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ADP-Ribosylation Factor-1 Is Sensitive to *N*-Ethylmaleimide[1]

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The treatment of normal rat kidney cells with *N*-ethylmaleimide caused the release of β-COP, a component of coatomer, from the Golgi apparatus without causing disassembly of the organelle. The release of β-COP, which was not due to depolymerization of microtubules, was markedly blocked by the activation of GTP-binding proteins by aluminum fluoride or a nonhydrolyzable analogue of GTP. To determine which component is *N*-ethylmaleimide-sensitive, we reconstituted the recruitment of coatomer from the bovine brain cytosol onto the Golgi apparatus in digitonin-permeabilized cells. In cells treated with *N*-ethylmaleimide before permeabilization, β-COP was still recruited onto the Golgi apparatus. In contrast, β-COP was not recruited when *N*-ethylmaleimide-treated bovine brain cytosol was used. These results suggest that the *N*-ethylmaleimide-sensitive factor(s) are present in the cytosol. It is known that coatomer and ADP-ribosylation factor-1 (ARF1) are the only cytoplasmic proteins needed for the assembly of Golgi-derived coated vesicles. *N*-Ethylmaleimide treatment of a coatomer-rich fraction did not affect the binding of β-COP to the Golgi apparatus, whereas the same treatment of an ARF-rich fraction abolished β-COP binding. Similar results were obtained using purified recombinant ARF1. Concomitant with inactivation, 0.85 mol of *N*-ethylmaleimide was incorporated into 1 mol of ARF1. ARF1 contains only one cysteine residue (Cys-159), which is located near the base moiety of the bound guanine nucleotide.

Key words: ADP-ribosylation factor, coat protein, Golgi apparatus, *N*-ethylmaleimide.

In eukaryotic cells, the Golgi apparatus, consisting of ordered stacks of cisternae, is located in the perinuclear region *via* association with the microtubule organizing center, and plays important roles in the sorting and transport of newly synthesized proteins (*1, 2*). COPI-coated vesicles mediate protein transport in both the anterograde direction through the Golgi stack and the retrograde direction from the Golgi apparatus to the endoplasmic reticulum (*3, 4*). COPI consists of coatomer, comprising 7 subunits, and a small GTP-binding protein, ARF (*5-7*). These cytosolic proteins are necessary and sufficient for the formation of COPI-coated vesicles (*8*). ARF is recruited onto Golgi membranes in the presence of a nonhydrolyzable GTP analogue, GTPγS, *in vitro* (*6*). The recruitment of ARF onto Golgi membranes *in vivo* occurs as a consequence of the replacement of bound GDP with GTP in ARF, which is mediated by ARF-specific guanine nucleotide exchange factor (*9, 10*). AlF_4^- also promotes the recruitment of ARF onto Golgi membranes by activating heterotrimeric G proteins (*11, 12*) or directly inhibiting the GTPase activity of the ARF associated with ARF GAP (*13*). ARF is a prerequisite for the binding of coatomer to Golgi membranes (*14, 15*), although its role is a matter of controversy (*6, 13, 16*).

Previously, we showed that NDGA renders heterotrimeric G proteins inactive, thereby causing disassembly of the Golgi apparatus in NRK cells (*17*). During the course of that study, we found that the treatment of NRK cells with 1 mM NEM blocks the NDGA-induced Golgi disassembly and promotes the release of β-COP, a component of coatomer, from the Golgi apparatus. In the present study, we examined which component is sensitive to NEM among the proteins involved in the association of COPI with Golgi membranes. We found that the treatment of ARF with NEM results in loss of its ability to bind to Golgi membranes.

EXPERIMENTAL PROCEDURES

Materials—NDGA was purchased from Biomol Research Laboratories and freshly dissolved in dimethyl sulfoxide

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Abbreviations: ARF, ADP-ribosylation factor; BFA, brefeldin A; COP, coat protein; DTNB, 5,5′-dithiobis (2-nitrobenzoic acid); GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; NEM, *N*-ethylmaleimide; NDGA, nordihydroguaiaretic acid; NSF, *N*-ethylmaleimide sensitive factor; NRK, normal rat kidney.

before use. NEM, $AlCl_3$, NaF, and DTNB were obtained from Wako Chemicals. Monoclonal antibodies against man II (clone 53FC3), α-tubulin (clone DM1A), γ-adaptin, and ARF were purchased from BAbCo, BioMakor, Transduction Laboratories, and Affinity Bioreagents, respectively. A polyclonal antibody against β-COP was raised against a synthetic peptide (residues 496-513 of β-COP). A polyclonal antibody against man II was raised against its lumenal 42-kDa region close to the transmembrane domain. Recombinant myristoylated ARF1 was prepared as described previously (*18*). Rat liver Golgi membranes were isolated as described previously (*19*).

