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ADP ribosylation factor regulates spectrin binding to the Golgi complex

ANNA GODI[†], IVANA SANTONE[†], PAOLO PERTILE[†], PRASAD DEVARAJAN[‡], PAUL R. STABACH[§], JON S. MORROW[§], GIUSEPPE DI TULLIO[†], ROMAN POLISHCHUK[†], TAMARA C. PETRUCCI[¶], ALBERTO LUINI[†], AND MARIA ANTONIETTA DE MATTEIS[†‖]

[†]Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro (Chieti), Italy 66030; Departments of [§]Pathology and [‡]Pediatrics, Yale School of Medicine, New Haven, CT 06520; and [¶]Laboratory of Cell Biology, Istituto Superiore di Sanità, Rome, Italy 00161

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ABSTRACT **Homologues of two major components of the well-characterized erythrocyte plasma-membrane-skeleton, spectrin (a not-yet-cloned isoform, $\beta I\Sigma^*$ spectrin) and ankyrin (Ank_{G119} and an ≈195-kDa ankyrin), associate with the Golgi complex. ADP ribosylation factor (ARF) is a small G protein that controls the architecture and dynamics of the Golgi by mechanisms that remain incompletely understood. We find that activated ARF stimulates the *in vitro* association of $\beta I\Sigma^*$ spectrin with a Golgi fraction, that the Golgi-associated $\beta I\Sigma^*$ spectrin contains epitopes characteristic of the $\beta I\Sigma 2$ spectrin pleckstrin homology (PH) domain known to bind phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$), and that ARF recruits $\beta I\Sigma^*$ spectrin by inducing increased $PtdInsP_2$ levels in the Golgi. The stimulation of spectrin binding by ARF is independent of its ability to stimulate phospholipase D or to recruit coat proteins (COP)-I and can be blocked by agents that sequester $PtdInsP_2$. We postulate that a PH domain within $\beta I\Sigma^*$ Golgi spectrin binds $PtdInsP_2$ and acts as a regulated docking site for spectrin on the Golgi. Agents that block the binding of spectrin to the Golgi, either by blocking the PH domain interaction or a constitutive Golgi binding site within spectrin's membrane association domain I, inhibit the transport of vesicular stomatitis virus G protein from endoplasmic reticulum to the medial compartment of the Golgi complex. Collectively, these results suggest that the Golgi-spectrin skeleton plays a central role in regulating the structure and function of this organelle.**

Despite the early recognition of a key role for the small G protein ADP ribosylation factor (ARF) among the molecules controlling the architecture of the Golgi complex (1), and recent advances in identifying structural components of this organelle (2–3), the mechanisms by which such control is effected remain obscure. Recently, homologues of two major components of the well-characterized erythrocyte plasma-membrane-skeleton, spectrin (a not-yet-cloned isoform, hence designed as $\beta I\Sigma^*$ spectrin) and ankyrin (Ank_{G119} and a ≈195-kDa ankyrin) have been identified in the Golgi complex (4–7), and a Golgi-targeting sequence has been identified in spectrin (7). The molecular mechanisms controlling such localization and its functional role remain incompletely understood.

We now demonstrate a mechanism controlling Golgi spectrin association and investigate the acute effects of the loss of Golgi spectrin binding on intracellular membrane traffic. Spectrin uses at least two sites to bind to Golgi fractions *in vitro*. One site involves spectrin's membrane association domain (MAD) 1, in accord with studies identifying a Golgi-targeting sequence in $\beta I\Sigma^*$ spectrin (7). The other site involves a pleckstrin homology (PH) domain within the MAD2 of $\beta I\Sigma^*$ spectrin. Binding at MAD2 is regulated by ARF, which recruits spectrin by increasing Golgi phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$) levels. Inhibitors of spectrin binding to Golgi block endoplasmic reticulum (ER) to Golgi transport of vesicular stomatitis virus (VSV)-G protein. Collectively these results identify a pathway regulating the assembly of the Golgi spectrin-ankyrin skeleton and demonstrate the importance of this complex for Golgi function. Portions of this work have been previously presented in abstract form (8).

METHODS

Immunofluorescence. NRK cells were left intact or incubated with 1 unit/ml of Streptolysin O (BioMerieux, Charbonnier les Bains, France) for 8 min at 0°C, washed and permeabilized for 15 min at 37°C in 25 mM Hepes-KOH, pH 6.95/125 mM KOAc/2.5 mM $Mg(OAc)_2$/10 mM glucose/1 mM DTT/1 mM ATP/2 mM creatine phosphate/7.3 units/ml creatine phosphokinase/2 mg/ml rat brain or NRK cell cytosol. Cells were fixed in 1.9% paraformaldehyde and permeabilized with 0.01% saponin before immunostaining (7).

