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Trimeric G proteins modulate the dynamic interaction of PKAII with the Golgi complex

María Esther Martín*, Josefina Hidalgo*, Francisco M. Vega and Angel Velasco‡

Department of Cell Biology, Faculty of Biology, University of Seville, 41012-Seville, Spain
*The first two authors contributed equally to this study
‡Author for correspondence (e-mail: avelasco@cica.es)

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SUMMARY

The Golgi complex represents a major subcellular location of protein kinase A (PKA) concentration in mammalian cells where it has been previously shown to be involved in vesicle-mediated protein transport processes. We have studied the factors that influence the interaction of PKA typeII subunits with the Golgi complex. In addition to the cytosol, both the catalytic (Cα) and regulatory (RIIα) subunits of PKAII were detected at both sides of the Golgi stack, particularly in elements of the *cis*- and *trans*-Golgi networks. PKAII subunits, in contrast, were practically absent from the middle Golgi cisternae. Cell treatment with either brefeldin A, AlF_4^- or at low temperature induced PKAII dissociation from the Golgi complex and redistribution to the cytosol. This suggested the existence of a cycle of association/dissociation of PKAII holoenzyme to the Golgi. The interaction of purified RIIα with Golgi membranes was studied in vitro and found not to be affected by brefeldin A while it was sensitive to modulators of heterotrimeric G proteins such as AlF_4^-, GTPγS, βγ subunits and mastoparan. RIIα binding was stimulated by recombinant, myristoylated $G\alpha_{i3}$ subunit and inhibited by cAMP. Pretreatment of Golgi membranes with bacterial toxins known to catalyze ADP-ribosylation of selected Gα subunits also modified RIIα binding. Taken together the data support a regulatory role for Golgi-associated Gα proteins in PKAII recruitment from the cytosol.

Key words: Protein Kinase A, Golgi complex, Trimeric G protein

INTRODUCTION

The protein kinase A (PKA) holoenzyme is a heterotetramer composed of a regulatory (R) subunit dimer that binds two catalytic (C) subunits. Binding of cAMP to each R subunit results in dissociation of the C subunits, which then become free and catalytically active to phosphorylate a variety of protein substrates on serine or threonine residues present in the consensus sequence Arg-Arg-X-Ser/Thr or Lys-Arg-X-X-Ser/Thr. Depending on the particular ligand that triggers PKA activation, enzymes, transcription factors, and membrane-bound ion channels can be phosphorylated (Taylor et al., 1990).

In mammalian cells, several isoforms of both C (Cα, Cβ, Cγ) and R (RIα, RIβ, RIIα, RIIβ) subunits exist that differ in subcellular localization, kinetic characteristics, and sensitivity to inhibitors and cAMP. Two holoenzyme subtypes named type I and type II are formed by the combination of either RI or RII with C subunits. While type I holoenzyme is predominantly cytosolic a significant proportion of the type II holoenzyme is found associated with subcellular structures such as the centrosome, actin cytoskeleton, microtubules, endoplasmic reticulum, plasma membrane, mitochondria, and peroxisomes (Dell'Acqua and Scott, 1997). Association occurs throughout the specific interaction of RII subunits with particular A kinase-anchoring proteins (AKAPs). It is believed that AKAPs help to organize the cellular responses to an increase in cAMP intracellular concentration by bringing together PKA enzyme and substrates (Colledge and Scott, 1999). Tethering of PKA to a particular subcellular location in close proximity to its substrates may explain the phosphorylation of selective protein targets despite the broad specificity exhibited by this kinase (Pawson and Scott, 1997).

Several reports indicate that PKA regulates vesicle-mediated protein transport processes along the exocytic (Hansen and Casanova, 1994; Pimplikar and Simons, 1994; Mostov and Cardone, 1995; Jilling and Kirk, 1996; Muñiz et al., 1996; Zegers and Hoekstra, 1997; Valenti et al., 1998) and endocytic (Bradbury and Bridges, 1992; Eker et al., 1994; Hansen and Casanova, 1994; Goretzki and Mueller, 1997) pathways. PKA is associated with the Golgi complex in a number of cell types (Nigg et al., 1985b; De Camilli et al., 1986; Griffiths et al., 1990; Dohrman et al., 1996; Feliciello et al., 1996). We have recently shown that PKA enzymatic activity is required for the production of constitutive transport vesicles from the *trans*-Golgi network (Muñiz et al., 1997). An 85-kDa Golgi membrane protein with properties of AKAP has also been identified (Rios et al., 1992). Taken together these studies suggest a pivotal role for PKA activity in Golgi structural organization and function. Golgi association, on the other hand, is not

permanent. Following an increase in cAMP intracellular concentration the free C subunits redistribute to the nucleus while R subunits remain associated with the Golgi (Nigg et al., 1985a; Dohrman et al., 1996). Moreover, RIIα has been recently shown to dissociate from the Golgi-centrosomal region during mitosis (Keryer et al., 1998). Also, in thyroid cells RIIβ redistributed from the Golgi to the cytosol following Ras or protein kinase C activation (Feliciello et al., 1996). In this report, we have investigated the factors that influence the interaction of PKAII subunits with the Golgi membranes. Results obtained in cells incubated with brefeldin A (BFA) or at low temperature indicate that PKAII holoenzyme continuously cycles between the cytosol and the Golgi. This cycle seems to be modulated by heterotrimeric G proteins present in the Golgi membranes. Although considerable attention has received the influence of these trimeric G proteins in Golgi structural organization (Hidalgo et al., 1995; Denker et al., 1996; Jamora et al., 1997; Yamaguchi et al., 1997) and transport activities (Donaldson et al., 1991; Stow et al., 1991; Bomsel and Mostov, 1992; Colombo et al., 1992; Ktistakis et al., 1992; Leyte et al., 1992; Schwaninger et al., 1992; Pimplikar and Simons, 1993; Helms et al., 1998), little is known about the way these modulators exert their actions. In this respect, our results suggest that Golgi trimeric G proteins control PKAII recruitment from the cytosol which in turn could determine protein export from this organelle.