Cell Culture and Immunofluorescence Analysis—NRK cells obtained from the ATCC were grown on glass coverslips in α-minimum essential medium supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum at 37°C in an incubator containing 5% CO_2 and 95% air. Immunofluorescence analysis was performed as described previously (*20*).

Permeabilization and Incubation Conditions—NRK cells were washed twice with KHM buffer [25 mM HEPES/KOH (pH 7.0) containing 110 mM potassium acetate and 2.5 mM magnesium acetate], then permeabilized with 40 μg/ml digitonin in KHM buffer at 0°C for 5 min. After washing with KHM buffer, the cells were incubated with KHMG buffer [25 mM HEPES/KOH (pH 7.0) containing 110 mM potassium acetate, 2.5 mM magnesium acetate, and 1 mg/ml glucose] in the presence of an ATP-regenerating system (5 mM creatine phosphate, 0.25 mM UTP, 0.05 mM ATP, and 12 IU/ml creatine phosphokinase) and bovine brain cytosol (0.8–1 mg/ml).

Preparation of ARF-Rich and Coatomer-Rich Fractions—Bovine brain cytosol was prepared as described previously (*21*). ARF-rich and coatomer-rich fractions were prepared from the bovine brain cytosol as described previously (*14*). Briefly, 3 ml of cytosol was applied to a Sephacryl S-300 HR column in a FPLC system (Pharmacia Biotechnology), then the column was developed at the flow rate of 0.5 ml/min with 25 mM Tris-HCl (pH 8.0) containing 0.1 M KCl. Fractions of 1.25 ml were collected, and the fractions containing ARF or coatomer were examined by immunoblotting. Appropriate fractions were collected, concentrated 5 times, and stored at −80°C until use.

Binding of Coatomer Proteins to Isolated Golgi Membranes—Mixtures containing rat liver Golgi membranes (10 μg), bovine brain cytosol (3 mg/ml protein), and an ATP regenerating system were preincubated at 37°C for 10 min with or without 1 mM NEM in 100 μl of buffer A [25 mM HEPES/KOH (pH 7.0) containing 25 mM KCl and 2.5 mM $MgCl_2$]. GTPγS was added at a final concentration of 100 μM, and the mixtures were further incubated at 37°C for 15 min. They were then layered on top of 200 μl of buffer A containing 0.25 M sucrose and centrifuged at 4°C for 15 min at 15,000 rpm. The membrane pellets were suspended in 12 μl of SDS sample buffer, then subjected to SDS-polyacrylamide electrophoresis. Membrane-bound β-COP was detected by immunoblotting with an ECL system. To remove the probe, the blot was washed in phosphate-buffered saline containing 0.02% Tween 20, then incubated with 62.5 mM Tris-HCl (pH 6.7) containing 2% SDS and 100 mM 2-mercaptoethanol at 50°C for 30 min. It was then incubated with the antibody against man II and developed with an ECL system.

Determination of the Sulfhydryl Content—The amount of sulfhydryl groups was determined using DTNB. The purified recombinant ARF1 in KHM buffer was incubated with or without 1 mM NEM at 32°C for 10 min. To remove excess NEM, the reaction mixture was passed through a spin column (Bio-Rad P6-DG) equilibrated with 0.1 M Tris-HCl (pH 8.0) containing 6 M guanidine-HCl and 0.01 M EDTA. DTNB was added to the flow-through fraction, and the absorbance at 412 nm was measured with a Beckman DU640 spectrophotometer. The molar absorption coefficient at 412 nm of the reaction product of DTNB in 6 M guanidine-HCl was assumed to be 13,380 $M^{-1} \cdot cm^{-1}$ (*22*).

