ADP-Ribosylation Factor and Coatomer Couple Fusion to Vesicle Budding

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Abstract. The coat proteins required for budding COP-coated vesicles from Golgi membranes, coatomer and ADP-ribosylation factor (ARF) protein, are shown to be required to reconstitute the orderly process of transport between Golgi cisternae in which fusion of transport vesicles begins only after budding ends. When either coat protein is omitted, fusion is uncoupled from budding—donor and acceptor compartments pair directly without an intervening vesicle. Coupling may therefore result from the sequestration of fusogenic membrane proteins into assembling coated vesicles that are only exposed when the coat is removed after budding is complete. This mechanism of coupling explains the phenomenon of "retrograde transport" triggered by uncouplers such as the drug brefeldin A.

How is membrane fusion coupled to vesicle budding? A transport vesicle must fuse with its target only after its budding from the parental membrane is completed. Otherwise, the various membrane-bound compartments connected by vesicle shuttles would fuse and the topological organization of the endomembrane system in cells would be destroyed. The basis for coupling can be more precisely formulated. As each transport vesicle presumably carries with it certain membrane proteins derived from the parental membrane that will later be needed to attach to receptors at the target membrane to initiate fusion, there must be a coupling mechanism to prevent these same "fusion" proteins from initiating premature (e.g., uncoupled) fusion while they still reside in the parental compartment.

One mechanism to ensure coupling would be to activate membrane fusion components as they enter transport vesicles, or at some later stage of vesicle maturation. A simpler mechanism is afforded to transport vesicles that bud with a cytoplasmic protein coat, such as the clathrin coat for endocytosis (Pearse and Robinson, 1990) or the COP coat for ER and Golgi transport (Rothman and Orci, 1992). The pertinent membrane fusion proteins of the parental membrane could simply be sequestered in the coats of budding vesicles until the coat is removed after completion of budding. A process that efficiently packages fusion proteins in assembling vesicles must be a part of any budding mechanism, and is easy to envision for coated vesicles.

COP-coated vesicles form from Golgi stacks incubated with cytosol and a source of energy, ATP (Balch et al., 1984;

Orci et al., 1989). Purification of COP-coated vesicles (Malhotra et al., 1989; Serafini et al., 1991a,b) revealed that their coats consist of a small GTP-binding protein (ADP-ribosylation factor, ARF)[1] and a complex of seven distinct proteins termed coatomer (Waters et al., 1992a; Stenbeck et al., 1993), whose subunits are α, β, β′, γ, δ, ε, and ζ-COPs. Coatomer and ARF exist separately in cytosol, but coassemble to form coats. Assembly is initiated when the GTP form of ARF (which must be N-myristylated) binds to a membrane receptor following a brefeldin A- (BFA) sensitive nucleotide exchange step (Donaldson et al., 1992b; Helms and Rothman, 1992; Helms et al., 1993). This membrane-bound form of ARF affords a binding site for coatomers, (Donaldson et al., 1992a; Palmer et al., 1993) and budding occurs as the coatomers bind (Orci et al., 1993a,b). Coatomer and ARF are the only cytosolic proteins that appear to be necessary for budding (Orci et al., 1993a). The coat disassembles when ARF is triggered to hydrolyze bound GTP (Tanigawa et al., 1993), most likely at the target membrane.

A body of strong correlative evidence (reviewed in Rothman and Orci, 1992) from cell-free transport reactions led to the view (Orci et al., 1989) that COP-coated vesicles mediate protein transport from ER to Golgi and between successive cisternae of the Golgi stack. This conclusion has recently been confirmed in living cells by both biochemical and genetic methods. Microinjection of an antibody to β-COP blocked transport of the VSV-G protein up to but not beyond the *trans*-Golgi (Pepperkok et al., 1993), and a

1. *Abbreviations used in this paper:* ARF, ADP-ribosylation factor; BFA, brefeldin A; NSF, N-ethylmaleimide-sensitive fusion; RF, releasing factor; SNAP, soluble NSF attachment protein.

secretion gene in yeast (sec21) was found to encode a homologue of γ-COP (Stenbeck et al., 1992) and also to be a subunit of a complex isolated from yeast cytosol that contains subunits of similar size to animal coatomer (Hosobuchi et al., 1992). When the two genes encoding ARF in yeast are deleted, secretion stops and cells die (Stearns et al., 1990a,b).

The cell-free Golgi transport assay that defined COP-coated vesicles and delineated their role in transport involves the coincubation of two types of Golgi fractions in the presence of ATP and cytosol-derived fractions (Fries and Rothman, 1980; Balch et al., 1984). The donor population of Golgi membranes is obtained from VSV-infected CHO mutant (clone 15B) cells unable to add the sugar N-Acetylglucosamine (GlcNAc) to glycoproteins due to an enzyme deficiency, and the acceptor population of Golgi membranes is from uninfected wild-type CHO cells. A signal in the assay results when G protein from donor membranes is glycosylated by the GlcNAc-transferase from acceptor membranes recorded by transfer of [³H]GlcNAc from UDP-[³H]GlcNAc to VSV-G protein. In crude cytosol, the assay signal is principally due to COP-coated vesicles that carry VSV-G protein from cis-Golgi cisternae in the donor stacks to the medial-Golgi cisternae in the acceptor stacks that house GlcNAc Transferase I (Rothman et al., 1984; Orci et al., 1989; Rothman and Orci, 1992). However, in principle, or under different conditions, a direct (uncoupled) fusion among Golgi stacks would also result in an assay signal (Mellman and Simons, 1992).

Given the recently defined and clearly delineated roles of ARF and coatomer in COP-coated vesicle budding, and the confirmation of the requirement for these proteins in vivo, these proteins are expected to be required for vesicular transport of VSV-G protein in the cell-free system. We now report a surprising finding obtained as a result of systematic fractionation of cytosol (Block et al., 1988; Clary and Rothman, 1990; Waters et al., 1992b) to yield the minimum set of components needed for efficient reconstitution of an assay signal: coatomer and ARF are not among these components. In investigating this apparent discrepancy, we discovered that with these minimal components the assay signal now results from uncoupled, direct fusion of Golgi stacks that occurs when either ARF or coatomer is absent. Adding back both coatomer and N-myristylated ARF protein restores coupling. Now coated vesicles assemble, but cannot be consumed due to the lack of a releasing factor. When this releasing factor is also added back, the assay signal is restored, now due to vesicular transport as fusion is once again coupled to budding, as in crude cytosol. These findings offer clear confirmation of the role of ARF and coatomer in cell-free transport, and also define a novel role for the coat proteins, coupling of fusion to budding. This new role allows a straightforward explanation of the phenomenology associated with brefeldin A (BFA) in its effects on "retrograde transport" from Golgi to ER (Klausner et al., 1992).