MATERIALS AND METHODS

Reagents

A plasmid containing the full length clone for N-terminal His-tag murine RIIα was a kind gift from Dr S. Taylor (University of California, San Diego, CA). Protein was expressed in *Escherchia coli* BL21 (DE3) cells according to the method of Cheng et al. (1998) and purified with Talon (Clontech, Palo Alto, CA) metal affinity resin according to the manufacturer's instructions. Protein was eluted with 0.5 M imidazole in 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, dialyzed extensively against PBS containing 1 mM PMSF and PBS/10% glycerol, and concentrated to 2-3 mg/ml final protein concentration. Antibodies against bacterially expressed His-tagged murine RIIα and Cα were raised in rabbits. Antisera were subjected to ammonium sulfate precipitation and affinity purification on recombinant protein coupled to activated Sepharose 4 (Pharmacia, Uppsala, Sweden). Western blot analysis showed that the specificity of these antibodies was similar to that of commercial antibodies raised against amino acid sequences from the carboxy terminus of human Cα and RIIα proteins (Santa Cruz Biotechnology, CA). Rabbit polyclonal antibody against an N-terminal peptide of mouse RIIβ was purchased from Biomol (Plymouth Meeting, PA) and tested on immunoblots containing total cellular proteins prepared from rat brain. G1/133 (Linstedt and Hauri, 1993), CTR453 (Bailly et al., 1989), and 1D3 mouse monoclonal antibodies against giantin, centrosome, and KDEL were kindly provided by Dr H. P. Hauri (Biozentrum, Basel, Switzerland), Dr M. Bornens (CNRS, Paris, France), and Dr S. Fuller (European Molecular Biology Laboratory, Heidelberg, Germany) respectively. Transducin βγ subunits were a gift from Dr Y. K. Ho (University of Illinois at Chicago, IL). BFA, ATP, GTPγS, cAMP and M3A5 mouse monoclonal antibody against β-COP were purchased from Sigma Chemical Co. (St Louis, MO). Goat anti-rabbit or anti-mouse IgG secondary antibodies conjugated to either fluoresceine or rhodamine were from TAGO (Burlingame, CA). Protein A (Pharmacia) was coupled to 8 nm colloidal gold particles according to the method of Slot and Geuze (1985). Bacterial toxins and rat, recombinant $G\alpha_{i3}$ were acquired from Calbiochem (San Diego, CA). Mastoparan was obtained from Fluka (Buchs, Switzerland).

Cell culture

COS-7 cells were maintained in high glucose DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Cytosol, Golgi, and microsomal preparations

Bovine brain cytosol was obtained as described previously (Hidalgo et al., 1995). Cytosol was dialyzed against several changes of 25 mM Hepes-KOH, pH 7.2, 25 mM KCl, 2.5 mM $MgCl_2$; aliquots (9-10 mg protein/ml) were frozen in liquid nitrogen, and stored at −80°C. Golgi stacks were prepared from rat liver according to the method of Slusarewicz et al. (1994). The preparation was enriched 80- to 90-fold over the initial homogenate as judged by immunodetection of mannosidaseII. Golgi membranes were incubated on ice with 3 M KCl for 10 minutes, recovered by centrifugation (12,000 ***g***, 20 minutes) 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. To prepare total microsomal membranes, COS-7 cells were resuspended in a small (2-3 ml) volume of ice-cold 0.1 M phosphate buffer, pH 7.2, containing 1 mM PMSF, 5 mM benzamidine, 100 μg/ml soybean trypsin inhibitor, 20 μg/ml aprotinin, and 10 μg/ml of each leupeptin, antipain, and pepstatin A. They were homogenized by several passes throughout a ball-bearing homogenizer. The postnuclear supernatant was centrifuged at 100,000 ***g*** in a TLA-100 ultracentrifuge and both the supernatant containing soluble proteins and the microsomal pellet were processed for SDS-PAGE and immunoblotting.

RIIα binding assay

To monitor binding of cytosolic RIIα, Golgi membranes (20 μg) were incubated with 3 mg/ml bovine brain cytosol, 1 mM ATP, and 1 mM GTP in a final volume of 50 μl of 25 mM Hepes-KOH, pH 7.2, 25 mM KCl, 2.5 mM $MgCl_2$, 1 mM DTT (assay buffer). Alternatively, 20 μg Golgi membranes were incubated with 0.5 mg/ml recombinant RIIα in 50 μl assay buffer. Incubation in both cases was carried out in siliconized microfuge tubes at 37°C for 15 minutes. Samples were diluted with 1 ml ice-cold assay buffer and transferred to new microfuge tubes. Membranes were recovered by centrifugation (12,000 ***g***, 20 minutes) at 4°C on a 30 μl 2 M sucrose cushion, transferred to new microfuge tubes, rinsed twice with assay buffer, and finally resuspended in electrophoresis sample buffer.

Electrophoresis and immunoblotting

Reduced proteins were resolved in 12.5% acrylamide gels run in the presence of SDS according to the method of Laemmli (1970). They were then transferred to Immobilon-P (Millipore, Bedford, MA) membranes which were blocked with 5% dry milk/1% Tween-20 in TBS, pH 8.0, and incubated with primary antibody diluted in the same buffer. Membranes were revealed by enhanced chemiluminiscence (Amersham, Buckinghamshire, UK) according to the instructions of the manufacturer. Bands were quantitated by scanning densitometry.

Immunocytochemistry

Cells cultured on round glass coverslips were used for immunofluorescence. They were fixed in 3% formaldehyde, prepared from paraformaldehyde, in PBS, rinsed with PBS, and incubated in 0.5% bovine serum albumin/0.05% saponin in PBS (PBS/BSA/saponin). Cells fixed in cold methanol were used to localize centrosome with CTR453 antibody. Antibodies were diluted in PBS/BSA/saponin. Incubation with antibodies was performed in a moist chamber at 37°C for 30 minutes. Coverslips were rinsed with PBS and mounted in PBS/glycerol. For immunogold labeling, cells were fixed for 1 hour in 3% formaldehyde/0.01% glutaraldehyde in

0.1 M phosphate buffer, pH 7.4. They were rinsed with PBS, infiltrated with 2.3 M sucrose/20% polyvinylpyrrolidone, and frozen in liquid nitrogen. Cryosections were recovered on nickel grids and rinsed extensively with PBS first and then with PBS/BSA. Incubation with primary antibodies and Protein A-gold complexes, both diluted in PBS/BSA, was performed in a moist chamber at room temperature for 2 hours and 1 hour, respectively. Sections were stained with a mixture (1:9) of 3% uranyl acetate and 2% methyl cellulose. For quantitation, micrographs were printed at a final magnification of ×70,000. Five random selected Golgi areas were analysed for each protein. The *trans* side of the Golgi stack was identified by the presence of clathrin-coated buds. Each compartment was delimited with an electronic pen that automatically converted the length of the closed outline into surface area. The number of gold particles within each compartment was counted and used to calculate the density of labeling.

Binding of [^{35}S]GTPγS to Gα_{i3}

100-200 ng of Gα_{i3} were incubated for 1 hour at 37°C in 50 μl of 25 mM Hepes, pH 7.0, 50 mM potassium acetate, 2.5 mM magnesium acetate, containing 1 mM EDTA, 0.5 mM ATP, 1 mg/ml BSA, 2 μM GTPγS, 250 pmoles [^{35}S]GTPγS (1000 Ci/mmol). The reaction was stopped with 1 ml cold buffer and the sample rapidly filtered through nitrocellulose. Filters were rinsed with 5× 1 ml ice-cold buffer and dried before liquid scintillation counting.