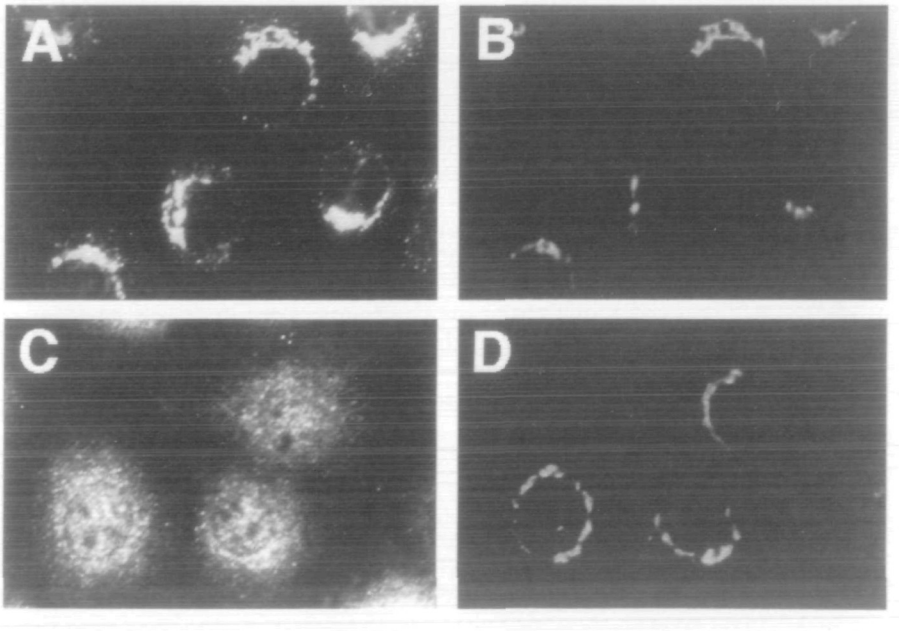

Fig. 1. **NEM causes the release of β-COP from the Golgi apparatus without affecting the Golgi morphology in NRK cells.** NRK cells were incubated without (A and B) or with (C and D) 1 mM NEM at 37°C for 10 min. β-COP (A and C) and a *medial* Golgi-resident protein, man II (B and D), were immunostained.

RESULTS AND DISCUSSION

β-COP is Released from the Golgi Apparatus by NEM-Treatment—In a previous study, we showed that NDGA causes disassembly of the Golgi apparatus by affecting heterotrimeric G proteins (*17*). During the course of that study, we found that the treatment of NRK cells with 1 mM