Materials and Methods

Materials

BFA, obtained from Epicenter Technologies Corp. (Madison, WI) was dissolved in methanol. ATP, UTP, palmitoyl-Coenzyme A, and DTT were

from Boehringer Mannheim Corp. (Indianapolis, IN). Creatin phosphokinase, creatine phosphate, and nucleotide monophosphate kinase were obtained from Sigma Chemical Co. (St. Louis, MO). Affinity-purified antiserum to ε-COP was a gift of Dr. S. Kuge, and a monoclonal antibody against β-COP (M3A5) was kindly donated by Dr. T. E. Kries. Affinity purified antibodies against ARF were prepared as described (Palmer et al., 1993). Donor and acceptor membrane fractions were prepared from VSV-infected CHO 15B and wild type CHO cells, respectively, as described previously (Balch et al., 1984). Salt extracted Golgi membranes were prepared as described (Waters et al., 1992b). Bovine brain cytosol was prepared by the method of Malhotra et al. (1989).

Cis- to Medial-Transport Assay

The assay is a modification of that previously described by Waters et al. (1992b). 2.5 μg CHO 15B Golgi membranes (lacking N-acetylglucosamine transferase) infected with VSV (donor) were mixed with 2.5 μg wild-type CHO Golgi (acceptors) and incubated for 1 h at 37°C in the presence of 25 mM Hepes-KOH, pH 7.2, 40 mM KCl, 2.5 mM Mg(OAc)₂, 100 μM ATP, 250 μM UTP, 5 mM creatine phosphate, 8 IU/ml creatine phosphate kinase, 10 μM palmitoyl-Coenzyme A, 40 μg/ml nucleotide monophosphate kinase, 200 mM sucrose, 0.6 μCi UDP-[³H]-N-acetylglucosamine ([³H]GlcNAc). The reaction mixtures were supplemented with either 10 ng His₆-NSF (Whiteheart et al., 1993), 60 ng His₆-α-SNAP (Soluble NSF attachment protein) (Whiteheart et al., 1993), 0.5 μg p115 (Waters et al., 1992b), 60 μg fraction Iα and 60 μg fraction Iβ or with 80 μg bovine brain cytosol, in a total volume of 50 μl. [³H]GlcNAc incorporated into VSV-G protein was detected by immunoprecipitation of VSV-G protein and scintillation counting (Balch et al., 1984).

Purification of Different Cytosolic Factors

Fractions Iα and Iβ were obtained by chromatography of 100 ml bovine brain cytosol (10 mg/ml) on a 20 ml Mono Q column equilibrated with 25 mM Tris-HCl, pH 7.4, 50 mM KCl, 1 mM DTT, and 10% glycerol (Waters et al., 1992b). The column was washed with 40 ml of the same buffer and then eluted with 50–500 mM KCl gradient in 160 ml. 3-ml fractions were collected, dialyzed to reduce the KCl concentration to 50 mM, and assayed for transport activity in the presence of 0.5 μg p115, 10 ng His₆-NSF, 60 ng His₆-α-SNAP and 5 μg 1 M KCl-extracted Golgi membranes. Activities corresponding to Iα were eluted at 210 mM KCl and to Iβ at 280 mM KCl. During the course of this study we have also used a mixture of Iα and Iβ (termed Iα+β) which corresponds to a pool of the fractions eluted from the Mono Q column between 200 mM and 290 mM KCl.

Recombinants His₆-NSF and His₆-α-SNAP were prepared as described (Whiteheart et al., 1993). Recombinants mARF1 and ARF1 were purified as described (Weiss et al., 1989) and p115 was purified from bovine liver cytosol as described (Waters et al., 1992b).

Western Blot Analysis

Proteins were fractionated on SDS-PAGE and electroblotted on to nitrocellulose in 25 mM Tris, 192 mM glycine, and 20% methanol at 22V/cm for 1 h. ARF and ε-COP were detected using affinity purified IgG (1:2,000) and peroxidase conjugated anti-rabbit IgG (diluted 1:2,000). β-COP was detected using the mouse monoclonal M3A5 (0.4 μg/ml, (Allan and Kreis, 1986)). Peroxidase labeling was detected by chemiluminescence using the ECL reagent (Amersham Corp., Arlington Heights, IL). β-COP signals were quantitated by imaging with a Scan Jet Plus scanner (Hewlett Packard, CA) and integration of images using the ScanAnalysis software from BioSoft (Cambridge, United Kingdom). β-COP and ARF were immunodetected on the top and the bottom halves of nitrocellulose blots, respectively.

Results

Fusion Uncoupled from Budding

We have previously reported that an assay signal is reconstituted to (1M KCl) salt-washed Golgi membranes by NSF and SNAPs in combination with three distinct cytosol-derived factors, termed Iα, Iβ, and Iγ, and that purification of Factor Iγ reveals it to be a trimer of 115-kD subunits (p115) (waters et al., 1992b). This mixture of components

efficiently (~60%) reconstitutes the assay signal of crude cytosol to both KCl-washed and untreated Golgi membranes (Fig. 1 a). The requirements for each component or fraction are less complete with untreated than KCl-washed membranes presumably because the membranes contribute an adsorbed pool of each component that is removed by the salt wash. When the amounts of fractions Iα, Iβ, and p115, and of Golgi membranes (not salt washed) that are used in standard assays (Fig. 1 a) were analyzed by Western blotting, no coatomer or ARF were detected (Fig. 1 b). The NSF and α-SNAP proteins used were recombinant from *E. coli* and could not provide ARF or coatomer. Standard (not KCl-washed) Golgi membranes are used throughout this study, although similar results are obtained with KCl-washed membranes.