RESULTS

Golgi PKAII localization

Since different cell lines differ in the type of R subunit associated with the Golgi (Rios et al., 1992; Dohrman et al., 1996; Keryer et al., 1998) we first determined the presence of either RIIα or RIIβ in COS-7 cells. Western blots containing total cellular proteins were incubated with antisera raised against either Cα, RIIα or RIIβ (Fig. 1A). A single band of molecular mass~53 kDa was recognized by antibody against RIIα (Fig. 1A, lane 2) while no specific immunoreactivity was detected with anti-RIIβ antibody (Fig. 1A, lane 3) or preimmune sera (not shown). Anti-Cα antibody reacted with a 40 kDa protein which is the expected size for the catalytic subunit (Fig. 1A, lane 1). In fixed cells processed for indirect immunofluorescence both antibodies strongly stained the perinuclear Golgi area identified with a monoclonal antibody against the resident Golgi protein giantin (Linstedt and Hauri, 1993) (Fig. 2). In addition, a diffuse, cytoplasmic staining was observed with both antibodies. However, neither Cα nor RIIα colocalized completely with giantin. Staining with this Golgi marker was more restricted to the perinuclear area than that of the PKAII subunits. Long membrane processes emerging from the Golgi and reaching the peripheral cytoplasm were frequently observed with both anti-Cα and anti-RIIα antibodies (see Fig. 8A). Also, both antibodies stained one or two perinuclear brilliant spots. Staining with the monoclonal antibody CTR453 (Bailly et al., 1989) indicated that these structures corresponded to the centrosome (see Fig. 4). That both PKAII subunits existed in soluble and membrane-associated forms was confirmed by subcellular fractionation. Comparison by immunoblotting of cytosolic and microsomal proteins indicated that almost 55% of RIIα and 30% of Cα were found associated with microsomal membranes (Fig. 1B). These values are probably underestimated since cells were homogenized on ice and membranes centrifuged at 4°C (see below). Both proteins were also localized to the same Golgi compartments by immunogold labeling. These included both sides of the Golgi complex with little staining of the medial

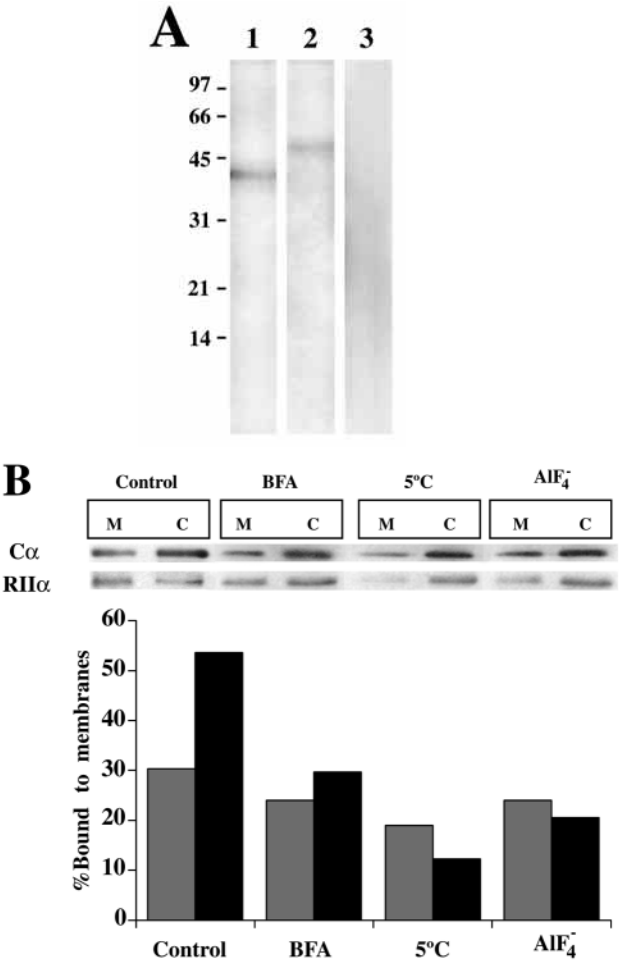

Fig. 1. Immunodetection of PKAII subunits in COS-7 cells. (A) Total cellular proteins (15 μg per lane) were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against Cα (lane 1), RIIα (lane 2), and RIIβ (lane 3). Molecular mass markers are indicated in kDa. (B) Cells were incubated for 30 minutes at 37°C with medium containing either no addition (control), 10 μM BFA or 50 μM $AlCl_3$ and 30 mM NaF (AlF_4^-). Alternatively, cells incubated for 30 minutes at 5°C were also processed. They were homogenized and fractionated to obtain total microsomal and cytosolic proteins. These were analyzed by immunoblotting (7 μg per lane) with anti-Cα and anti-RIIα antibodies. Results derived from band quantitation by scanning densitometry are shown.

Table 1. Quantitation of the immunogold labeling of PKAII subunits in the Golgi area of COS-7 cells

	Cα	RIIα
Cis tubulo-vesicular structures	597±66 (*n*=47)	427±120 (*n*=33)
The cis-most cisterna	167±78 (*n*=6)	143±28 (*n*=8)
2-3 medial cisternae	27±30 (*n*=10)	37±22 (*n*=13)
The trans-most cisterna	152±21 (*n*=8)	78±45(*n*=9)
TGN elements	376±42 (*n*=85)	743±140 (*n*=50)

Data (mean ± s.e.m.) indicate number of gold particles/μm^2.

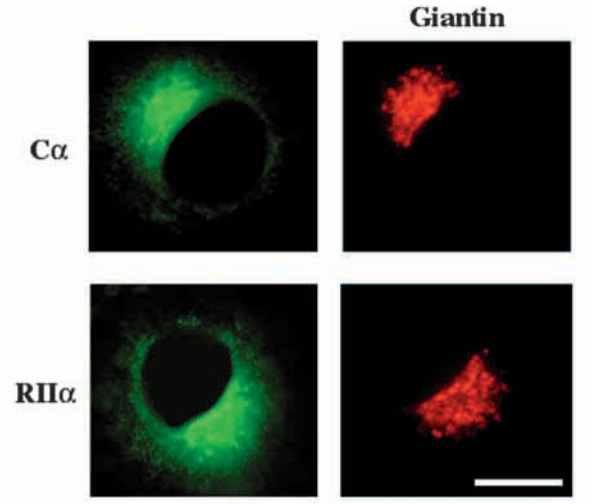

Fig. 2. Immunofluorescence localization of PKAII subunits. Cells were double stained with antibodies against either Cα or RIIα subunits and the Golgi marker giantin. Bar, 18 μm.

cisternae (Fig. 3). 1-2 *trans*-Golgi cisternae and vesicles and tubular elements of the *trans*-Golgi network were heavily labeled. Also, vesicular-tubular structures close to the proximal, *cis* side of the Golgi stack were stained with both antibodies (Fig. 3). These observations were corroborated by quantitation of the density of labeling (Table 1). Labeling of the medial Golgi cisternae was thus similar to that of mitochondria (9±7 gold particles/μm^2 with either anti-Cα or anti-RIIα antibody) or endoplasmic reticulum cisternae (28±13) while it was negligible in the case of plasma membrane or the outer membrane of the nuclear envelope. Collectively, the data indicated that the *trans*- and *cis*-Golgi networks represent major subcellular locations of PKAII anchoring.

