C-PKA on ARF1 binding as well. In order to strictly evaluate β-COP incorporation, a cytosol preparation enriched in high molecular weight proteins and practically devoid of ARF1 was obtained. As shown in Fig. 1 (compare *lanes* 1 and *3*) β-COP and C-PKA were present at similar concentrations in both crude and ARF-depleted cytosols, whereas the amount of ARF1 in the latter was reduced from 230 μg/ml in crude cytosol to 23 μg/ml. Golgi membranes were preincubated with recombinant ARF1-FLAG first and then incubated with ARF-depleted cytosol (Fig. 11*B*). As expected, β-COP efficiently bound to those Golgi membranes that had been preincubated with ARF1-FLAG (Fig. 11*B, lane 2*) but not to those lacking ARF1-FLAG bound (Fig. 11*B, lane 1*). This corroborates the statement that coatomer binding requires previous ARF1 incorporation (49, 50). The effects of adding PKA modulators during the second incubation step were then evaluated. When samples preincubated with ARF1-FLAG were incubated with incubation medium containing ARF-depleted cytosol and supplemented with either pure C-PKA (Fig. 11*B, lane 4*) or PKI (Fig. 11*B, lane 3*), no stimulatory or inhibitory effect on β-COP binding was observed. This indicated that PKA activity had no effect on β-COP binding. This conclusion was also supported by the observation that apyrase decreased β-COP recruitment when added during the first incubation step (not shown) but not when added to the second incubation step (Fig. 11*B, lane 5*), indicating that in contrast to ARF1 recruitment (Fig. 7*B*) ATP depletion does not affect β-COP incorporation.

DISCUSSION

We show in this report that ARF1 association to the Golgi complex is regulated by PKA activity. Pure catalytic subunits of PKA (C-PKA) promoted recruitment of both endogenous, cytosolic ARF1 and a recombinant form of human ARF1, ARF1-FLAG, onto purified Golgi membranes. Conversely, inhibition of endogenous PKA activity present in the cytosol with either PKI or RIIα decreased ARF1 recruitment. Cytosol depletion with anti-C-PKA also diminished ARF1 binding. In fact, the stimulatory effect of C-PKA on ARF1 recruitment from cytosol was best shown when C-PKA-depleted cytosol was used. In this case, a significant 30-fold increase occurred. Furthermore, ARF1 recruitment was stimulated following an increase in cAMP concentration. *In vivo* this resulted in ARF1 redistribution from cytosol to the perinuclear Golgi region. Collectively, these data indicate that ARF1 association to Golgi membranes is positively modulated by PKA activity. One would anticipate that the PKA stimulatory effect would be reverted by phosphatases present in the cytosol. Indeed, ARF1 incorporation was increased after cytosol preincubation with okadaic acid, a specific serine/threonine phosphatase inhibitor.

A stimulatory effect of PKC activity on ARF1 binding has been previously reported (27). Whereas it is possible that ARF1 recruitment to intracellular membranes including the Golgi