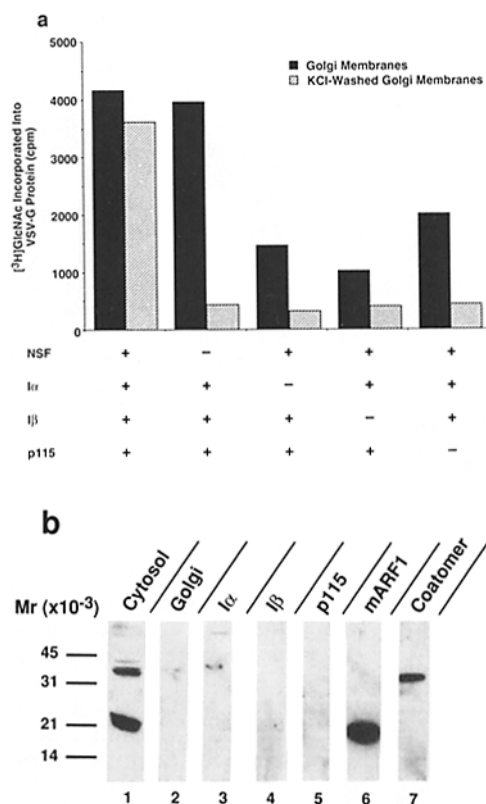Because coatomer and ARF are both required for coated vesicles to bud from Golgi membranes (Orci et al., 1993a),

Figure 1. (*a*) Cytosolic factors required for reconstitution of the cell free assay. CHO 15B Golgi membranes were mixed with wild-type CHO Golgi and incubated for 1 h at 37°C in the presence of [³H]GlcNAc, 10 ng His₆-NSF, 60 ng His₆-α-SNAP 0.5 μg p115, 60 μg fraction Iα, and 60 μg fraction Iβ as indicated. Shown are results using standard CHO-Golgi membranes (■) or 1M KCl extracted CHO-Golgi (▨). (*b*) Western blotting analysis of the different cytosolic fractions. 48 μg bovine brain cytosol (lane *1*), 5 μg CHO Golgi (lane 2), 60 μg fraction Iα (lane *3*), 60 μg fraction Iβ (lane *4*), 0.5 μg p115 (lane *5*), 0.5 μg recombinant mARF (Weiss et al., 1989; Duronio et al., 1990) (lane *6*), and 1 μg purified coatomer (Waters et al., 1992a) (lane *7*) were fractionated on SDS-PAGE using 10% gel under reducing conditions. Coatomer and ARF were detected by Western blotting as described in Materials and Methods.

and because coated vesicles (and derived uncoated vesicles) are the only products of budding observed in the cell-free system (Orci et al., 1993b), the simplest interpretation of this surprising result is that the assay signal in the presence of this coat protein-deficient mixture of cytosol components (NSF, SNAP, p115, Iα, Iβ; hereafter termed "presumed fusion components") is due to a direct fusion between the donor and acceptor Golgi membranes, not involving transport vesicles.

When coatomer and myristylated ARF1 (mARF1) protein are added back with the presumed fusion components, the assay signal is inhibited at least 70% (Fig. 2 a, ●). Inhibition of the presumed direct fusion reaction requires both coatomer and mARF1 (Fig. 2 a, □) and ARF must be myristylated (Fig. 2 a, ○). These conditions inhibiting presumed direct fusion are precisely those that allow coated vesicles to form (Orci et al., 1993b), and indeed coated vesicles and coated buds accumulate (Fig. 2 c) under the same conditions in which the presumed direct fusion reaction is blocked. When Golgi are incubated with presumed fusion components and mARF1 in the absence of coatomer, conditions in which presumed direct fusion can proceed (Fig. 2 a), electron microscopy (Orci et al., 1993a,b) reveals that there are (per μm² of Golgi area) 2.8 ± 0.6 uncoated vesicles, 3.0 ± 0.9 coated vesicles, and 3.0 ± 0.7 coated buds. However, when coatomer (2 μg) is also added, inhibiting presumed direct fusion to 30% of the maximal value (Fig. 2 a), now there are 2.0 ± 0.4 uncoated vesicles, 10.1 ± 1.0 coated vesicles, and 11.4 ± 1.8 coated buds (10 Golgi areas examined in each case). The limited production of coated vesicles in the absence of added coatomer may reflect trace levels endogenous to the fractions used.

Consistent with a causal connection between the assembly of coated buds and coated vesicles and the inhibition of the presumed direct fusion, adding GTPγS, which further accumulates coated vesicles (Melançon et al., 1987), potentiates the coatomer and mARF1-dependent inhibition of presumed direct fusion (Fig. 2 a, ■). Furthermore, BFA, a drug that blocks the assembly of coated vesicles (Orci et al., 1991) by inhibiting GTP–GDP exchange (Donaldson et al., 1992b; Helms and Rothman, 1992), thereby preventing the binding of ARF and coatomer to Golgi membranes (Donaldson et al., 1992a; Palmer et al., 1993), prevents the inhibition of the presumed direct fusion reaction by coatomer and mARF1 (Fig. 2 a, △).