Cytosol and Golgi Fraction Preparation. Golgi fractions were obtained from NRK cells or rat liver (9–10). The Golgi-rich fraction from the postnuclear supernatant contained >90% of the mannosidase II (Golgi marker), 4% of NaK-ATPase or ecto-ATPase HA4 (plasma-membrane marker, ref. 11), and 3% of CaBP1 (an ER marker) (12). Cytosol was obtained from rat brain, NRK, or RBL cells (9). ARF-depleted cytosol was prepared (13). Coatomer-depleted cytosol was prepared by a 20-min 37°C incubation of rat brain cytosol with excess Golgi and 100 μM GTPγS in the absence of ATP and an ATP-regenerating system. Residual coatomer was assessed in cleared cytosol by using anti-β-coat protein (COP) antibodies. ARF was purified from bovine brain cytosol (14).

Antibodies and Recombinant Polypeptides. Antibodies used were mAbVIIIC7 (15) and antibody 993 (Chemicon) against $\beta I\Sigma 1$ spectrin, and three antibodies against the PH domain of $\beta I\Sigma 2$ spectrin. Specifically used were: MUS1, against a 17-residue peptide ($\beta I\Sigma 2$-A, ref. 16); MUS2, against -LEGPNKKASNR-SWNN..GGC- representing the variable loop between the first and second β-sheet of the PH domain (J.S.M. and C. D. Cianci, unpublished work); and PAB-$\beta I\Sigma 2_{DIII}$, against region III of $\beta I\Sigma 2$ spectrin (J.S.M. and S. A. Weed, unpublished work). Other antibodies were PAB-jasmin against the Golgi ankyrin

Abbreviations: ARF, ADP ribosylation factor; MAD, membrane association domain; PtdIns, phosphatidylinositol; PH, pleckstrin homology; ER, endoplasmic reticulum; VSV, vesicular stomatitis virus; COP, coat protein(s); BFA, brefeldin A; GST, glutathione *S*-transferase; Btk, Bruton's tyrosine kinase; PLD, phospholipase D; OSBP, oxysterol-binding protein.

‖To whom reprint requests should be addressed. e-mail: dematteis@cmns.mnegri.it.

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Ank_{G119} (5); PAB-10D against βII spectrin (17); anti-β-COP (M3A5, Sigma), anti-actin (Sigma), anti-centractin (provided by E. Holzbaur, University of Pennsylvania, Philadelphia); and anti-ARF (1D9, provided by R. Kahn, Emory University, Atlanta). Recombinant peptides generated as fusions with glutathione *S*-transferase (GST) included various βI spectrin peptides (7), βII spectrin-PH domain (17), the oxysterol-binding protein (OSBP)-PH domain (18), and the Bruton's tyrosine kinase (Btk)-PH domain (amino acids 6–217) (19).

Binding to Golgi. Golgi and cytosol were incubated 25 min at 37°C in binding buffer (25 mM Hepes-KOH, pH 7/0.2 M sucrose/25 mM KCl/2.5 mM $MgCl_2$/1 mM ATP/5 mM creatine phosphate/10 units/ml creatine phosphokinase/1 mM DTT, and 20 μM GTPγS for the last 10 min). Membranes were pelleted and analyzed by SDS/PAGE and Western blotting. βIΣ* spectrin binding also was assayed on flotation gradients. Samples were made 1.24 M sucrose, overlaid with 1 M sucrose, 0.5 M sucrose, 10 mM Tris·HCl, pH 7.4 and centrifuged 120 min at 90,000 × *g* at 4°C. Interfacial material between 0.5 M and 1 M sucrose was analyzed. βIΣ* spectrin was taken as a protein of 220 K_d recognized by the βI, but not βII, reactive antibodies.

Other. Phospholipids were extracted in chloroform/methanol and analyzed by TLC (20), and radioactive products were quantified by using a Packard Instant-Imager. ER-Golgi transport of the G protein of ts045 VSV was measured in semi-intact VSV-infected NRK cells (20–22).

RESULTS

Golgi-Associated Spectrin Shares a Close Antigenic Similarity to βIΣ2 Spectrin. All βI spectrin antibodies labeled Golgi-like perinuclear reticular and punctate structures in NRK cells (Fig. 1). The Golgi-associated spectrin underwent a diffuse redistribution after treatment with brefeldin A (BFA), a fungal toxin that rapidly disassembles the Golgi complex (4, 23, 24). The Golgi-like distribution detected by the PH domain-specific spectrin antibodies and the BFA sensitivity suggested that Golgi spectrin must contain a PH domain antigenically similar to that of βIΣ2 spectrin. The distribution of Ank_{G119}, a Golgi-associated ankyrin isoform (5), paralleled that of spectrin in control and BFA-treated cells (data not shown). The distribution of both βIΣ* spectrin and Ank_{G119} overlapped that of giantin (a 376-kDa Golgi-specific membrane protein) (3, 25), with areas of colocalization and additional regions enriched in spectrin but devoid of giantin (Fig. 1).