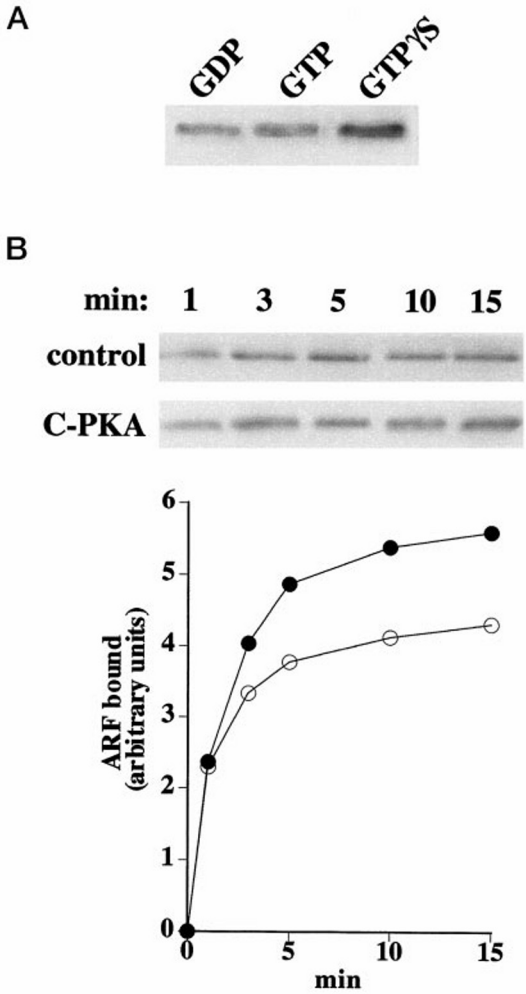

FIG. 10. **Effect of C-PKA addition on the guanine nucleotide-independent recruitment of ARF1-FLAG.** The ARF1-FLAG binding assay was scaled up 2-fold. *A,* binding in the presence of the indicated guanine nucleotides. *B,* time course of the association of ARF1-FLAG to Golgi membranes in the absence of guanine nucleotides added and in the presence (●) or not (○) of 8 units of C-PKA. Data are average of two different experiments.

complex is regulated by more than one protein kinase, the data here described do not seem to be related to PKC activation. Thus, PKC activity is not expected to be affected by reagents such as C-PKA, RIIα, PKI, and cAMP which are natural, specific modulators of PKA activity. In addition, C-PKA stimulated ARF1 recruitment in the presence of calphostin C, a potent PKC inhibitor. On the other hand, the stimulatory effect observed with C-PKA was saturable with respect to both dose and time. This and results obtained with PKI and RIIα argue against the possibility that the observed effects merely reflect the introduction of negative charges onto the membranes that could affect ARF1 binding nonspecifically. Instead, the results support a specific role of PKA activity in ARF1 recruitment from cytosol onto the Golgi membranes.

ARF1 is an ubiquitous, abundant protein involved in multiple cellular functions (5). As far as transport activities are concerned, ARF1 mediates recruitment of COPI (49, 50), AP-1 (21, 55), and AP-3 (56) complexes that accounts for the involvement of this small GTP-binding protein in a variety of transport pathways including anterograde and retrograde transport between the endoplasmic reticulum and the Golgi (57, 58), intra-Golgi transport (45), and transport from the Golgi to either endosomes or the plasma membrane (59, 60). Also, endosome fusion (61) and nuclear envelope fusion (62) as well as the biogenesis of peroxisomes (63) and secretory (59, 64) and synaptic (65) vesicles have been reported to occur in an ARF1-dependent manner. In addition, a growing body of evidence suggests that ARF1 is involved in signal transduction events (29–33), cell growth and division (66), cytoskeleton rearrangements (67–70), cell adhesion (71), and the maintenance of organelle structure (72–76). It is evident from the multiplicity of functional roles assigned to this protein, the number of different molecules interacting with it, and from the diversity of ARF1-mediated transport steps that mechanisms must exist that determine the particular effects in each case. In this respect, ARF1 recruitment to particular subcellular locations most likely determines downstream effects. In the case of the Golgi membranes two different pools of prebound ARF1 have been characterized (20). One was a loosely bound, nonsaturable pool of molecules extractable with liposomes, and the other was a tightly bound pool of ARF1 molecules that resisted liposome extraction. The latter pool was hypothesized to comprise ARF1 molecules interacting with a putative membrane receptor (20). Recruitment of AP-1 adaptor complex onto the trans-Golgi network (TGN) has also been described to require previous ARF1 interaction with a docking protein (21, 22). In support of this model is our finding that Golgi membranes incubated with proteinase K exhibited a decreased ability to recruit ARF1 (Fig. 2). Nevertheless, the fact that ARF1 can bind native, untreated Golgi membranes *in vitro* suggests that if a receptor is required it is already present in the purified membranes in a functional conformation. According to our data the receptor would be a protein that is tightly associated to the membrane since it resisted high salt extraction and its interaction with ARF1 would be modulated by phosphorylation. Thus, ARF1 recruitment was dramatically decreased following dephosphorylation with alkaline phosphatase of high salt-washed Golgi membranes. The fact that addition of pure C-PKA reestablished the capacity of previously dephosphorylated Golgi membranes to recruit ARF1 suggests that this kinase plays a relevant role in the activation of the putative receptor. It is also important to note that as expected from the interaction of soluble ARF1 with a PKA-sensitive receptor the stimulatory effect of adding C-PKA to the binding assay was saturable.