Whereas coatomer and mARF1 inhibit the assay signal produced by the presumed fusion components, the coat proteins have no inhibitory effect upon the signal produced by crude cytosol (Fig. 2 b). This implies that crude cytosol provides an additional factor (which we term RF, for "releasing factor") that is not needed for the presumed direct fusion reaction (and was thus removed during the fractionation to yield the minimum set of components needed for an assay signal), but which is needed for the assay to operate in the presence of coatomer and mARF1. The postulated releasing factor has been identified in fractions of crude cytosol on the basis of its ability to allow an assay signal in the presence of the presumed fusion components, coatomer, and mARF1. Fractionation on a Superose 6 column leads to clear resolution and partial purification of RF activity (Fig. 3 a) eluting at approximately 14,000 native molecular weight. This RF fraction does not contain coatomer, NSF, p115, ARF, α-SNAP,
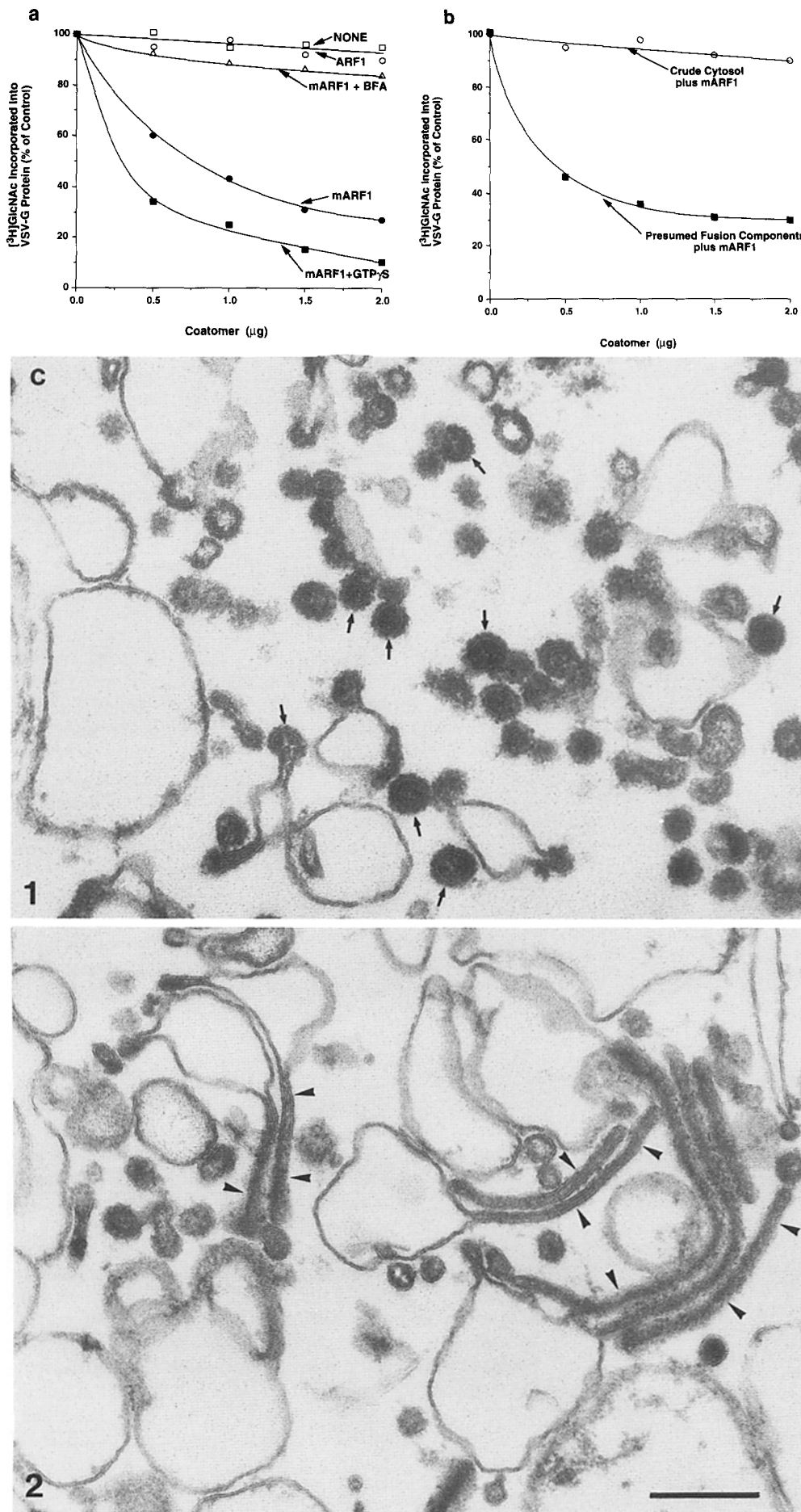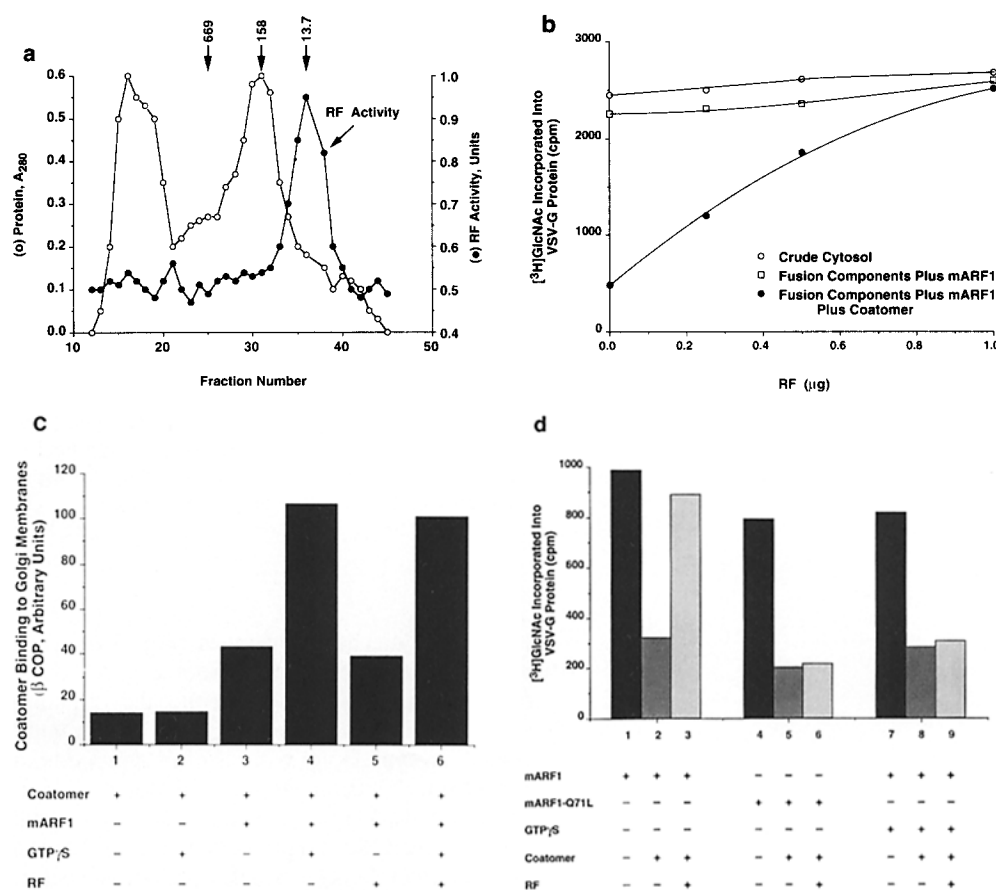
Figure 2. (*a*) Coatomer and mARF inhibit the assay signal reconstituted with presumed fusion components. Purified coatomer was titrated into 50 μl reactions containing 2.5 μg each donor and acceptor Golgi, and presumed fusion components (10 ng His$_6$-NSF, 60 ng His$_6$-α-SNAP, 0.5 μg p115, and 60 μg fraction I α+β) (□). 0.5 μg ARF1 (○), 0.5 μg mARF1 (●), 0.5 μg mARF1 plus 10 μM GTPγS (■), 0.5 μg mARF1 plus 150 μM brefeldin A (△) were added in addition to the presumed fusion components. Incorporation of [³H]GlcNAc into VSV-G protein was determined after 1 h of incubation at 37°C. 100% corresponds to 1950 cpm (□), 2350 cpm (○), 2250 cpm (●), 1,820 cpm (△), and 2085 cpm (■). (*b*) Coatomer and mARF do not inhibit transport reconstituted with crude bovine brain cytosol. Golgi membranes were either incubated with presumed fusion components (as in *a*) plus 0.5 μg mARF1 (■) or with 80 μg bovine brain cytosol plus 0.5 μg mARF1 (○) and the indicated amount of coatomer. 100% corresponds to 2,650 cpm (■) and 4,230 cpm (○). (*c*) Electron microscopy of Golgi fractions. 100 μl Golgi membranes were incubated (in final volume of 1 ml) with presumed fusion components (as in *a*) and 5 μg mARF in the presence (*1*) or the absence (*2*) of 20 μg coatomer for 20 min at 37°C. The membranes were then pellet, fixed and processed as described (Orci et al., 1986, 1991). (*1*) Presumed fusion components, mARF and coatomer. Note the accumulation of non clathrin-coated buds and vesicles (*arrows*). (*2*) Same as *1* but coatomer was omitted. Note the predominance of flat cisternal elements (*arrowheads*). Bars: (*C*, 1) 0.24 μm; (*C*, 2) 0.26 μm.