The Small GTPase ARF Regulates the Association of a Spectrin-Ankyrin Skeleton to the Golgi. The association between spectrin and the Golgi complex was characterized in permeabilized NRK cells. Golgi βIΣ* spectrin partially redistributed into the cytoplasm upon permeabilization, but regained its perinuclear location in the presence of the G protein activator GTPγS (Fig. 1), mirroring the behavior of β-COP and giantin. This effect of GTPγS was blocked by BFA (Fig. 1), indicating that spectrin localized to the Golgi in a G protein-dependent and BFA-sensitive manner. These sensitivities paralleled those of ARF, a small G protein activated by nucleotide exchange on Golgi fractions (26, 27) and implicated ARF in the control of spectrin-Golgi binding. To assess the role of ARF, the GTPγS- and BFA-sensitive binding of βIΣ* spectrin to isolated Golgi fractions was reconstituted and characterized *in vitro* by using subcellular fractions both from NRK cells and rat brain or liver (9, 10). When Golgi fractions were incubated with cytosol, a discrete set of proteins at ≈ 220, 170, 110, 108, 43, 30, and 20 kDa was recruited in a GTPγS-dependent and BFA-sensitive manner (Fig. 2*A*, *). GTP also was able to recruit the same set of proteins onto Golgi fractions in a BFA-sensitive manner, but with lower potency and efficacy compared with GTPγS (not shown). The 20- and 110-kDa proteins were identified by immunoblotting as ARF and β-COP, respectively. The 220-kDa protein was identified as βIΣ* spectrin, recognized by the panel of anti-βI spectrin antibodies, including two antibodies specific for the Σ2 splicing variant of βI spectrin but not by the anti-βII spectrin antibody. The profile of βIΣ* spectrin binding to subcellular membranes precisely overlapped with the profile of the Golgi marker mannosidase II and clearly was dissociated from plasma membrane or ER markers (not shown). The 108- and 43-kDa proteins were identified immunologically as Ank_{G119} and actin, respectively. Centractin was associated with the Golgi fractions as previously noted (7), but its binding was not stimulated by activated ARF (not shown). The GTPγS-dependent and BFA-sensitive association of the above proteins with Golgi was confirmed in experiments in which the membranes were recovered by flotation on sucrose gradient (Table 1).

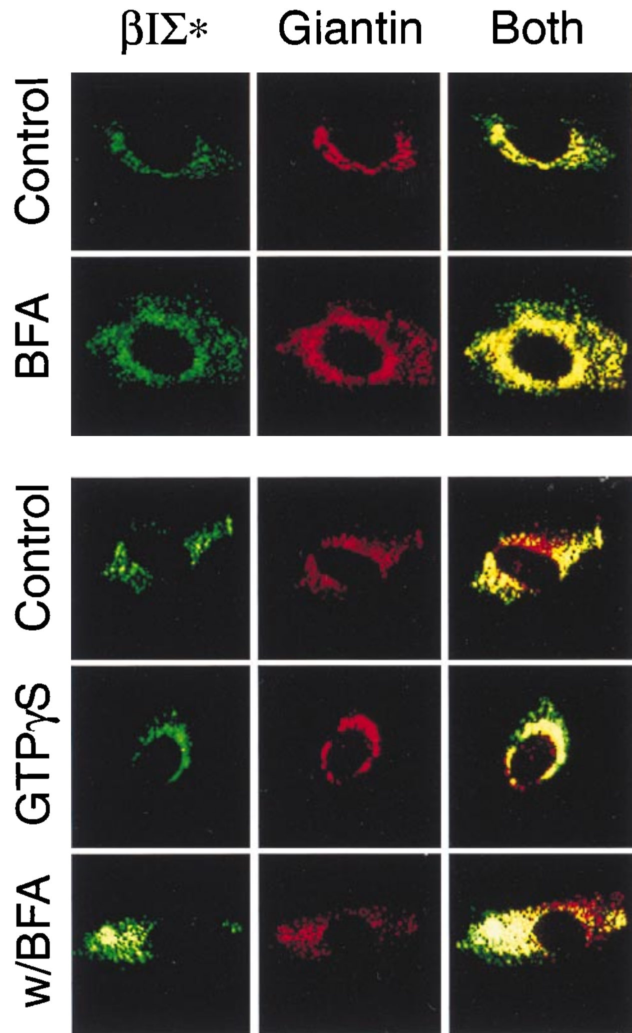

FIG. 1. Golgi βIΣ* spectrin contains PH domain epitopes and binds the Golgi complex in a GTPγS-dependent fashion. Four anti-spectrin antibodies were used to assess the distribution of the Golgi-associated spectrin. Two of these antibodies, MUS1 and MUS2, were specific for the PH domain of βIΣ2 spectrin. All gave similar distributions in NRK cells; the pattern of MUS1 staining is shown. This pattern largely overlaps that of giantin and is disrupted by BFA, and its reassembly is stimulated by GTPγS in permeabilized NRK cells. (*Upper*) Intact NRK cells were treated with 5 μg/ml of BFA for 5 min. (*Lower*) Streptolysin O-permeabilized NRK cells were treated with 50 μM GTPγS or with 1 μg/ml BFA for 5 min before and during permeabilization in the presence of 50 μM GTPγS (BFA). The immunofluorescence patterns shown are representative of the average pattern present in at least 80% of the cells for each treatment and observed in at least three experiments run in duplicate. Both represents the superimposition of the fluoresceine and Cy3 images, with yellow depicting the areas of overlap of the two antigens.