C-PKA slightly increased the exchange of GDP for GTP on ARF1 in the presence of Golgi membranes. It is therefore possible that PKA plays a role in ARF1 activation either by somehow stimulating spontaneous nucleotide exchange or, alternatively, throughout the regulation of a still uncharacterized GEF protein associated to the Golgi membranes. Although this issue remains open at present, additional data indicate that the primary PKA effect is exerted on ARF1 binding. This step was shown to be independent on guanine nucleotides and therefore could be analyzed separate from the activation reaction. In the absence of guanine nucleotides, addition of C-PKA increased ARF1 binding without modifying significantly the kinetic of recruitment. This suggests that new ARF1 high affinity binding sites were generated following the C-PKA-mediated phosphorylation of selective protein targets. Additionally, these observations support a model by which binding of ARF1-GDP to the membrane would precede the nucleotide exchange reaction (4, 77). According to our data the former step would be regulated by PKA activity.

ARF1 binding to a particular membrane is the prerequisite that triggers coat assembly and ultimately evagination of a transport intermediate (1). It has been shown previously that interaction of AP-1 and AP-2 adaptor proteins with clathrin is modulated by serine phosphorylation (78). This along with the finding that *in vivo* the coatomer proteins β-COP and δ-COP exhibit considerable charge heterogeneity due to variable ser-

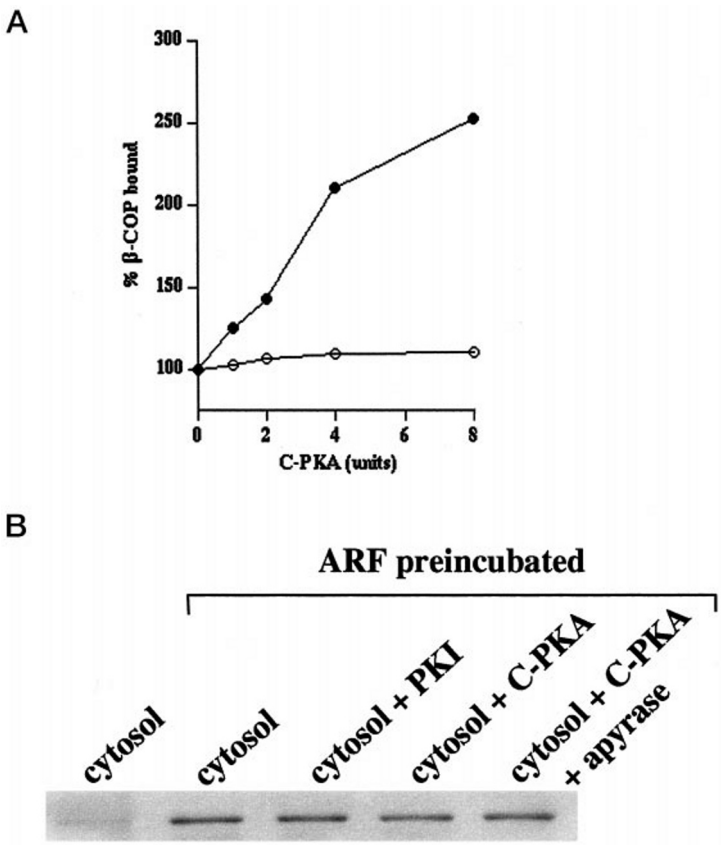

FIG. 11. **Effect of PKA activity on β-COP recruitment from cytosol.** *A,* high salt-washed Golgi membranes (10 μg) were incubated at 37 °C for 15 min with bovine brain cytosol, ATP-regenerating system, GTPγS, and the indicated amounts of pure C-PKA added. Membranes were reisolated, rinsed with buffer, and bound β-COP detected by immunoblotting with M3A5 antibody. Data (average of three different experiments) obtained by scanning densitometry of β-COP incorporated from both crude (○) and C-PKA-depleted (●) cytosol are expressed as percentage of the amount of β-COP bound in the absence of C-PKA. *B,* Golgi membranes were preincubated at 37 °C for 10 min with buffer containing ATP-regenerating system, GTPγS, and either 2 μg ARF1-FLAG or no further addition (sample not preincubated with ARF). ARF-depleted cytosol was then added to all samples and incubation continued at 37 °C for 15 min in the absence of additional components (cytosol) or, alternatively, in the presence of 1 μM PKI, 8 units of C-PKA, or 8 units of C-PKA plus 0.6 units/ml apyrase.

ine phosphorylation (54) led us to think that in addition to ARF1 incorporation coatomer recruitment could also be affected by PKA activity. Indeed, addition of C-PKA to the binding assay increased β-COP recruitment. However, additional data obtained with ARF-depleted cytosol and Golgi membranes containing prebound ARF1-FLAG revealed that PKA does not influence β-COP recruitment. Thus, addition of either C-PKA or PKI did not affect β-COP binding when this association was evaluated in an ARF1-independent way. These data, therefore, indicate that PKA activity stimulates evagination of transport intermediates by primarily increasing ARF1 recruitment.