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β-SNAP, or γ-SNAP, as judged by Western blotting, nor does it yield a signal when assayed alone with Golgi membranes at concentrations that can reverse coatomer and mARF inhibition (data not shown). Moreover, while adding RF to presumed direct fusion reactions that are blocked by coatomer and mARF1 restores the assay signal, RF has no effect on transport assays conducted with crude cytosol (Fig. 3 b).

Given that assembly of coatomer with mARF1 into coats on Golgi membranes blocks the presumed direct fusion reaction, RF could in principle allow an assay signal either by preventing coat assembly in the first place, or by allowing already assembled coated vesicles to be productively consumed (in the simplest view by helping to remove the coat after assembly/budding). We can eliminate the first possibility because RF does not prevent coat assembly: mARF-dependent coatomer binding to Golgi membranes (a direct measure of coated vesicle assembly) (Orci et al., 1993a,b; Palmer et al., 1993) is not affected by RF under the same conditions in which RF restores the assay signal (Fig. 3 c). That RF acts after coat assembly is confirmed by the finding

that two conditions that prevent uncoating of coated vesicles (use of GTPγS [Melançon et al., 1987] or a mutant of ARF-Q71L [Tanigawa et al., 1993] that binds but does not hydrolyze GTP) permit coatomer and mARF1-dependent inhibition of the presumed fusion reaction, but do not allow RF to relieve this inhibition (Fig. 3 d).

To summarize, the simplest interpretation is that in the absence of coatomer and ARF, Golgi membranes can fuse directly with each other in a reaction uncoupled from vesicle budding that nonetheless uses the same set of cytosolic fusion components (NSF, SNAP, p115, Iα, Iβ) as do transport vesicles. When coatomer and ARF are added back, this direct fusion reaction is blocked by a mechanism requiring coat assembly. However, despite the presence of fusion components, the accumulated transport vesicles (containing VSV-G protein) cannot fuse with the GlcNAc transferase-containing acceptor Golgi due to the lack of a final cytosolic factor (RF) which is not needed for membrane fusion per se, but is needed to make the vesicles encased in coats available. Thus, adding back RF restores the assay signal as accumulated coated vesicles (and perhaps those produced in later

Figure 3. (a) Chromatography of bovine brain cytosol on Superose 6. 0.3 ml of bovine brain cytosol (16 mg/ml) were loaded on a 24 ml Superose 6 (HQ 10/30, Pharmacia Fine Chemicals, Piscataway, NJ) column equilibrated with 25 mM Tris-HCl, pH 7.4, 150 mM KCl, 1 mM DTT, and 10% glycerol. 0.5-ml fractions were collected and 10 μl samples were added to transport assays (as described in Fig. 2) in the presence or the absence of 1 μg coatomer. A unit of RF activity is defined as the ratio of cpm incorporated in the presence of coatomer to that incorporated in its absence (●). Arrows indicate the elution position (OD$_{280}$) of molecular weight markers of 669, 158, and 13.7 kD. (b) Cytosolic releasing factor (RF) reverses the inhibition by coatomer and mARF1. Fractions 36–38 eluted from the Superose 6 column were pooled and titrated into 50-μl transport reactions (as described in Fig. 2) in the presence of 80 μg bovine brain cytosol (○), pre-

sumed fusion factors and 0.5 μg mARF1 (□), or the same plus 1 μg coatomer (●). (c) Lack of effect of RF on mARF dependent coatomer binding. Golgi membranes were incubated with presumed fusion components (Fig. 2) and with 2 μg coatomer for 10 min at 37°C. Myristylated ARF1 (0.5 μg) (lane 3–6), RF (1 μg) (lanes 5 and 6) and 20 μM GTPγS (lines 2, 4, and 6) were included in the binding reaction when indicated. Golgi membranes were then pelleted through a 15% (wt/wt) sucrose cushion, proteins were fractionated on 10% SDS-PAGE under reducing conditions, and coatomer was detected by Western blotting using the M3A5 antibody (directed against β-COP). (d) RF does not prevent coat assembly. RF was assayed (50 μl final volume) in the presence of: mARF1 (0.5 μg) (lanes 1–3), mARF1-Q71L (0.5 μg) (Glu was changed to Leu at codon 71 in human recombinant ARF1 by site directed mutagenesis to reduce GTP hydrolysis) (Tanigawa et al., 1993) (lanes 4–6) or mARF1 and 20 μM GTPγS (lanes 7–9). Transport activity was measured without coatomer (lanes 1, 4, and 7), with coatomer (2 μg) (lanes 2, 5, and 8), or in the presence of coatomer and RF (1 μg) (lanes 3, 6, and 9).

rounds) can now access the fusion pathway. Now the assay signal (like that with crude cytosol) is due to transport by coated vesicles, rather than to direct (uncoupled) fusion of Golgi membranes (as in the absence of the coat proteins).

Distinguishing Vesicular Transport from Uncoupled Fusion

This interpretation has been confirmed by two direct and independent tests. In the first method, which is empirical, we take advantage of the fact that the only mechanism by which GTPγS can block the cell-free assay is by trapping mARF in its GTP-bound form, preventing uncoating of coated vesicles (Tanigawa et al., 1993). Therefore, inhibition of the assay signal by GTPγS is the sine qua non of a reaction using coated vesicles. The assay signal produced by Golgi membranes in the presence of crude cytosol is profoundly inhibited by GTPγS (Fig. 4, ■), while that produced by the presumed fusion components is not inhibited by GTPγS (Fig. 4, ●). The signal restored by RF when uncoupled fusion is prevented by coatomer and mARF1 (Fig. 4, o) is as inhibited by GTPγS as is the signal produced by crude cytosol, diagnostic of the use of coated transport vesicles. As would be predicted, further adding BFA to prevent coated vesicles from assembling makes the signal resulting from fusion components, mARF, coatomer, and RF resistant to GTPγS (□); now, even though coat proteins are present, they cannot assemble into coats.