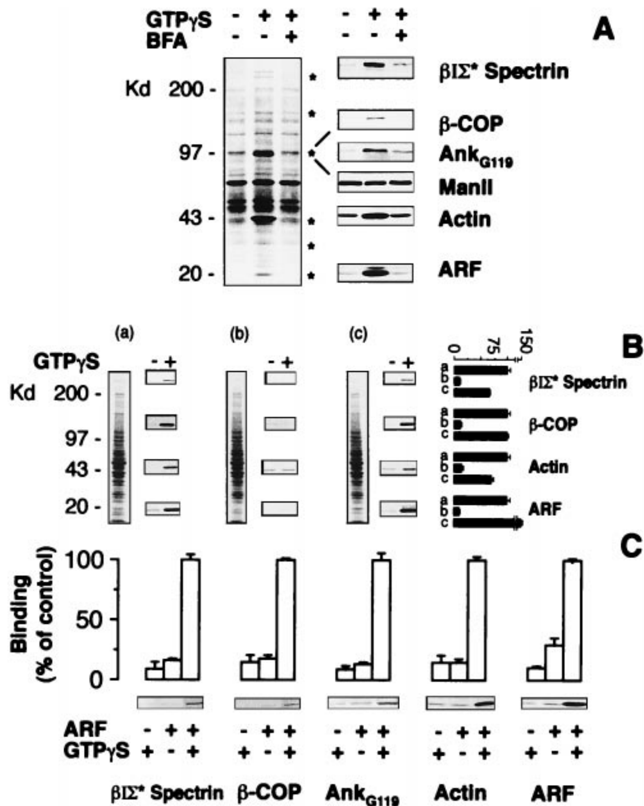

FIG. 2. ARF regulates the association of spectrin with Golgi fractions. (*A*) Isolated Golgi fractions were incubated with cytosol for 20 min with or without 20 μM GTPγS. Where indicated, 40 μg/ml of BFA was added at the beginning of the incubation. Note that GTPγS induced the recruitment of a discrete set of at least six cytosolic proteins (*), but did not affect integral membrane proteins such as the resident Golgi enzyme mannosidase II (ManII). (*B*) The proteins present in control cytosol (*a*), ARF-depleted cytosol (*b*), or ARF-depleted cytosol reconstituted with 1 μM purified bovine ARF (*c*) were separated by SDS/PAGE, transferred on nitrocellulose filters, and stained with Ponceau red. This analysis verified the loss of the ARF band at $K_d \approx 20$. For each cytosol preparation the binding of βIΣ* spectrin, β-COP, actin, and ARF to Golgi in the absence (−) or presence (+) of GTPγS then was evaluated as in *A*. When ARF is depleted from the cytosol, GTPγS loses its ability to recruit βIΣ* spectrin, β-COP, or actin [Western blots shown, quantified, and expressed in the bar graph on the right as percent of GTPγS effect in the presence of control (*a*) cytosol]. Data represent the average of three experiments; error bars represent ±1 SD. (*C*) Golgi fractions were first incubated with 20 μM GTPγS or 1 μM purified bovine ARF or with both for 15 min at 37°C. Membranes then were pelleted and rinsed, and incubated ARF depleted cytosol for 15 min at 37°C. The binding, expressed as percent of controls (ARF + GTPγS present in the first step), was determined as in *A*. Data represent the average of five experiments (±1 SD). Note that only when ARF is activated by GTPγS is there a significant binding of spectrin, β-COP, Ank$_{G119}$, or actin.

The ARF requirement for binding between spectrin and Golgi was tested by using two approaches: the depletion of ARF and the selective activation of ARF achieved through a two-step incubation. In the presence of an ARF-depleted cytosol (Fig. 2*B*), GTPγS lost its ability to recruit spectrin, β-COP, or actin to Golgi; this activity was restored by purified ARF (14) (Fig. 2*B*). The two-step binding experiments confirmed the ARF requirement for spectrin association with Golgi fractions (Fig. 2*C*). Golgi fractions, washed with 1 M KCl to assure the removal of residual ARF, were incubated with or without purified bovine ARF and/or GTPγS in the first step, and then incubated with cytosol in the absence of GTPγS in the second step. Only membranes preincubated with ARF and GTPγS recruited spectrin, β-COP, Ank$_{G119}$, and actin, indicating that the GTPγS-induced binding of spectrin (and several other proteins) is strictly and specifically ARF dependent (Fig. 2*C*). Interestingly, the recruitment efficiency of the ARF-preloading protocol vs. the single-step addition of GTPγS to the cytosol was similar for spectrin, β-COP, and ankyrin, but lower for actin, consistent with the possibility that cytosolic G proteins other than ARF also may promote actin binding and/or polymerization.

Table 1. ARF-dependent binding of βIΣ* spectrin to Golgi measured by pelleting vs. flotation assay

βIΣ*-spectrin binding to Golgi fractions	Pelleting, % of control ± SD	Flotation, % of control ± SD
One-step incubation		
Basal	15 ± 4	10 ± 3
GTPγS	100 ± 9	100 ± 8
BFA-GTPγS	20 ± 5	18 ± 4
Two step-incubation		
ARF	15 ± 2	13 ± 3
GTPγS	10 ± 3	8 ± 1
ARF + GTPγS	100 ± 8	100 ± 7

Data represent the average ± SD of three experiments.