PKA activity has been involved in a number of transport events including transport from the endoplasmic reticulum to the Golgi (37), across the Golgi stack (37), and from the TGN to the plasma membrane (35–37, 39). Also, exocytosis (79), endocytosis (40–42), and transcytosis (34, 39) have been described to be regulated by PKA activity. We have recently reported that PKA activity is required for the generation of TGN-derived constitutive transport vesicles containing the envelope glycoprotein of vesicular stomatitis virus (38). Very recently these vesicles have also been shown to be formed *in vitro* in an ARF- and coatomer-dependent manner (60, 80). By generalization, it can be speculated that PKA has a general, regulatory role in most membrane trafficking processes. That would be the formation of transport vesicles from a donor compartment by controlling the amount of ARF1 recruited onto the membrane. The data shown here provide evidence that PKA could do this by inducing the phosphorylation of an ARF1-specific receptor located in the membrane.

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REFERENCES

1. Donaldson, J. G., and Klausner, R. D. (1994) *Curr. Opin. Cell Biol.* **6,** 527–532
2. Boman, A. L., and Kahn, R. A. (1995) *Trends Biochem. Sci.* **20,** 147–150
3. Moss, J., and Vaughan, M. (1995) *J. Biol. Chem.* **270,** 12327–12330
4. Goldberg, J. (1998) *Cell* **95,** 237–248
5. Moss, J., and Vaughan, M. (1998) *J. Biol. Chem.* **273,** 21431- 21434
6. Roth, M. G. (1999) *Cell* **97,** 149–152
7. Donaldson, J. G., Finazzi, D., and Klausner, R. D. (1992) *Nature* **360,** 350–352
8. Helms, J. B., and Rothman, J. E. (1992) *Nature* **360,** 352–354
9. Chardin, P., and McCormick, F. (1999) *Cell* **97,** 153–155
10. Spang, A., Matsuoka, K., Hamamoto, S., Schekman, R., and Orci, L. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 11199–11204
11. Stamnes, M., Schiavo, G., Stenbeck, G., Söllner, T. H., and Rothman, J. E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 13676–13680
12. Zhao, L., Helms, J. B., Brügger, B., Harter, C., Martoglio, B., Graf, R., Brunner, J., and Wieland, F. T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 4418–4423
13. Serafini, T., Orci, L., Amherdt, M., Brunner, M., Kahn, R. A., and Rothman, J. E. (1991) *Cell* **67,** 239–253
14. Waters, M. G., Serafini, T., and Rothman, J. E. (1991) *Nature* **349,** 248–251
15. Orci, L., Palmer, D. J., Amherdt, M., and Rothman, J. E. (1993) *Nature* **364,** 732–734
16. Brown, H. A., Gutowski, S., Moomaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) *Cell* **75,** 1137–1144
17. Ktistakis, N. T., Brown, H. A., Waters, M. G., Sternweis, P. C., and Roth, M. G. (1996) *J. Cell Biol.* **134,** 295–306
18. Lanoix, J., Ouwendijk, J., Lin, C., Stark, A., Love, H. D., Ostermann, J., and Nilsson, T. (1999) *EMBO J.* **18,** 4935–4948
19. Godi, A., Pertile, P., Meyers, R., Marra, P., Di Tullio, G., Lurisci, C., Luini, A., Corda, D., and De Matteis, M. A. (1999) *Nat. Cell Biol.* **1,** 280–287
20. Helms, J. B., Palmer, D. J., and Rothman, J. E. (1993) *J. Cell Biol.* **121,** 751–760
21. Traub, L. M., Ostrom, J. A., and Kornfeld, S. (1993) *J. Cell Biol.* **123,** 561–573
22. Zhu, Y., Traub, L. M., and Kornfeld, S. (1998) *Mol. Biol. Cell* **9,** 1323–1337
23. Finazzi, D., Cassel, D., Donaldson, J. G., and Klausner, R. D. (1994) *J. Biol. Chem.* **269,** 13325–13330
24. Donaldson, J. G., Kahn, R. A., Lippincott-Schwartz, J., and Klausner, R. D. (1991) *Science* **254,** 1197–1199
25. Randazzo, P. A., Terui, T., Sturch, S., and Kahn, R. A. (1994) *J. Biol. Chem.* **269,** 29490–29494
26. Colombo, M. I., Inglese, J., D'Souza-Schorey, C., Beron, W., and Stahl, P. D. (1995) *J. Biol. Chem.* **270,** 24564–24571
27. De Matteis, M. A., Santini, G., Kahn, R. A., Di Tullio, G., and Luini, A. (1993) *Nature* **364,** 818–820
28. Randazzo, P. A. (1997) *J. Biol. Chem.* **272,** 7688–7692
29. Karnam, P., Standaert, M. L., Galloway, L., and Farese, R. V. (1997) *J. Biol. Chem.* **272,** 6136–6140