The second test is based on first principles (Fig. 5), using a competition experiment that distinguishes direct fusion from vesicular transport. For this purpose, we added a third Golgi population (from uninfected CHO clone 15B cells) to otherwise standard transport reactions. These 15B Golgi contribute neither VSV-G protein (being from uninfected cells) nor GlcNAc Transferase I (being from mutant cells), but can act as a "silent acceptor" of transport vesicles. Thus,
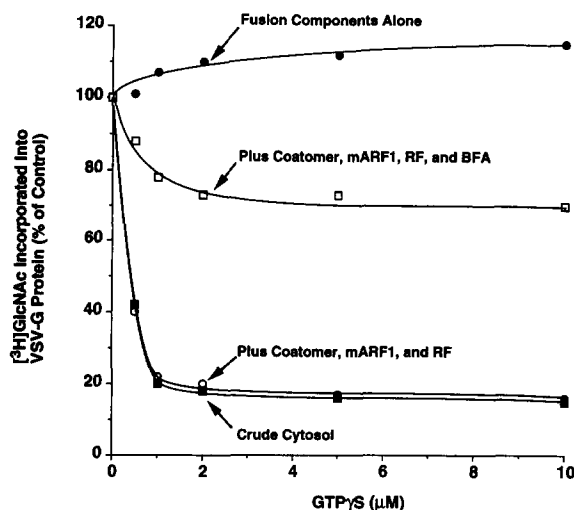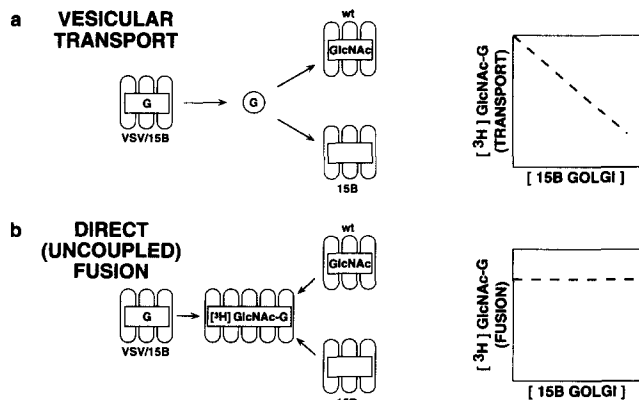

Figure 5. Competition of the in vitro assay signal by uninfected CHO 15B Golgi. (*a*) The assay signal resulting from vesicular transport is competed by uninfected CHO 15B Golgi. A diffusible intermediate (vesicle) that is involved in the transport of VSV-G protein in vitro will have a similar probability to dock to CHO wild-type and 15B Golgi. Thus, increasing concentrations of Golgi membranes derived from CHO mutant 15B will result in a decrease in VSV-G protein that is processed by the *N*-acetylglucosamine transferase in wild type Golgi. To calculate the predicted inhibition curve we have used the following equation:

$$\frac{S}{S_{50}} = 2\left(1 - \frac{15B}{WT + 15B}\right)$$

where S = predicted assay signal, S_{50} = signal measured with 1:1 protein weight ratio of donor and acceptor Golgi; $15B$ = amount (μg protein) of total 15B CHO Golgi present (donor and uninfected 15B Golgi); and WT = amount (μg protein) of wild-type CHO Golgi. (*b*) An assay signal resulting from direct fusion of Golgi cisternae is not competed by uninfected CHO 15B Golgi. Direct fusion between the Golgi cisternae could in principle result in glycosylation of VSV-G protein, but this signal would not be inhibited by uninfected 15B. Increasing concentrations of uninfected 15B would only dilute the concentrations of VSV-G protein and *N*-acetylglucosamine transferase in mixed fused Golgi membranes but would not affect the extent of glycosylation of G protein (e.g., S/S_{50} = 1). These models assume that there is a single round of vesicular transport in the cell free system, an assumption for which there is considerable evidence (for example see Rothman et al., 1984; and Orci et al., 1988).

Figure 4. GTPγS inhibition of Golgi protein transport. Increasing concentrations of GTPγS were added to assays (50 μl) reconstituted with 80 μg bovine brain cytosol (■), presumed fusion components (●), presumed fusion components plus 0.5 μg mARF1, 2 μg coatomer and 5 μg RF (o) or the same plus 150 μM brefeldin A (□). 100% corresponds to 4,507 cpm (■), 2,251 cpm (●), 1,851 cpm (o), and 3,016 cpm (□).

if a vesicle transport mechanism applies (Fig. 5 *a*), each VSV-G–containing vesicle emanating from a donor stack will have to choose, on a random basis, either the 15B competitor Golgi or the wild-type acceptor Golgi, but not both. Fusion with the wild-type Golgi will result in glycosylation and thus an assay signal; fusion with the 15B Golgi will not. As the proportion of 15B Golgi increases, the assay signal will monotonically decrease in a predictable fashion (as can be calculated from the relative amounts of donor, acceptor, and competitor Golgi present; the donor VSV-15B Golgi must be taken into account as they also lack GlcNAc Transferase I and therefore contribute to the silent acceptor population. See legend to Fig. 5 for details of calculation). However, should the donor and acceptor Golgi fuse directly together to generate the assay signal (Fig. 5 *b*), then the 15B Golgi will not compete, because the G protein (from the VSV-15B Golgi) and GlcNAc Transferase (from wild type CHO Golgi) will meet irrespective of whether 15B Golgi

join in, and because the 15B Golgi contribute neither the sugar transferase nor G protein. Of course, as additional neutral (15B) Golgi membrane is added, the rate of glycosylation may eventually slow, but in practice this effect has not been observed in the range of 15B membrane concentrations we have used, the extent of inhibition of the assay signal by 15B membranes is independent of the time of incubation beyond 1.5 h (not shown).

To provide confirmation of the validity of the method, we apply it to the assay signal resulting from crude cytosol, which much earlier evidence implies is due to coated transport vesicles (Orci et al., 1989; Rothman and Orci, 1992), and to a situation in which Golgi stacks are already known to fuse, e.g., in the presence of BFA (Orci et al., 1991). (In addition to preventing coated vesicles from assembling, adding BFA to cell-free incubations of Golgi membranes and crude cytosol results in extensive fusion of cisternae within a stack and between stacks [Orci et al., 1991].) When 15B Golgi are added to incubations of Golgi with crude cytosol, the assay signal is progressively reduced (Fig. 6 a, ●), in reasonable agreement with the line predicted for random choice competition for transport vesicles (dashed line in Fig. 6 a). However, when BFA is added under conditions known to result in extensive fusion, little competition resulted (o); a residual competition could be due to incomplete blockade of coat assembly by BFA (Orci et al., 1991). We also used an engineered mutant of ARF (expressed and N-myristylated in E. coli) in which residue 31 is changed from Thr to Asn, prepared in analogy to a dominant Ras mutant (S17N) that interferes with nucleotide exchange of wild-type Ras (Fransworth and Feig, 1991) to mimic the effect of BFA. Adding mARF1-T31N is equivalent to adding BFA in the competition assay (Fig. 6 a, □), and like BFA results in an assay signal that is resistant to GTPγS (data not shown).