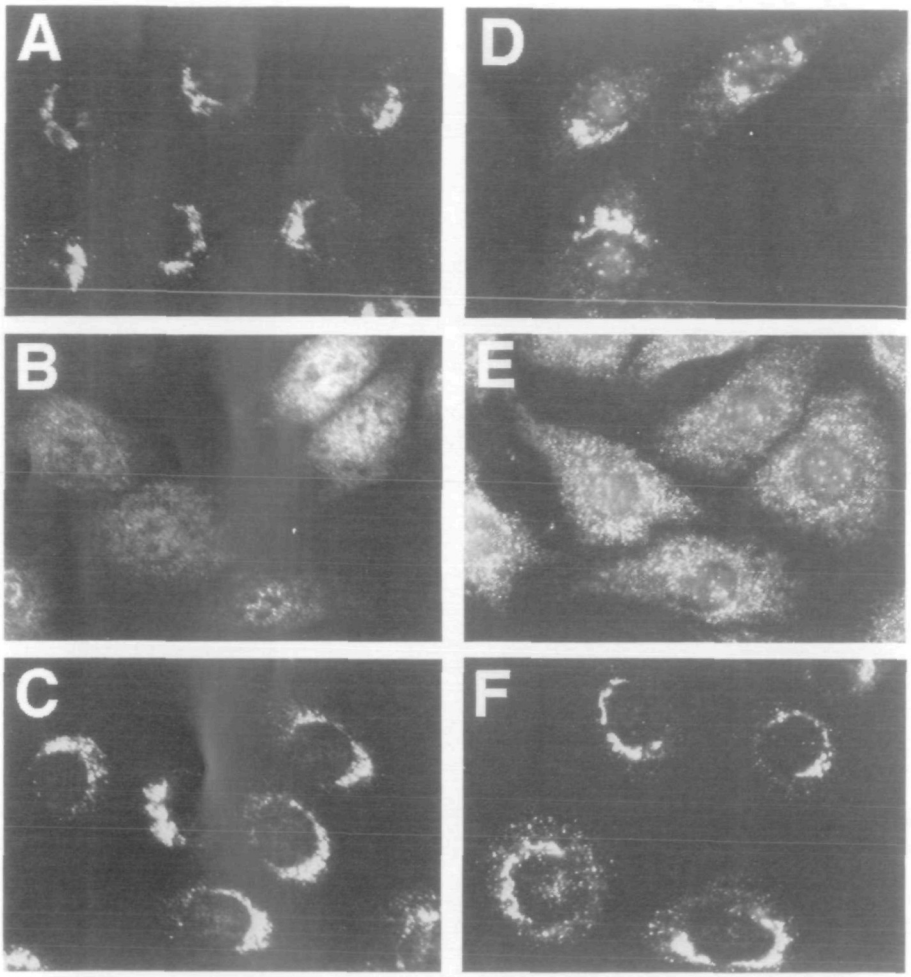

Fig. 2. **Activation of GTP-binding proteins prevents the NEM-induced release of β-COP from the Golgi apparatus.** NRK cells were preincubated in the absence (A and B) or presence of AlF_4^- (30 mM NaF, 50 μM $AlCl_3$) (C) at 37°C for 10 min, then incubated without (A) or with 1 mM NEM (B and C) at 37°C for 10 min. Alternatively, NRK cells were permeabilized with 40 μg/ml digitonin at 0°C for 5 min, then incubated at 32°C for 30 min in the presence of an ATP-regenerating system and bovine brain cytosol without NEM (D), with 1 mM NEM (E), or with 1 mM NEM plus 10 μM GTPγS (F). β-COP was immunostained.

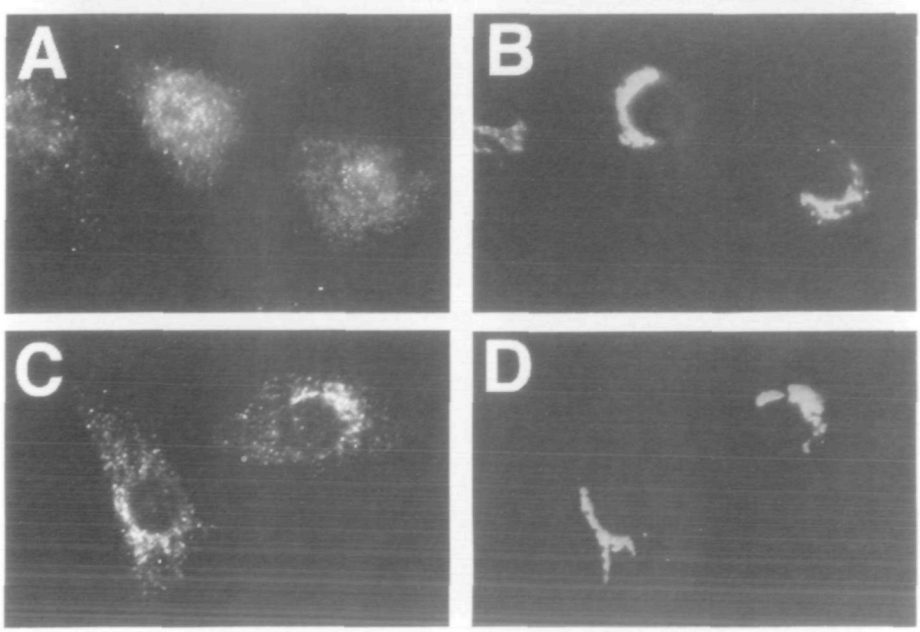

Fig. 3. **Treatment of Golgi membranes with NEM has no effect on the recruitment of β-COP onto the membranes.** NRK cells were preincubated with 1 mM NEM at 37°C for 10 min to promote the release of β-COP, then permeabilized with 40 μg/ml digitonin at 0°C for 5 min (A and B). The cells were then incubated at 32°C for 30 min in the presence of an ATP-regenerating system and bovine brain cytosol (C and D). β-COP (A and C) and man II (B and D) were immunostained.