Brefeldin A (BFA) effect

BFA is known to interfere with the association of COPI coat protein complex to the Golgi membranes which in turn causes Golgi disassembly and redistribution to the endoplasmic reticulum (Klausner et al., 1992). We examined the fate of Golgi-associated PKAII during the course of BFA treatment. As shown in Fig. 4 the perinuclear staining pattern characteristic of RIIα became diffuse and dispersed throughout the cytoplasm following incubation with 10 μM BFA. This redistribution, however, was slower than that of β-COP, a component of COPI vesicle coat. Thus, β-COP redistribution itself took place during the first minute of BFA treatment while RIIα redistribution became evident after 10-15 minutes of incubation. Importantly, Cα also redistributed and with the same kinetics as RIIα in the presence of BFA (not shown). In contrast, centrosome staining with either anti-Cα or anti-RIIα antibody was unaffected by BFA treatment (Fig. 4). Staining of the endoplasmic reticulum with an antibody against the KDEL sequence indicated that the BFA effect did not involve redistribution of the PKA subunits to this organelle. Thus, the reticular staining pattern obtained with anti-KDEL antibody was clearly distinguishable from the diffuse staining pattern obtained with either anti-Cα or anti-RIIα (Fig. 4) antibody. This suggested that PKA subunits dissociated from the Golgi membranes during BFA treatment. Indeed, the amount of both Cα and RIIα associated with microsomal membranes decreased following treatment with BFA (Fig. 1B).

To gain insight on the mechanism of PKAII redistribution we studied the in vitro association of cytosolic RIIα to purified, high salt-washed rat liver Golgi membranes (Fig. 5). In this assay, RIIα recruitment depended on the simultaneous presence of both cytosol and Golgi membranes and it was not inhibited by BFA. The amount of RIIα incorporated to Golgi membranes was similar in the absence or in the presence of 500 μM BFA while β-COP association was inhibited in samples incubated with BFA

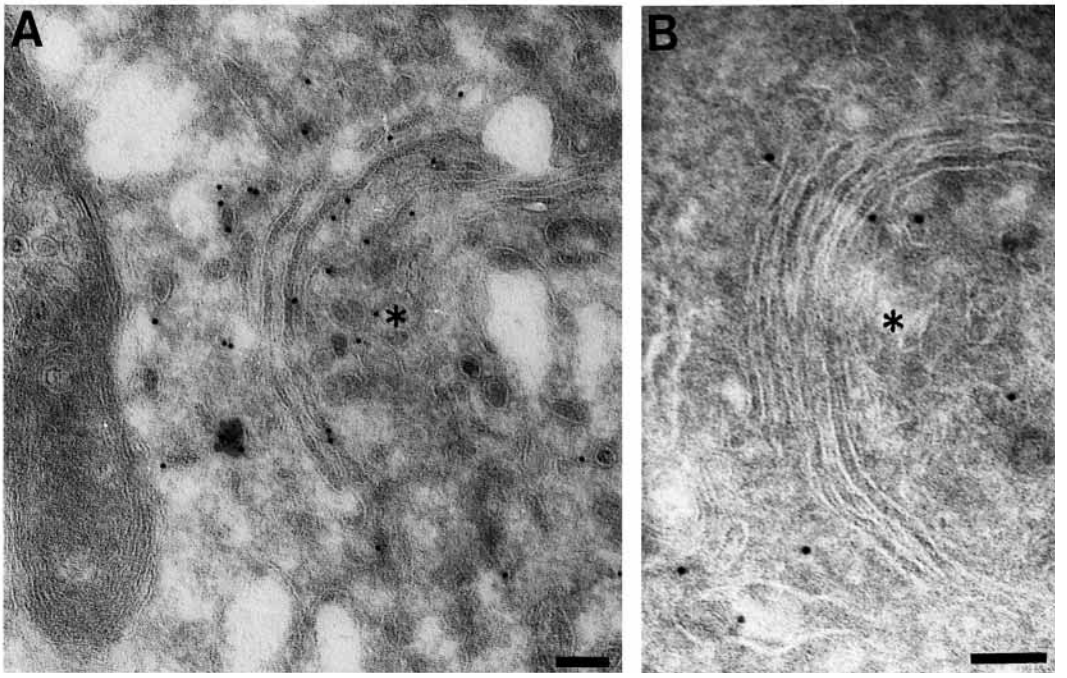

Fig. 3. Immunogold localization of PKAII subunits within the Golgi area. Detection of Cα (A) and RIIα (B) subunits. Labeling in both cases is mostly localized in vesicles and tubular elements at both sides of the Golgi stack, especially the *trans*-Golgi netwok (asterisks). Bars, 100 nm.

(Fig. 5). These results suggested that redistribution of PKAII subunits observed in cells exposed to BFA could be an indirect effect, derived from the absence of Golgi membranes which after some minutes of incubation with this agent would be mostly fused with the endoplasmic reticulum. To test this possibility we analyzed the