30. Shome, K., Vasudevan, C., and Romero, G. (1997) *Curr. Biol.* **7,** 387–396
31. Fensome, A., Whatmore, J., Morgan, C., Jones, D., and Cockcroft, S. (1998) *J. Biol. Chem.* **273,** 13157–13164
32. Luo, J.-Q., Liu, X., Frankel, P., Rotunda, T., Ramos, M., Flom, J., Jiang, H., Feig, L. A., Morris, A. J., Kahn, R. A., and Foster, D. A. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 3632–3637
33. Mitchell, R., McCulloch, D., Lutz, E., Johnson, M., MacKenzie, C., Fennell, M., Fink, G., Zhou, W., and Sealfon, S. C. (1998) *Nature* **392,** 411–414
34. Hansen, S. H., and Casanova, J. E. (1994) *J. Cell Biol.* **126,** 677–687
35. Pimplikar, S. W., and Simons, K. (1994) *J. Biol. Chem.* **269,** 19054–19059
36. Jilling, T., and Kirk, K. L. (1996) *J. Biol. Chem.* **271,** 4381–4387
37. Muñiz, M., Alonso, M., Hidalgo, J., and Velasco, A. (1996) *J. Biol. Chem.* **271,** 30935–30941
38. Muñiz, M., Martín, M. E., Hidalgo, J., and Velasco, A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 14461–14466
39. Zegers, M. M. P., and Hoekstra, D. (1997) *J. Cell Biol.* **138,** 307–321
40. Bradbury, N. A., and Bridges, R. J. (1992) *Biochem. Biophys. Res. Commun.* **184,** 1173–1180
41. Eker, P., Holm, P. K., van Deurs, B., and Sandvig, K. (1994) *J. Biol. Chem.* **269,** 18607–18615
42. Goretzki, L., and Mueller, B. M. (1997) *J. Cell Sci.* **110,** 1395–1402
43. Velasco, A., Hendricks, L., Moremen, K. W., Tulsiani, D. R. P., Touster, O., and Farquhar, M. G. (1993) *J. Cell Biol.* **122,** 39–51
44. Martín, M. E., Hidalgo, J., Vega, F. M., and Velasco, A. (1999) *J. Cell Sci.* **112,** 3869–3878
45. Taylor, T. C., Kahn, R. A., and Melançon, P. (1992) *Cell* **70,** 69–79
46. Slusarewicz, P., Nilsson, T., Hui, N., Watson, R., and Warren, G. (1994) *J. Cell Biol.* **124,** 405–413
47. Rosa, J. L., Casaroli-Marano, R. P., Buckler, A. J., Vilaró, S., and Barbacid, M. (1996) *EMBO J.* **15,** 4262–4273
48. Liang, J. O., and Kornfeld, S. (1997) *J. Biol. Chem.* **272,** 4141–4148
49. Donaldson, J. G., Cassel, D., Kahn, R. A., and Klausner, R. D. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 6408–6412
50. Palmer, D. J., Helms, J. B., Beckers, C. J. M., Orci, L., and Rothman, J. E. (1993) *J. Biol. Chem.* **268,** 12083–12089
51. Walsh, D. A., Angelos, K. L., vanPatten, S. M., Glass, D. B., and Garetto, L. P. (1990) in *Peptides and Protein Phosphorylation* (Kemp, B. E., ed) pp. 43–84, CRC Press, Inc., Boca Raton, FL
52. Taylor, S. S., Knighton, D. R., Zheng, J., Eyck, L. F. T., and Sowadski, J. M. (1992) *Annu. Rev. Cell Biol.* **8,** 429–462
53. Randazzo, P. A., Yang, Y. C., Rulka, C., and Kahn, R. A. (1993) *J. Biol. Chem.* **268,** 9555–9563
54. Sheff, D., Lowe, M., Kreis, T. E., and Mellman, I. (1996) *J. Biol. Chem.* **271,** 7230–7236
55. Stamnes, M. A., and Rothman, J. E. (1993) *Cell* **73,** 999–1005