Spectrin Binding to Golgi Fractions Occurs Independently of COPI and Involves MAD1 and MAD2. To assess whether the COPI coat played a role in the ARF-dependent binding of spectrin to Golgi, binding experiments were repeated by using a COPI-depleted cytosol. Using this β-COP (and hence COPI) depleted cytosol with fresh Golgi fractions and added ARF; spectrin, Ank$_{G119}$, and actin all bound just as well as with the control (undepleted) cytosol (Fig. 3*A*). Thus, ARF regulates the assembly of spectrin and coatomer complexes on Golgi by distinct mechanisms.

Spectrin can associate with membranes by both adapter-mediated and direct binding mechanisms (28). In βIΣ2 spectrin *in vitro* studies have identified two direct MADs, MAD1 and MAD2 (17). MAD1 is confined to spectrin repeat unit 1, whereas MAD2 encompasses most of spectrin's domain III and includes its PH domain (Fig. 3*B*). Because the Golgi-associated βIΣ* spectrin shares a close antigenic similarity to βIΣ2 spectrin, the role of MAD1 and MAD2 in the ARF-dependent recruitment of βIΣ* spectrin to Golgi fractions was of interest. Recombinant GST-fused polypeptides spanning MAD1 or MAD2 of βIΣ2 spectrin each individually inhibited the binding of βIΣ* spectrin (as well as that of Ank$_{G119}$ and actin) to Golgi fractions (Fig. 3*B*). mAbVIIIC7 also potently inhibited the binding of βIΣ* spectrin, Ank$_{G119}$, and actin ($IC_{50} \approx 0.2$ μg/ml). In contrast, none of the fusion proteins or mAbVIIIC7 affected the binding of β-COP (Fig. 3*B*) or ARF (not shown), suggesting that spectrin binding occurs independently of COPI coat assembly.

ARF Regulates the Association of Spectrin to the Golgi by Controlling the Levels of PtdInsP_2. Because polypeptides encompassing spectrin's MAD2 include a PH domain that binds PtdInsP_2 *in vitro* (29), we examined the involvement of PtdInsP_2 in Golgi-spectrin association. Recombinant GST-fused polypeptides containing PH domains known to bind PtdInsP_2 also were tested, including those from βII spectrin (30), the OSBP (18), and Btk (which only weakly binds PtdInsP_2 and preferentially binds PtdInsP_3, refs. 19 and 31). The PH domains from βII spectrin and OSBP both inhibited the association of βIΣ* spectrin to Golgi, whereas Btk-PH did not (Fig. 4*A*). The ineffectiveness of Btk-PH and the observation that 20 nM wortmannin (an inhibitor of PtdIns3 kinase) did not affect spectrin binding suggested a preferential role for PtdInsP_2 (vs. PtdInsP_3) in spectrin binding.

If PtdInsP_2 was involved in mediating the spectrin-Golgi complex interaction, ARF might control this interaction by modulating the levels of PtdInsP_2 in Golgi membranes. To test