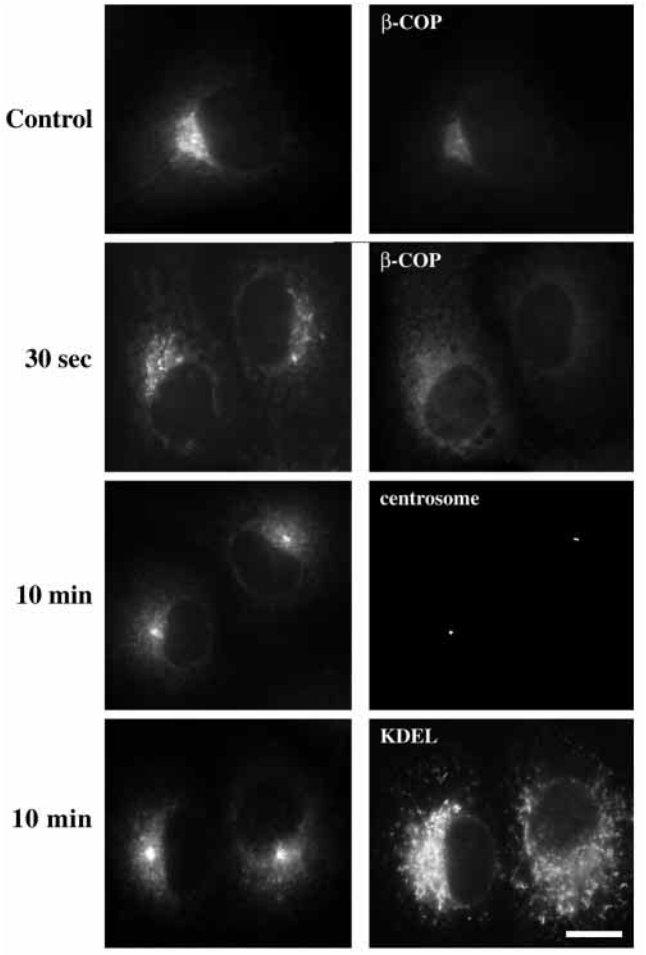

Fig. 4. Effect of BFA on RIIα immunofluorescence localization. Cells were incubated or not (control) at 37°C with 10 μM BFA for the indicated time periods before fixation and double staining with antibodies against RIIα (left panels) and either β-COP, centrosome, or the KDEL sequence. Bar, 18 μm.

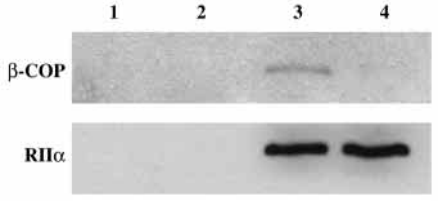

Fig. 5. Effect of BFA on RIIα recruitment from cytosol. Golgi membranes (20 μg) and cytosol (3 mg/ml) were incubated at 37°C for 15 minutes in the absence (lane 3) or presence of 500 μM BFA (lane 4). Membranes were rinsed and processed for SDS-PAGE and immunoblotting with antibodies against β-COP and RIIα. Samples lacking either cytosol (lane 1) or Golgi membranes (lane 2) were similarly processed.

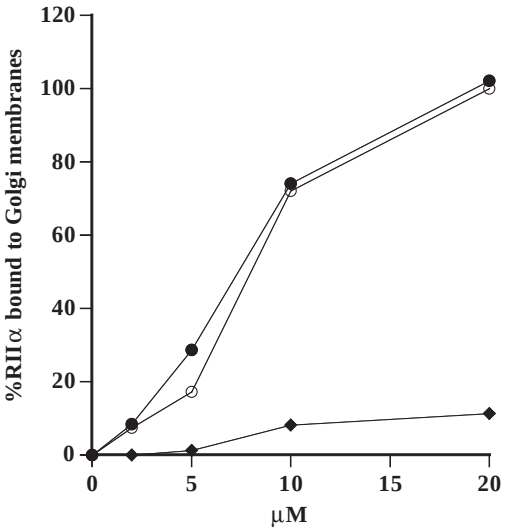

Fig. 6. Association of recombinant RIIα to Golgi membranes. Golgi membranes (20 μg) were incubated with the indicated concentrations of purified RIIα for 15 minutes at 5°C (◆) or, alternatively, at 37°C in the absence (control, ○) or presence of 500 μM BFA (●). Membranes were rinsed and processed for SDS-PAGE and immunoblotting. The amount of RIIα incorporated was quantitated by scanning densitometry.

association of pure, recombinant RIIα to Golgi membranes (Fig. 6). In the continuous presence of Golgi membranes BFA did not affect RIIα binding which at the different concentrations tested was comparable to that of control, untreated samples nonincubated with this agent (Fig. 6). Whereas these data exclude the possibility of BFA interfering directly with PKA recruitment they suggest that in vivo this holoenzyme continuously cycles between the cytosol and the Golgi membranes. This cycle would be interrupted in BFA-treated cells simply because of the absence of acceptor Golgi membranes.

Low temperature effect

Localization of PKAII to the Golgi complex depended on the temperature of incubation. Golgi immunofluorescence staining was less extensive in cells incubated at either 20°C or 15°C than in cells incubated at 37°C (Fig. 7A). Apparently, PKAII subunits gradually redistributed from the Golgi to the peripheral cytoplasm as temperature decreased. For instance, following incubation for 30 minutes at 5°C no Golgi staining was observed. Instead, both Cα (not shown) and RIIα (Fig. 7A) subunits exhibited a cytoplasmic distribution although they remained concentrated at the centrosome. This observation correlated with a dramatic decrease in the amount of these proteins associated with microsomal membranes (Fig. 1B). Under these conditions, only 19% of Cα and 12% of RIIα remained membrane-associated which in the case of RIIα accounted for a 4.3-fold decrease with respect to cells preincubated at 37°C. At 5°C redistribution occurred rapidly and it did not involve changes in Golgi structural organization or dissociation of a peripheral Golgi protein such as β-COP (Fig. 7B). Interaction in vitro of RIIα with Golgi membranes was

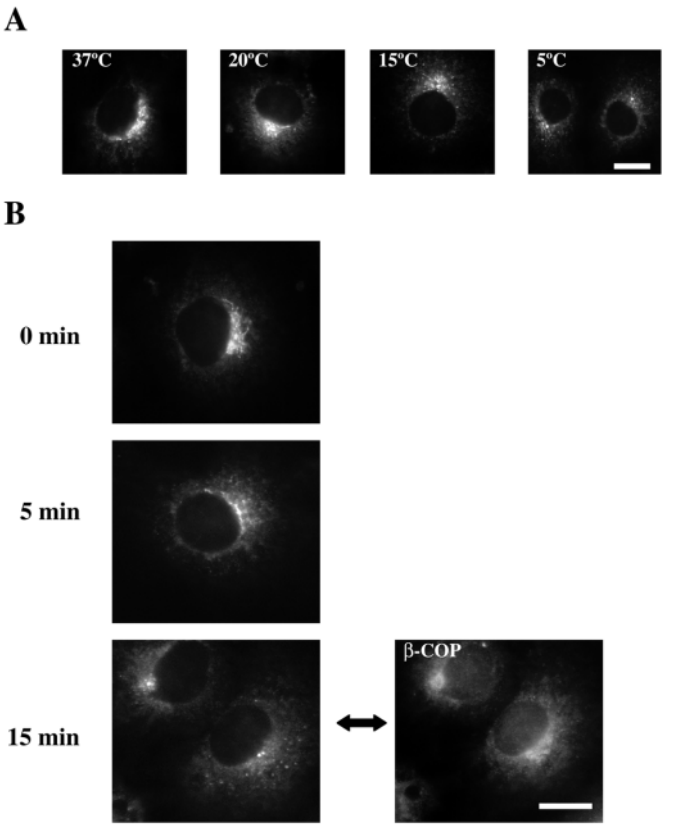

Fig. 7. Temperature-dependent redistribution of RIIα. Cells were incubated for 30 minutes at the indicated temperatures (A) or, alternatively, incubated at 5°C for the indicated time periods (B). They were fixed and processed for indirect immunofluorescence with anti-RIIα antibody. Double staining with anti-β-COP is also shown in B. Bars, 18 μm.