56. Ooi, C. E., Dell'Angelica, E. C., and Bonifacino, J. S. (1998) *J. Cell Biol.* **142,** 391–402
57. Balch, W. E., Kahn, R. A., and Schwaninger, R. (1992) *J. Biol. Chem.* **267,** 13053–13061
58. Letourneur, F., Gaynor, E. C., Hennecke, S., Démollière, C., Duden, R., Emr, S. D., Riezman, H., and Cosson, P. (1994) *Cell* **79,** 1199–1207
59. Nickel, W., Huber, L. A., Kahn, R. A., Kipper, N., Barthel, A., Fasshauer, D., and Söling, H.-D. (1994) *J. Cell Biol.* **125,** 721–732
60. Simon, J.-P., Ivanov, I. E., Shopsin, B., Hersh, D., Adesnik, M., and Sabatini, D. D. (1996) *J. Biol. Chem.* **271,** 16952–16961
61. Lenhard, J. M., Kahn, R. A., and Stahl, P. D. (1992) *J. Biol. Chem.* **267,** 13047–13052
62. Boman, A. L., Taylor, T. C., Melançon, P., and Wilson, K. L. (1992) *Nature* **358,** 512–514
63. Passreiter, M., Anton, M., Lay, D., Frank, R., Harter, C., Wieland, F. T., Gorgas, K., and Just, W. W. (1998) *J. Cell Biol.* **141,** 373–383
64. Chen, Y.-G., and Shields, D. (1996) *J. Biol. Chem.* **271,** 5297–5300
65. Faúndez, V., Horng, J.-T., and Kelly, R. B. (1997) *J. Cell Biol.* **138,** 505–515
66. Zhang, C., Cavenagh, M. M., and Kahn, R. A. (1998) *J. Biol. Chem.* **273,** 19792–19796
67. Radhakrishna, H., Klausner, R. D., and Donaldson, J. G. (1996) *J. Cell Biol.* **134,** 935–947
68. Frank, S. R., Hatfield, J. C., and Casanova, J. E. (1998) *Mol. Biol. Cell* **9,** 3133–3146
69. Blader, I. J., Cope, M. J. T. V., Jackson, T. R., Profit, A. A., Greenwood, A. F., Drubin, D. G., Prestwich, G. D., and Theibert, A. B. (1999) *Mol. Biol. Cell* **10,** 581–596
70. Radhakrishna, H., Al-Awar, O., Khachikian, Z., and Donaldson, J. G. (1999) *J. Cell Sci.* **112,** 855–866
71. Norman, J. C., Jones, D., Barry, S. T., Holt, M. R., Cockcroft, S., and Critchley, D. R. (1998) *J. Cell Biol.* **143,** 1981–1995
72. Zhang, C.-J., Rosenwald, A. G., Willingham, M. C., Skuntz, S., Clark, J., and Kahn, R. A. (1994) *J. Cell Biol.* **124,** 289–300
73. Gaynor, E. C., Chen, C.-Y., Emr, S. D., and Graham, T. R. (1998) *Mol. Biol. Cell* **9,** 653–670
74. Godi, A., Santone, I., Pertile, P., Devarajan, P., Stabach, P. R., Morrow, J. S., Tullio, G. D., Polishchuk, R., Petrucci, T. C., Luini, A., and Matteis, M. A. D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 8607- 8612
75. Monier, S., Chardin, P., Robineau, S., and Goud, B. (1998) *J. Cell Sci.* **111,** 3427–3436
76. Lorra, C., and Huttner, W. B. (1999) *Nat. Cell Biol.* **1,** E113–E115
77. Franco, M., Chardin, P., Chabre, M., and Paris, S. (1996) *J. Biol. Chem.* **271,** 1573–1578
78. Wilde, A., and Brodsky, F. M. (1996) *J. Cell Biol.* **135,** 635–645
79. Takuma, T., and Ichida, T. (1994) *J. Biol. Chem.* **269,** 22124- 22128
80. Simon, J.-P., Shen, T.-H., Ivanov, I. E., Gravotta, D., Morimoto, T., Adesnik, M., and Sabatini, D. D. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 1073–1078