NEM at 37°C for 10 min blocks NDGA-induced Golgi disassembly (data not shown) and promotes the release of β-COP, a subunit of coatomer, from the Golgi apparatus (Fig. 1C). The release of β-COP started 5 min after the addition of NEM, and once released it did not return to the Golgi apparatus after extensive washing to remove NEM, implying that the effect of NEM is irreversible. The effect of NEM was temperature-dependent. No release of β-COP was observed when cells were incubated with NEM at 1 mM at 0°C for 20 min. β-COP released upon NEM treatment at 37°C was not distributed equally throughout the cytoplasm, but rather in a scattered punctate pattern (Fig. 1C). This may suggest that the released β-COP is associated with membrane structures or accumulates in aggregates. BFA is known to cause the rapid release of β-COP from the Golgi apparatus, which is followed by redistribution of Golgi components into the endoplasmic reticulum (for a review, see Ref. *23*). Although BFA treatment causes β-COP to be distributed equally throughout the cytoplasm in most cells, β-COP accumulates in large cytoplasmic aggregates in several types of cells (*24-26*). The scattered punctate distribution of β-COP observed upon NEM treatment may correspond to β-COP aggregates, but we did not investigate further.

In contrast to β-COP, the localization of man II, a *medial* Golgi-resident protein, was not affected by NEM treatment (Fig. 1D), implying that the reagent does not cause disassembly of the Golgi apparatus. In this regard the effect of NEM is different from that of BFA. However, this difference can be partly explained by the fact that NEM treatment at 37°C causes inactivation of not only proteins

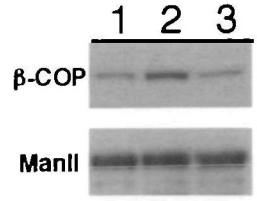

Fig. 5. **NEM blocks the binding of β-COP to isolated Golgi membranes.** Rat liver Golgi membranes were preincubated at 37°C for 10 min in the presence of an ATP-regenerating system and bovine brain cytosol without (lanes 1 and 2) or with (lane 3) 1 mM NEM, then further incubated without (lane 1) or with (lanes 2 and 3) 100 μM GTPγS at 37°C for 15 min. The reaction mixtures were layered on top of buffer containing 0.25 M sucrose and centrifuged. The membrane pellets were suspended in the sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Membrane-bound β-COP was visualized by immunoblotting using anti-β-COP with an ECL system. After detection of β-COP, the blot was washed to remove the probe, then man II was detected using its polyclonal antibody with an ECL system.