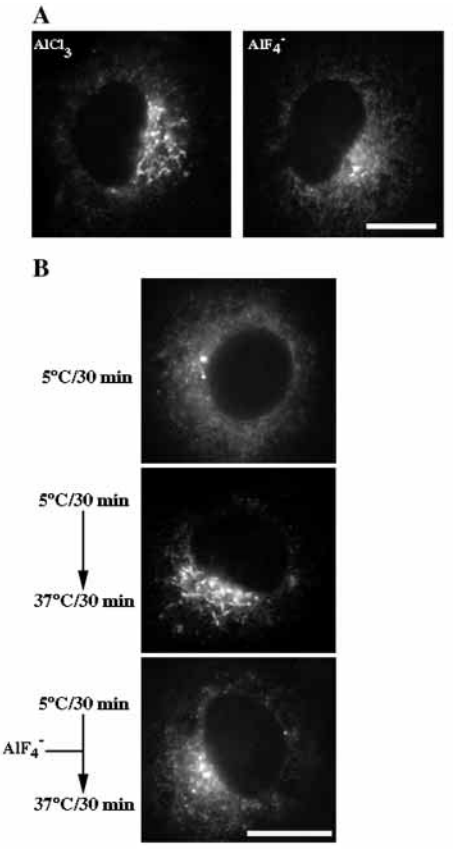

Fig. 8. Effect of AlF_4^- on RIIα immunofluorescence localization. (A) Cells were incubated at 37°C for 15 minutes with either 50 μM $AlCl_3$ alone or 50 μM $AlCl_3$ and 30 mM NaF (AlF_4^-) before fixation and processing for indirect immunofluorescence. (B) Following incubation at 5°C for 30 minutes, cells were returned at 37°C and additionally incubated for 30 minutes in the presence or absence of AlF_4^-. Bars, 18 μm.

inhibited at 5°C (Fig. 6) accounting for the redistribution observed in cells exposed to low temperature. These data therefore also support the existence of an itinerant cycle of PKAII between the Golgi and the cytosol.

Modulation by trimeric G proteins

The steady-state association of PKAII with the Golgi complex was not affected by treatment with 1 μM of either staurosporine, a broad range protein kinase inhibitor, or okadaic acid, a serine/threonine phosphatase inhibitor, as well as with 30 μM H-89, a specific PKA inhibitor. In addition, 50 μM of either zinc chloride or pervanadate which are inhibitors of tyrosine phosphatases did not alter PKAII localization. In contrast, cells incubated with AlF_4^- which activates trimeric G proteins (Kahn, 1991) showed dissociation of RIIα from the Golgi complex but not from the centrosome (Figs 8A, 1B). AlF_4^- was formed by addition of $AlCl_3$ and NaF to final concentrations of 50 μM and 30 mM, respectively. When used alone at those concentrations, neither $AlCl_3$ (Fig. 8A) nor NaF (not shown) alone modified PKAII localization. Interestingly, AlF_4^- inhibited reassociation of RIIα to the Golgi following incubation at low temperature. As shown in Fig. 8B such a reassociation occurred efficiently at 37°C in the absence of AlF_4^- but not in the presence of this agent. Taken together these observations pointed to a role of trimeric G proteins in RIIα recruitment.

The effects of different modulators of trimeric G proteins were evaluated in a series of in vitro experiments (Fig. 9). First, the assay that reproduces the association of RIIα to Golgi membranes was supplemented with agents that directly activate or inhibit trimeric G proteins (Fig. 9A). In agreement with the effect observed in vivo, AlF_4^- decreased the incorporation of RIIα to the Golgi to a 50-60% of the control value. A similar inhibition was observed with 25-100 μM GTPγS, a poorly hydrolyzable analog of GTP. These two compounds, however, could potentially activate low-molecular-mass, monomeric G proteins in addition to trimeric G proteins. A more specific reagent would be a complex of βγ subunits purified from transducin. Free βγ subunits are expected to bind and therefore inactivate Gα subunits (Bomsel and Mostov, 1992). Association of RIIα was stimulated by 2-fold with 5-10 μM βγ (Fig. 9A).

Whereas these results suggested a negative regulatory role of trimeric G proteins on RIIα incorporation, additional data indicated that particular Gα proteins might play a stimulatory role. Thus, addition of recombinant, myristoylated $G\alpha_{i3}$ subunit at nanomolar concentrations