The signal presumed to be due to direct fusion when Golgi membranes are incubated with NSF, SNAP, Iα, and Iβ in the absence of coatomer and ARF can now be confirmed as such, as 15B Golgi do not reduce this signal (Fig 6 b, □). As expected, this direct fusion persists when mARF1 is added without coatomer (Fig. 6, o), but when coatomer and mARF1 are added together with RF, as expected the restored signal is due to vesicular transport (Fig. 6 b, ●), as was the case with crude cytosol (Fig. 6 a, ●). Examination by electron microscopy (not shown) of membrane pellets from reactions exhibiting direct fusion does not reveal evidence for the kind of extensive membrane fusion observed with BFA (Orci et al., 1991), in which numerous Golgi stacks and cisternae became fused into a single network. Fusion of individual stacks of cisternae would be difficult to detect by this crude method.

Discussion

The expected requirement of cell-free vesicular transport for coatomer and ARF proved difficult to elucidate in a straightforward manner, because of the dual role these coat components play in driving vesicle budding and also in coupling fusion to budding. When either coatomer or ARF is removed, or when present but prevented from assembling onto membranes (e.g., when BFA is added), a process of uncoupled fusion is unleashed that creates an assay signal that mimics the vesicular transport signal in quantity but not in its under-

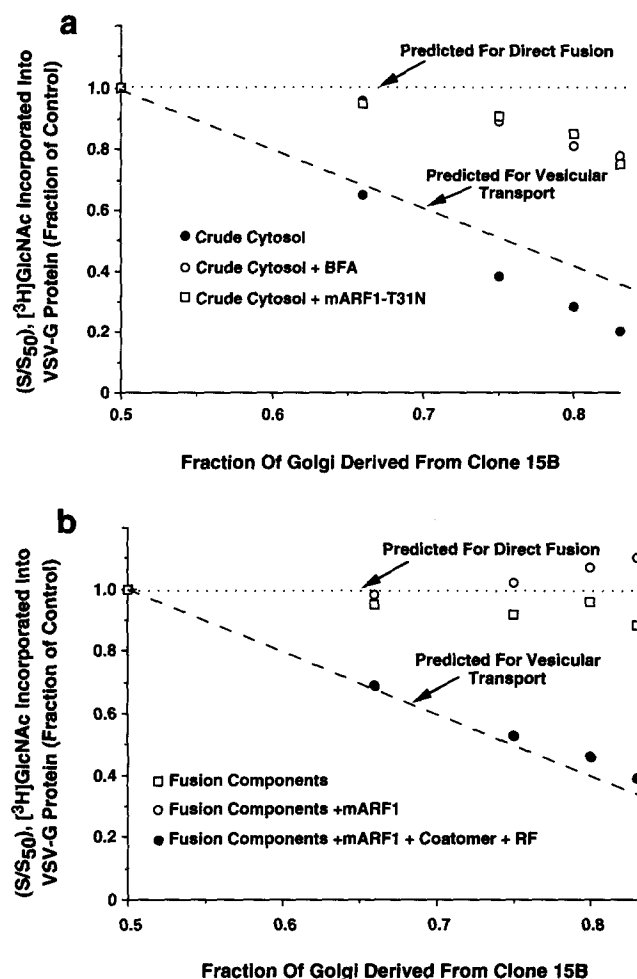Fraction Of Golgi Derived From Clone 15B

Figure 6. (a) Brefeldin A and mARF1-T31N induce direct fusion between Golgi cisternae. The ratio of 15B Golgi (from donor plus uninfected 15B) to wild-type Golgi plus 15B derived Golgi was varied from 50 to 83% by diluting an assay reaction that had a standard 1:1 (wt/wt) mixture of donor and acceptor membranes with increasing amounts of a correspondingly prepared reaction containing only uninfected 15B Golgi (100 μg/ml). All reactions contained 1.6 mg/ml bovine brain cytosol with no addition (●), or with 150 μM brefeldin A (o) or with 72 μg/ml mARF1-T31N (□). (The ARF1 mutant was constructed by site-directed mutagenesis [Thr to Asn at codon 31], expressed in the pET11d vector, and produced in E. coli that coexpressed N-myristoyltransferase.) Reactions were incubated for 90 min at 37°C, and [3H]GlcNAc incorporated into VSV-G protein was determined (S) and expressed as fraction of the value (100%) observed with the standard reaction (S50). The results for the standard assay (1:1 VSV-15B donor to wt acceptor), or S50 to which all other data normalized are: 4239 cpm (bovine brain cytosol), 3240 cpm (cytosol plus BFA) and 5,200 cpm (cytosol plus mARF1-T31N). The dashed lines represent the predicted results for vesicles transport and direct fusion. (b) Coatomer and mARF1 couple fusion to vesicle budding. A competition experiment was performed as described above. Assays (performed as in a) contain presumed fusion components (□), the same plus 10 μg/ml mARF1 (o), or plus 10 μg/ml mARF1, 40 μg/ml coatomer, and 50 μg/ml RF (●). S50 are 2,252 cpm (□), 2,650 cpm (o), and 3,888 cpm (●).

lying nature (Figs. 4 and 6). As direct fusion predominates only when certain components (ARF and coatomer) that are normally present in cells are removed, (or when a drug, BFA is added), this event is properly regarded as an uncoupled partial reaction of the transport pathway.

It is often the case in biochemistry that removing a component from a closely coupled system results in uncoupled, partial reactions. A classic example is the coupling of ATP synthesis to electron transport resulting in oxidative phosphorylation by mitochondrial membranes (Racker, 1976). When a key component (the F1 ATPase) is removed (or if certain uncoupling drugs are added), ATP synthesis stops but respiration continues, now uncoupled from phosphorylation. Monitoring the wrong partial reaction (e.g., oxygen consumption) would lead to the erroneous conclusion that F1 is unimportant. (Our experience is also a nice illustration of Racker's "TAGFY" view, discussed in lecture 1 in his book [Racker, 1976]).