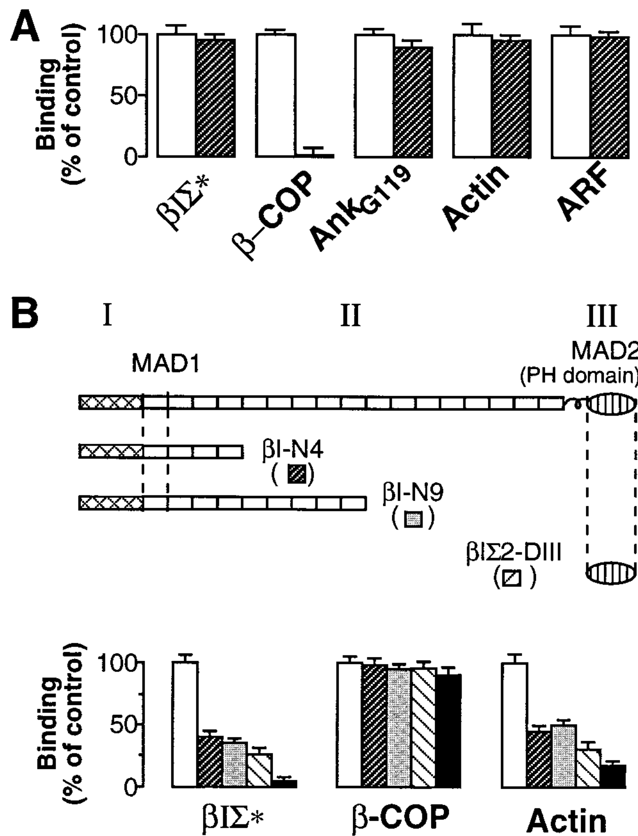

FIG. 3. Spectrin binding to Golgi fractions occurs independently of COPI binding and involves both MAD1 and MAD2. (*A*) Binding experiments similar to those in Fig. 2 were conducted by using cytosol depleted in COPI. Membranes were incubated with control cytosol (empty bars) or coatomer-depleted cytosol containing 1 μM ARF (striped bars). The binding of each component to Golgi fractions, relative to the binding in control cytosol, is shown. Data represent the average of three experiments (±1 SD). (*B*) Schematic representation of βIΣ2 spectrin: I, II, III indicate the spectrin domains, and MAD1 and MAD2 the regions of direct membrane association. Golgi (10 μg/sample) were incubated for 15 min at 37°C with cytosol (75 μg/sample) and 20 μM GTPγS with 3 μM GST (empty bars) or GST-fused polypeptides illustrated in the schematic representation of spectrin, i.e., $\beta I_{N\text{-}4}$ or $\beta I_{N\text{-}9}$ from βI spectrin (encompassing MAD1) or $\beta I\Sigma 2_{DIII}$ (encompassing MAD2, and hence the third domain of βIΣ 2 spectrin containing the PH domain) (7, 16) or the anti-βI spectrin mAb mAbVIIIC7 (0.4 μg/ml) (filled bars). Two control mAbs had no effect. The GTPγS-induced binding of ARF was not changed by any of the reported treatments (not shown). Binding is expressed as percent of control (GST alone). Data represent the average of four experiments (±1 SD). The IC_{50} measured for $\beta I\Sigma 2_{DIII}$ is 1 μM.

this, isolated Golgi were treated with or without GTPγS and/or purified ARF, washed, and incubated with cytosol in the presence of ATP-γ [^{32}P] (2 μCi/sample) to label phospholipids (Fig. 4*B*). The presence of ARF and GTPγS in the first step (but not GTPγS or ARF alone) elicited a 5-fold increase in $PtdInsP_2$ during the second step (Fig. 4*B*). Next, we tested to see whether the $PtdInsP_2$ synthesized in response to ARF activation was required for the association of βIΣ* spectrin with Golgi. The antibiotic neomycin, which sequesters $PtdInsP_2$ with high affinity (32), abolished ARF-induced binding of spectrin to Golgi fractions with an IC_{50} of ≈150 μM (Fig. 4*C*) but did not affect the binding of ARF or β-COP (not shown). The inhibition by neomycin appeared to be caused by $PtdInsP_2$ sequestration, because it could be completely reversed by the administration of exogenous $PtdInsP_2$. Interestingly, $PtdInsP_2$ was insufficient alone (in the absence of activated ARF) to recruit spectrin (Fig. 4*C*). Adenosine (a