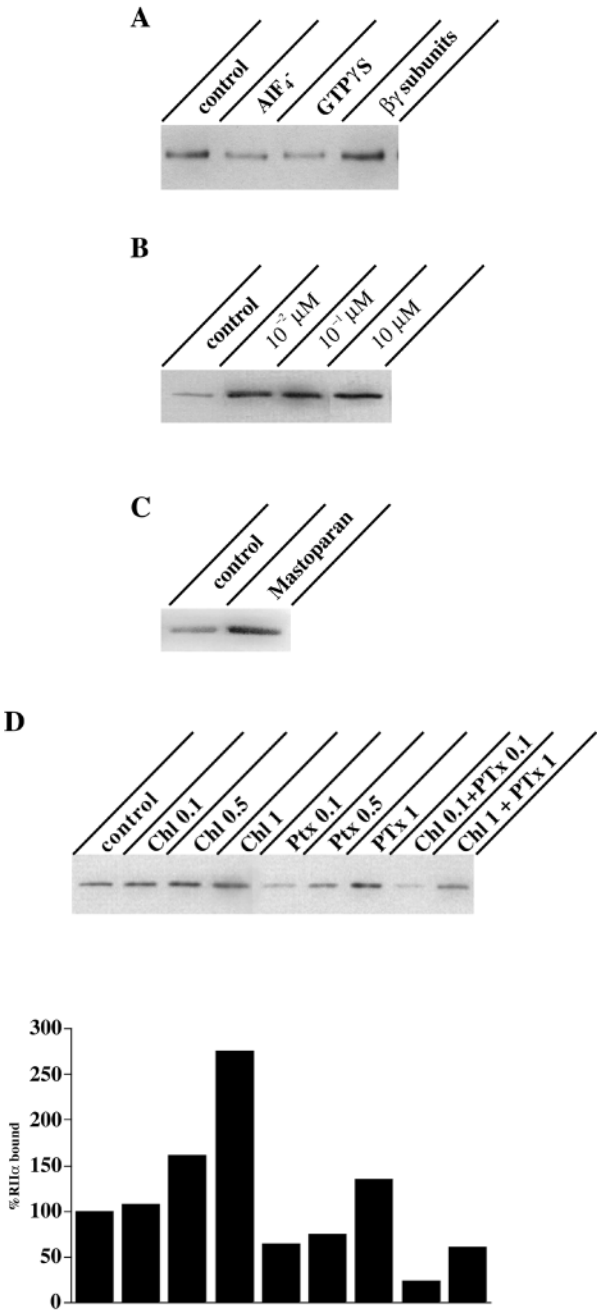

Fig. 9. Effects of trimeric G proteins modulators on RIIα recruitment. Golgi membranes (20 μg) were incubated for 15 minutes at 37°C with 10 μM recombinant RIIα and processed as described in Fig. 6. (A) The incubation medium contained no further addition (control) or, alternatively, was supplemented with 50 μM $AlCl_3$ and 30 mM NaF (AlF_4^-), 50 μM GTPγS, or 10 μM transducin βγ subunits. (B) The incubation medium contained or not (control) the indicated concentrations of pure, myristoylated Gαi3 subunit. (C) 2 μM mastoparan was included or not (control) in the incubation medium. (D) Golgi membranes were preincubated for 1 h at 37°C with 0 (control), 0.1, 0.5 or 1 μg/ml of each one cholera toxin (Chl) or pertussis toxin (Ptx) diluted in assay buffer. Alternatively, they were treated with both toxins simultaneously. They were reisolated, rinsed, and incubated in the standard incubation medium. Bound RIIα was quantitated and expressed as percentage of the amount incorporated under control conditions.

induced a significant, 3-4-fold increase in RIIα recruitment (Fig. 9B). The $G\alpha_{i3}$ preparation was biologically active as shown by its ability to bind [^{35}S]GTPγS (132 pmol GTPγS/nmol protein). Addition to the assay of 2-4 μM mastoparan, an amphipathic peptide that activates $G\alpha_i$ and $G\alpha_o$ subunits (Bomsel and Mostov, 1992), also stimulated binding of RIIα to the Golgi membranes (Fig. 9C). Furthermore, we made use of Golgi membranes which had been preincubated with bacterial toxins known to catalyze ADP-ribosylation of particular Gα subunits (Bomsel and Mostov, 1992) (Fig. 9D). RIIα incorporation was increased following pretreatment of Golgi membranes with cholera toxin which selectively activates Gαs. Depending on the toxin concentration and duration of the treatment, either 1 hour or 4 hours, the amount of RIIα bound was increased 1.6- to 2.7-fold with respect to control membranes that had not been preincubated with the toxin. By contrast, pretreatment with pertussis toxin decreased RIIα binding when low concentrations, 0.1-0.5 μg/ml, of the toxin were used whereas at higher concentrations, 1 μg/ml, a 1.3-fold stimulatory effect was observed. Pertussis toxin ADP-ribosylates a number of Gα proteins including $G\alpha_i$, $G\alpha_o$, and $G\alpha_t$ whose differential inactivation could explain these opposite effects (Bomsel and Mostov, 1992). Interestingly, when Golgi membranes were preincubated with both toxins simultaneously RIIα recruitment decreased independently of the concentration used (Fig. 9D). Collectively, these data support the involvement of different Golgi-located Gα proteins in the control of PKA recruitment.

cAMP effect

A different potential factor affecting PKA interaction with the Golgi would be cAMP since each R subunit contains two cAMP-binding sites. In fact, it was shown previously that in response to an increase in cAMP intracellular concentration Cα dissociates from the Golgi and redistributes to the nucleus whereas RIIα would remain Golgi-associated (Nigg et al., 1985a). We reasoned that since the above data indicated that RIIα is not permanently anchored to the Golgi but cycles between the Golgi and the cytosol binding of cAMP to RIIα might also affect recruitment. We therefore analyzed the association of RIIα to Golgi membranes in the presence of increasing concentrations of cAMP. RIIα recruitment was decreased by 3-fold with 1 μM cAMP with maximal inhibition, 8- to 10-fold decrease, taking place at concentrations above 10 μM cAMP (Fig. 10). Since 20 μM cAMP is required to fully activate all the RIIα molecules present in the assay these results reflect the inhibitory effect caused by the occupancy of the cAMP-binding sites. Thus, binding of cAMP to RIIα decreases the ability of this protein to interact with the Golgi membranes.

Fig. 10. Effect of cAMP on RIIα recruitment. The assay that monitors binding of recombinant RIIα to Golgi membranes was supplemented with the indicated concentrations of cAMP. Samples were incubated for 15 minutes at 37°C and processed as described in Fig. 6.

DISCUSSION

PKAII localization within the Golgi

We have previously shown that PKA enzymatic activity regulates membrane protein transport along the secretory pathway (Muñiz et al., 1996) and it is required for the formation of constitutive transport vesicles from the *trans*-Golgi network (Muñiz et al., 1997). The ultrastructural localization of PKAII subunits is consistent with such a role. Both the *cis*- and *trans*-Golgi networks were shown to be major subcellular locations of PKAII anchoring by immunogold labeling (Fig. 3). In contrast, medial-Golgi cisternae showed little labeling if any (Table 1). The mechanism of protein transport across the Golgi complex, whether mediated by vesicles/tubules or taking place by cisternal maturation, is controversial at present (Mironov et al., 1997; Farquhar and Palade, 1998). It is well established, however, that transfer of proteins and lipids between the endoplasmic reticulum and the Golgi as well as export from the Golgi requires cargo selection and packing within some kind of membrane-bound carrier intermediates (Schekman and Orci, 1996; Bannykh and Balch, 1997). The *trans*-Golgi network is the departure site for soluble and membrane proteins whose final destination is the plasma membrane, the extracellular space or endosomes and lysosomes (Traub and Kornfeld, 1997). The *cis*-Golgi network, on the other hand, receives molecules from the endoplasmic reticulum and selects those to be returned back to the endoplasmic reticulum by retrograde transport (Aridor and Balch, 1996). The fact that PKAII is concentrated at both the entrance and exit sites of the Golgi stack emphasizes the importance of this kinase in the control of transport activities. PKA could be involved in an initial step in the formation of transport intermediates such as coat assembly and/or cargo selection. Other kinases like protein kinase C (Simon et al., 1996), tyrosine kinase (Austin and Shields, 1996) and phosphatidylinositol-3-kinase (Jones and Howell, 1997) might also participate in this highly regulated process.