In the case of reconstituted transport, exclusively monitoring processing of the cargo protein can now be expected to lead to false negatives regarding the requirements for coatomer and ARF because this signal will persist in an uncoupled fusion reaction. In light of our new findings, in retrospect it would have been wiser to have defined the GTPγS-sensitive component (Melançon et al., 1987) of VSV-G protein processing to be the authentic signal in the cell-free system. As a result, cytosol fractions providing ARF, coatomer, and RF would have been retained during the systematic fractionation to yield the minimum set of required components and no doubt these components would have been discovered in this context. Our original finding that inhibition by GTPγS was most pronounced as cytosol concentration is increased (Melançon et al., 1987) can now be understood to mean that fusion becomes uncoupled as cytosol concentration is lowered because coat proteins are limiting in crude cytosol. Because the cytosol and Golgi concentrations used in cell-free assays are ~200-fold lower and 10-fold lower than in a cell, respectively, the ratio of cytosol to Golgi is ~20-fold lower in vitro than in cells. Thus, increasing the concentration of cytosol in the cell-free system is in the physiological direction; lowering the concentration is nonphysiological. The report that reducing ARF concentration in cytosol does not affect the Golgi assay signal (Taylor et al., 1992) is now easily explained, and the inhibition of in vitro nuclear envelope fusion by mARF1 can now be rationalized by assembly of coats on these membranes (Boman et al., 1992).

NSF (Block et al., 1988; Malhotra et al., 1989), SNAP (Clary et al., 1990), and p115 (Waters et al., 1992b) are all required for vesicular transport in crude cytosol and for uncoupled transport. Because uncoupled fusion uses the same cytosolic fusion machinery as coupled (vesicle) fusion, the simplest possibility is that in the case of uncoupled fusion the donor and acceptor membranes now pair directly, without involving an intervening vesicle. In terms of the SNARE hypothesis (Söllner et al., 1993) for vesicle targeting, this direct pairing model would mean that the same v-SNARE that would normally first be packaged into a transport vesicle and only then be able to interact with a t-SNARE in the target membrane would now interact while still in the donor membrane. We envision that as soon as a free v-SNARE or other donor membrane-derived fusion protein appears in a donor

membrane, it rapidly assembles itself into coated buds, and is thereby sequestered and prevented from productively engaging t-SNAREs until the coat is removed when ARF's bound GTP is hydrolyzed (Tanigawa et al., 1993) after budding is finished, thereby assuring that fusion follows budding. When coat assembly is prevented, free v-SNAREs would accumulate at the donor membrane, with nothing to prevent them from pairing directly with t-SNAREs at the target membrane and then using the general cytosolic machinery to trigger fusion. In the absence of RF, and the presence of excess coat proteins, coated vesicles containing the full supply of v-SNAREs would accumulate, preventing uncoupled fusion, but the accumulated coated vesicles could not be consumed. When these coats cannot be removed (as when GTPγS, or the ARF GTPase mutant is used), even RF cannot enable these vesicles to engage in fusion. This mechanism can easily explain why in earlier work (Orci et al., 1991) preincubation of Golgi membranes with GTPγS and cytosol prevented the BFA-induced fusion of Golgi membranes. To be efficiently packaged into coated vesicles, v-SNAREs (and other membrane proteins) need only bind to ARF or to the postulated (Helms et al., 1993) ARF receptor.

With our new results, a straightforward explanation of the two principal effects of BFA on whole cells (Klausner et al., 1992) and the cell-free system (Orci et al., 1991) seems at hand. These are a block in protein secretion, and fusion of Golgi cisternae to each other and to the ER, a process termed "retrograde transport" (Lippincott et al., 1989). The block to secretion is explained by the block of COP-coated vesicle assembly secondary to the inhibition of nucleotide exchange of ARF (Donaldson et al., 1992b; Helms and Rothman, 1992). We now propose that the phenomenon of "retrograde transport" is due to uncoupled fusion between successive pairs of donor and acceptor compartments along the secretory pathway that are normally connected by COP-coated transport vesicle shuttles (involving only those compartments housing BFA-sensitive ARF nucleotide exchange factors). In terms of the SNARE hypothesis, perhaps v-SNAREs in the ER would now pair directly with t-SNAREs in the *cis*-Golgi, those in *cis* with those in *medial*, etc., until all of these compartments have collapsed into the ER. Specificity must be inherent in this massive fusion event, for while ER and Golgi membranes are engaged, nearby late endosomes and TGN are typically not involved (Chege and Pfeffer, 1990; Hunziker et al., 1991; Lippincott et al., 1991; Wood et al., 1991). Because the ER is further away from Golgi cisternae than the cisternae are from each other, fusion of ER with *cis* Golgi should be the slowest step, and hence the one most easily visualized by microscopy (Lippincott et al., 1990). Portions of the Golgi membrane can take the shape of tubules before this fusion reaction, perhaps because microtubule motor binding sites normally sequestered in coats are exposed (Pelham, 1991b). However, neither microtubules nor the membrane tubules are necessary for the ER–Golgi fusion process, as disassembling microtubules abolishes membrane tubules and slows the rate but does not affect the extent of ER–Golgi fusion (Lippincott et al., 1990).

An additional kind of retrograde transport from Golgi to ER surely exists to recycle key membrane proteins, and has been directly demonstrated in the case of KDEL receptors that retrieve escaped ER resident proteins (Pelham, 1991a)

and certain ER membrane proteins (Nilsson et al., 1993). It seems doubtful that any low level of uncoupled fusion that might occur physiologically (without BFA) could be relevant to selective retrograde movements like KDEL and KKXX-dependent retrieval, which are by definition signal-dependent; direct fusion of ER and proximal Golgi would have to be unselective, returning an entire cisterna at a time. However, it cannot be excluded that uncoupled fusion could contribute a basal mechanism for recycling Golgi membrane for re-sorting or repair, and this process could well explain the occasional continuities among ER and Golgi cisternal membranes that have been noted (Cooper et al., 1990; Rambourg and Clermont, 1990). The remarkable fact that the intermixing of ER and Golgi triggered by BFA is fully reversible (Lippincott et al., 1989) reveals that low levels of uncoupled fusion can no doubt be tolerated by cells even if this process is not gainfully employed, allowing a relatively imprecise coupling mechanism to suffice.

The cytosol requirement for vesicular transport seems to be fulfilled by a relatively small number of components. Coatomer and N-myristylated ARF are required for vesicle budding, RF is needed in a process following budding (perhaps to help trigger uncoating, since locking coats in place with GTPγS or the ARF mutant prevents RF action) and only NSF, SNAP, p115, Iα, and Iβ are required for membrane fusion. With the recent discovery of the SNAP receptors in both vesicle and target membranes (Sollner et al., 1993), a clear outline of the machinery and mechanisms of intracellular transport is beginning to emerge.

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