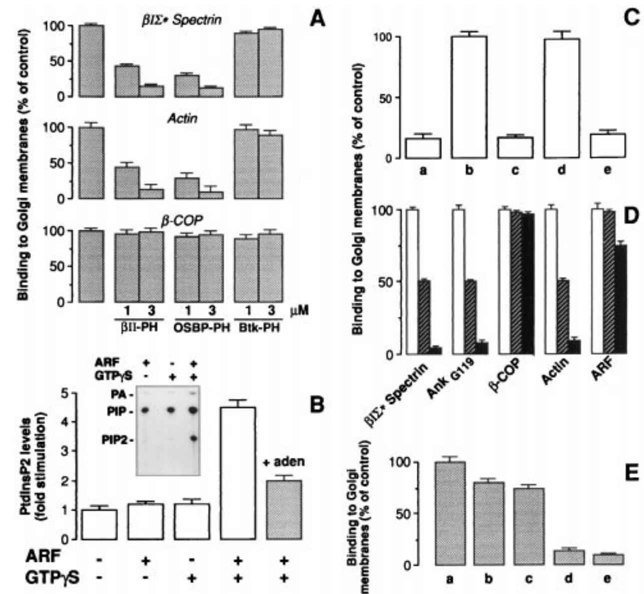

FIG. 4. ARF stimulation increases $PtdInsP_2$ in Golgi fractions, and $PtdInsP_2$ is required for the binding of βIΣ* spectrin. (*A*) Golgi fractions were incubated with cytosol in the presence of 20 μM GTPγS and 3 μM GST or the indicated concentrations of GST-fused polypeptides containing the PH domains of βII spectrin (βII-PH), OSBP (OSBP-PH), or Btk (Btk-PH). Results (binding to Golgi) are expressed as percent of control binding (GST alone). Data represent the average of four experiments (±1 SD). (*B*) Golgi were incubated in two steps: first (as indicated) with control buffer or 1 μM ARF or with 20 μM GTPγS alone or GTPγS and 1 μM ARF for 15 min at 37°C. In the second step, membranes were pelleted, rinsed, and incubated with cytosol and 2 μCi/sample of ATP-γ [^{32}P]. Where indicated (filled bar), 2.5 mM adenosine was added during both the first and second incubation steps to block PtdIns4 kinase activity. At the end of the incubation, phospholipids were extracted and analyzed by TLC. The level of ^{32}P-labeled $PtdInsP_2$ is expressed relative to unstimulated control. Data represent the average of eight experiments (error bars ±1 SD). (*Inset*) A representative TLC with the positions of phosphatidic acid (PA), PtdIns4P (PIP), and PtdIns4,5P2 (PIP2) standards marked. (*C*) Golgi fractions were incubated with control buffer (*a* and *e*), 20 μM GTPγS (*b*), or 20 μM GTPγS and 250 μM neomycin (*c* and *d*). Membranes were pelleted, incubated for 30 min at 4°C with phospholipid liposomes containing phosphatidylethanolamine (PE, *a–c*) or $PtdInsP_2$/PE (1:5 mol/mol) (*d* and *e*), and incubated with cytosol for 15 min at 37°C. Results (βIΣ* spectrin binding to Golgi) are expressed as percent of controls (GTPγS alone). The data represent the average of four experiments (±1 SD). (*D*) Golgi fractions were incubated in the first step with 1 μM ARF and 20 μM GTPγS, then pelleted, rinsed, and incubated with cytosol in the second step. The buffer used in both steps contained 500 μM ATP (empty bars), 500 μM ATP and 2.5 mM adenosine (striped bars), or no ATP (filled bars). Results (binding to Golgi) are expressed as percent of controls (500 μM ATP alone), averaged over four experiments (error bars ±1 SD). (*E*) Golgi were incubated with GTPγS and ARF under control conditions (*a*), in the presence of 1% 1-butanol (*b*), or 2-butanol (inactive as PLD substrate) (*c*). Membranes were pelleted and incubated with cytosol and 1-butanol (*b*) or 2-butanol (*c*). Golgi fractions were incubated with 0.2 units/ml of PLD from *Streptomyces chromofuscus* in the absence (*d*) or in the presence of 100 μM Ca^{2+} (*e*), pelleted, rinsed, and incubated with cytosol and PLD in the second step in the absence (*d*) or in the presence of 1 μM Ca^{2+} (*e*). In parallel experiments (not shown) the levels of phosphatidic acid levels were measured as in *B*, and a 10-fold increase in phosphatidic acid was measured in samples treated with exogenous PLD compared with control samples. Results (βIΣ* spectrin binding to Golgi) are expressed as percent of controls (ARF and GTPγS). Each data represent the average of four experiments (±1 SD).

blocker of the PtdIns4 kinase responsible for the synthesis of PtdIns4P, the precursor of PtdIns4,5P_2, ref. 33) also inhibited ARF-dependent $PtdInsP_2$ generation and spectrin binding (Fig. 4 *B* and *D*). Broad spectrum protein kinase inhibitors

such as staurosporine, H89, and genistein had no effect on spectrin's binding (not shown). Consistent with a requirement for neo-synthesized $PtdInsP_2$ is the observation that the binding of spectrin, but not that of ARF or β-COP, to Golgi fractions was strictly ATP dependent (Fig. 4*D*).

A moderate stimulatory effect of ARF on $PtdInsP_2$ synthesis has been reported in HL-60 cells (34) and has been attributed to ARF stimulation of phospholipase D (PLD). However, PLD stimulation did not appear to be the mechanism by which ARF induced spectrin recruitment in the system studied here, because neither 1-butanol or ethanol (inhibitors of the formation of phosphatidic acid by PLD) (35) specifically affected the ARF stimulation of binding, nor did the addition of exogenous PLD (36) stimulate binding (Fig. 4*E*).

Spectrin Binding to the Golgi Complex Is Required for ER to Golgi Transport of the VSV-G Protein. Given that at least two sites by which spectrin binds to Golgi had been identified, it was of interest to determine whether binding at either or both sites is required for Golgi function. To this end we used the above-described recombinant peptides or antibodies to block the assembly of native Golgi spectrin in semi-intact VSV-infected NRK cells and measured the ER to Golgi transport of VSV-G protein. Under control conditions 60% and 80% of VSV-G protein was transported at 32°C from the ER to the Golgi after 60 and 90 min, respectively (Fig. 5). These values are similar to those observed by others (21). With the exception of Btk, all fusion proteins and mAbVIIIC7 suppressed transport of VSV-G from the ER to medial Golgi (Fig. 5*A*). The MAD1-encompassing polypeptide was the most effective inhibitor of transport, in agreement with *in vivo* transfection data obtained in MDCK cells (7). None of the above agents, added after 60 min at 32°C under control conditions (to let the VSV-G protein reach the medial Golgi), affected the trans-Golgi/trans-Golgi network (TGN) arrival of VSV-G (Fig. 5*B*). Thus, it appears that spectrin binding to the Golgi is required for VSV-G transport from the ER to Golgi, but not for its subsequent transport to the trans-Golgi/TGN.