PKAII association/dissociation cycle

Cells incubated with BFA (Figs 1B, 4) or at low temperature (Figs 1B, 7) showed redistribution of PKAII subunits from the Golgi to the cytosol. This suggests that Golgi association is not permanent but instead PKAII would continuously cycle between the cytosol and the Golgi. Data obtained in vitro support this conclusion. Interaction of recombinant RIIα subunit with Golgi membranes did not occur at low temperature whereas it was not affected by the presence of BFA in the assay medium (Fig. 6). Both effects can be explained if in cells incubated with BFA or exposed at low temperature RIIα can dissociate from the Golgi membranes but it is unable to reassociate with them. In the case of BFA this would be due to the alteration induced by this agent in the organization of the Golgi complex (Klausner et al., 1992). It was shown previously that in response to an increase in cAMP intracellular concentration Cα dissociates from the Golgi and redistributes to the nucleus whereas RIIα would remain Golgi-associated (Nigg et al., 1985a). Our data, however, suggest that association of RIIα with the Golgi membranes is transient as well. In fact, RIIα has been described recently to become dissociated from the Golgi during mitosis (Keryer et al., 1998). While in this study the relationship between RIIα dissociation and the fragmentation undergone by the Golgi complex during mitosis was not addressed the data reported here indicate that both PKAII subunits continuously associate and dissociate from the intact Golgi complex of interphase cells.

We envisage a scenario in which the complete PKAII holoenzyme would be recruited from the cytosol. Interaction with the Golgi would involve the RIIα subunits and an AKAP protein localized in the Golgi membranes. Our data indicate that this acceptor molecule is resistant to treatment with high salt and therefore would be an integral membrane protein. The Golgi-associated RIIβ-binding protein identified previously was also described as an integral membrane protein (Rios et al., 1992). We are tempted to speculate that during the budding process a local increase in cAMP concentration would occur at the cytosolic side of the Golgi membrane. Binding of cAMP to the RIIα subunits would promote activation of Golgi-bound PKAII. The released Cα subunits would then be able to phosphorylate particular Golgi proteins that somehow would trigger membrane evagination. This would explain the stimulatory effect of adding free exogenous Cα subunits to an in vitro budding assay (Muñiz et al., 1997). The dimer formed by the two RIIα subunits and containing cAMP bound would also become dissociated from the Golgi as suggestted by the effect of cAMP on RIIα recruitment (Fig. 10). In this model, the RIIα subunits would funtion to bring the Cα subunits close to the sites of bud formation in the Golgi membrane. To test the model it will be necessary to prove that PKAII recruitment from cytosol indeed precedes budding of transport intermediates.

Regulation by trimeric G proteins

Incubation of intact cells with AlF_4^- gave rise to RIIα redistribution from the Golgi to the cytosol (Figs 1B, 8A). While GTPγS activates both trimeric and low-molecular-mass GTP-binding proteins AlF_4^- primarily activates trimeric G proteins (Kahn, 1991; Bomsel and Mostov, 1992). Although activation of certain classes of small GTP-binding proteins with AlF_4^- has been reported (Mittal et al., 1996; Reza et al., 1997; Hoffman et al., 1998) additional results obtained in vitro with purified βγ subunits (Fig. 9A), Gαi3 subunits (Fig. 9B), mastoparan (Fig. 9C), and bacterial toxins (Fig. 9C) support the involvement of trimeric G proteins in the regulation of PKAII recruitment.

Whereas the available data are consistent with trimeric G proteins controlling PKAII recruitment from cytosol it is unclear at present the role played by particular Gα subunits in the process. From the effects caused by general modulators it can be inferred a negative regulatory role of trimeric G proteins on RIIα incorporation. Thus, G proteins activation with either GTPγS or AlF_4^- inhibited RIIα recruitment whereas it was stimulated by addition of βγ subunits which should be able to complex with free Gα subunits and therefore would cause G proteins inactivation (Fig. 9A). However, RIIα recruitment was increased (3-fold at 10 nM) by addition of a recombinant, myristoylated form of $G\alpha_{i3}$ (Fig. 9B). This protein could bind [^{35}S]GTPγS and therefore was functionally active. Incorporation of RIIα was also increased by the peptide mastoparan (Fig. 9C), which mimics the cytoplasmic domain of an activated receptor giving rise to the activation of $G\alpha_i$ and $G\alpha_o$ proteins. Activation of Gαs following ADP-ribosylation with cholera toxin also stimulated RIIα binding (Fig. 9D).

Therefore, it seems that different Gα proteins with opposite effects would be involved in RIIα recruitment.

In recent years the involvement of heterotrimeric G proteins in the control of both Golgi structural organization (Hidalgo et al., 1995; Jamora et al., 1997; Yamaguchi et al., 1997) and transport activities (Stow et al., 1991; Bomsel and Mostov, 1992; Colombo et al., 1992; Leyte et al., 1992; Schwaninger et al., 1992; Pimplikar and Simons, 1993; Helms et al., 1998) has been examined in detail. Several Gα subunits including Gαs (Denker et al., 1996), XLαs (Kehlenbach et al., 1994), $G\alpha_{i2}$ (Montmayeur and Borrelli, 1994), $G\alpha_{i3}$ (Stow et al., 1991; Denker et al., 1996), and $G\alpha_{q/11}$ (Denker et al., 1996) have been characterized as Golgi residents but yet their mechanism of action remains unclear. AlF_4^-inhibits intra-Golgi transport in vitro by blocking the fusion of COPI-coated vesicles with acceptor membranes (Melançon et al., 1987; Helms et al., 1998). However, other data have implicated Gα proteins in coat assembly and thereby vesicle formation. Excess of βγ subunits inhibited association with the Golgi membranes of ADP-ribosylation factor (ARF) and β-COP (Donaldson et al., 1991). Mastoparan promoted β-COP binding to Golgi membranes and antagonized the effect of BFA (Ktistakis et al., 1992). Moreover, different Gα proteins with opposite effects seem to act coordinately to control protein export from the *trans*-Golgi network (Leyte et al., 1992; Pimplikar and Simons, 1993) and the endoplasmic reticulum (Schwaninger et al., 1992). The finding that recruitment of RIIα is regulated by Gα proteins present in the Golgi membranes implies that at least some of the processes controlled by these signaling molecules are PKA-mediated. In fact, evidences have been obtained of membrane traffic events that are regulated by Gα proteins through a mechanism that involves both cAMP and PKA (Hansen and Casanova, 1994; Valenti et al., 1998). Trimeric G proteins, PKA, and AKAP could then be part of a signaling pathway located at the Golgi membranes and destined to control Golgi structural integrity and function.

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