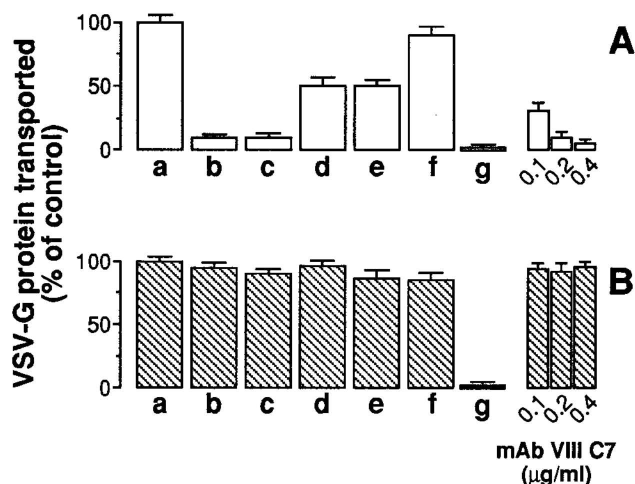

FIG. 5. Spectrin binding to Golgi is required for ER to Golgi transport of VSV-G. (*A*) ER-Golgi transport. VSV-infected NRK cells were pulse-labeled with [^{35}S]-methionine at 40°C, permeabilized, and incubated with cytosol and 3 μM GST (*a*) or 3 μM GST-fused polypeptides as illustrated in the schematic representation of spectrin in Fig. 3*B*, i.e., βI_{N-4} (*b*), βI_{N-9}(*c*), MAD2 (containing the PH domain) of $\beta I\Sigma 2$ spectrin (*d*), 3 μM GST-fused PH domains of βII spectrin (*e*), or Btk (*f*) for 90 min at 32°C. *g* indicates samples that, after the pulse at 40°C, were maintained on ice for the next 90 min. The anti-βI spectrin mAb mAbVIIIC7 (at the indicated concentrations) was preincubated with permeabilized cells for 45 min on ice after the 40°C incubation, before the shift to 32°C. Two control mAbs had no effect. Results are expressed as percent of controls (*a*). (*B*) Intra-Golgi transport. After pulse and permeabilization, NRK cells were shifted to 32°C for 60 min (to let the VSV-G protein reach the medial-Golgi), then transferred to 4°C, and after the addition of the agents specified in *A*, shifted again to 32°C for 90 min. *g* indicates samples that, after the pulse at 40°C and 60 min at 32°C, were kept on ice for the next 90 min. Results are expressed as percent of controls (*a*). Data represent the average of four experiments (±1 SD).

DISCUSSION

The association of spectrin with membranes is multivalent and cooperative and involves both direct and adapter-mediated membrane attachment sites (17). Based on the data presented here and previous data, we envisage that the binding of spectrin to Golgi membranes involves at least two binding domains, one (MAD1) bearing a targeting signal (7) and responsible for constitutive localization to the Golgi complex, and one (MAD2) acting to enhance the affinity of this binding and to render it sensitive to $PtdInsP_2$ regulation. Because the overall affinity of such a two-site interaction equals the product of the affinities of each individual site, it is conceivable that the most stable spectrin-membrane associations are achieved only at Golgi-specific docking sites where, and when, the local density of $PtdInsP_2$ reaches a threshold necessary to engage the spectrin PH domain (i.e., MAD2). It is also possible that interactions mediated by other adapter proteins, such as ankyrin, contribute to Golgi binding given their prominence as a membrane linker in the erythrocyte membrane skeleton. Finally, although a growing body of evidence indicates that phosphoinositides are essential to the function of the secretory apparatus (37), the molecular mechanisms and the targets of phosphoinositides remain uncertain. Our results indicate that $PtdInsP_2$ is required for the ARF-dependent association of $\beta I\Sigma^*$ spectrin to the Golgi complex.

The data reported here together with data reported in parallel studies in transfected cells (7) demonstrate that the association of spectrin to Golgi is required for ER to Golgi transport. However, the precise role of spectrin in Golgi function remains to be defined. Spectrin does not belong to any of the known classes of proteins so far implicated in the management of protein traffic in the secretory pathway. Two nonmutually exclusive models can be proposed at this time. Spectrin might function as a novel type of vesicular coat, mediating the capture and anterograde transport of specific cargo molecules between the ER and Golgi (7). In this model, spectrin also might mediate the interaction between Golgi membranes and the machinery of dynein-driven transport by direct binding to the dynactin complex (7, 38). Another attractive role of the spectrin-based Golgi skeleton, considering spectrin's established role at the plasma membrane to guarantee structural integrity and organize membrane domains, could be to organize incoming ER-derived membranes into a cis-Golgi compartment (39). Such a function would be particularly appealing in the context of recently proposed versions of the cisternal progression-maturation model (40, 41) that envisage the existence of a dynamic Golgi scaffold able to undergo rapid remodeling to integrate the new membranes coming from pre-Golgi compartments, and to release Golgi membranes destined to post-Golgi compartments. Within this framework it is difficult to distinguish a pure "structural" from a "functional" role of spectrin in the Golgi complex: the block of spectrin association, caused in our experiments by competing peptides and antibodies, would impair the organization and integration of incoming ER-derived membranes into the Golgi complex, thereby inhibiting transport of cargo molecules from the ER.

It has long been clear that some kind of matrix or skeleton must play a critical role in maintaining the structure and function of the Golgi complex. Studies with BFA and mitotic Golgi (1, 24, 42) indicate that the Golgi skeleton must be dynamic and controlled by ARF, able to quickly disassemble and reassemble. The studies reported reveal the identity of

such a skeleton, define one mechanism by which it is regulated, and define useful biochemical approaches to further dissect its function.

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