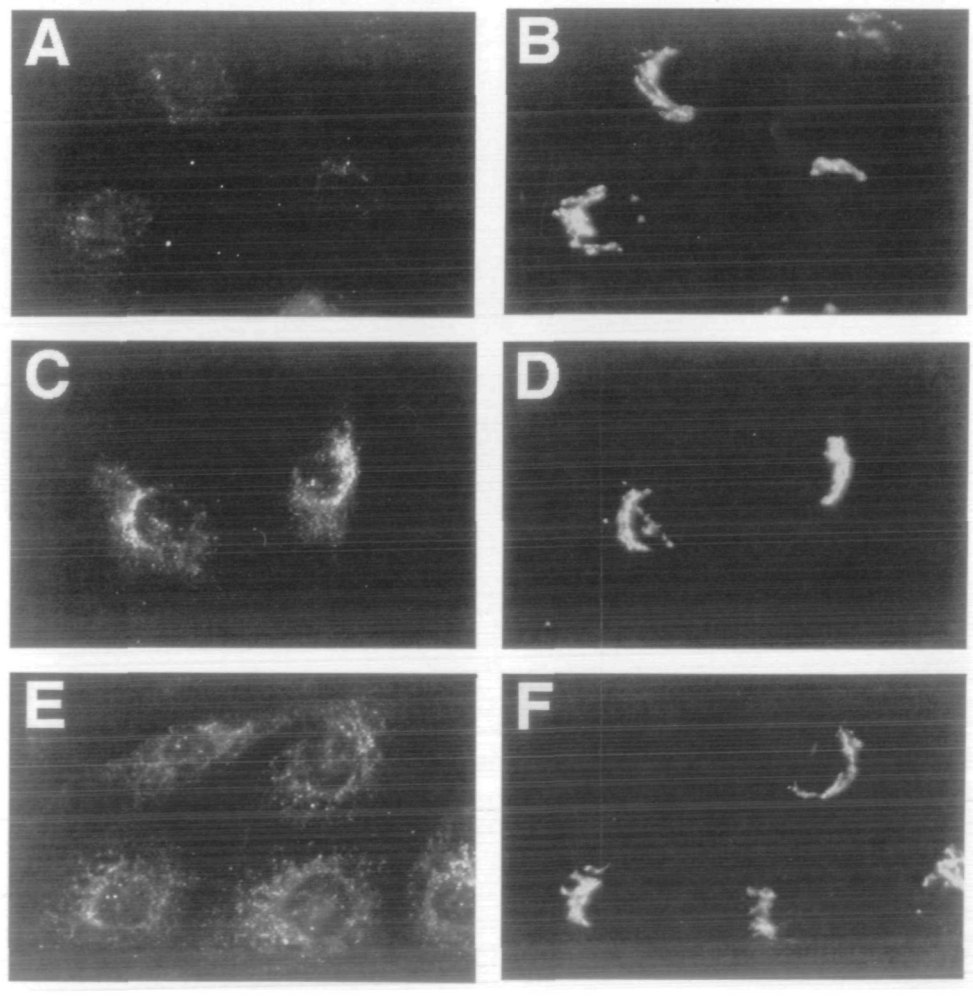

Fig. 4. **Reconstitution of the recruitment of β-COP onto the Golgi apparatus in permeabilized cells.** NRK cells were permeabilized with digitonin and incubated in the absence of an ATP-regenerating system at 32°C for 20 min to allow the release of β-COP (A and B). After washing the cells, incubation was continued at 32°C for 30 min in the presence of an ATP-regenerating system with cytosol (C and D) or NEM-treated cytosol (E and F). NEM-treated cytosol was prepared by the incubation of cytosol with 1 mM NEM for 10 min at 32°C, followed by treatment with 2 mM dithiothreitol to inactivate the excess NEM. The cells were double-labeled for β-COP (A, C, and E) and man II (B, D, and F).

involved in the binding of COPI but also *N*-ethylmaleimide-sensitive factor (NSF), whereas BFA may affect only the former proteins. A recent study showed that NSF activity is required for Golgi disassembly following the release of β-COP in BFA-treated cells (*27*). Treatment of cells with 1 mM NEM at 37°C must inactivate NSF because it is sensitive to 1 mM NEM at 0°C (*28*).

To clarify the mechanism underlying the NEM-induced release of β-COP from the Golgi apparatus, we first examined the effect of taxol on this reaction. β-COP was originally discovered as a protein in the Golgi apparatus that interacts with microtubules (*29*). Since microtubules are sensitive to NEM, β-COP may be released from the Golgi apparatus as a consequence of their depolymerization. To examine this possibility, NRK cells were preincubated with 10 μg/ml taxol for 1 h to stabilize the microtubules, then treated with NEM. Although the microtubules detected with anti-α-tubulin remained intact, β-COP was released by the NEM-treatment as observed for cells without taxol (data not shown).

Activation of Heterotrimeric G Proteins Markedly Prevents the NEM-Induced Release of β-COP from the Golgi Membranes—AlF_4^- activates ARF by modulating heterotrimeric G proteins (*11, 12*) or inhibiting the GTPase activity of the ARF associated with ARF GAP (*13*). If the redistribution of β-COP induced by NEM is due to the inactivation of proteins involved in the binding of COPI, AlF_4^- may affect this redistribution. When NRK cells were preincubated with AlF_4^- at 37°C for 10 min, the release of β-COP was almost completely blocked upon incubation with 1 mM NEM at 37°C for 10 min (Fig. 2C).

To confirm that this blockage occurs *via* GTP-binding proteins, we examined the effect of GTPγS, a nonhydrolyzable analogue of GTP, on the release of β-COP in permeabilized NRK cells. When NRK cells were permeabilized with 40 μg/ml digitonin, then incubated with 1

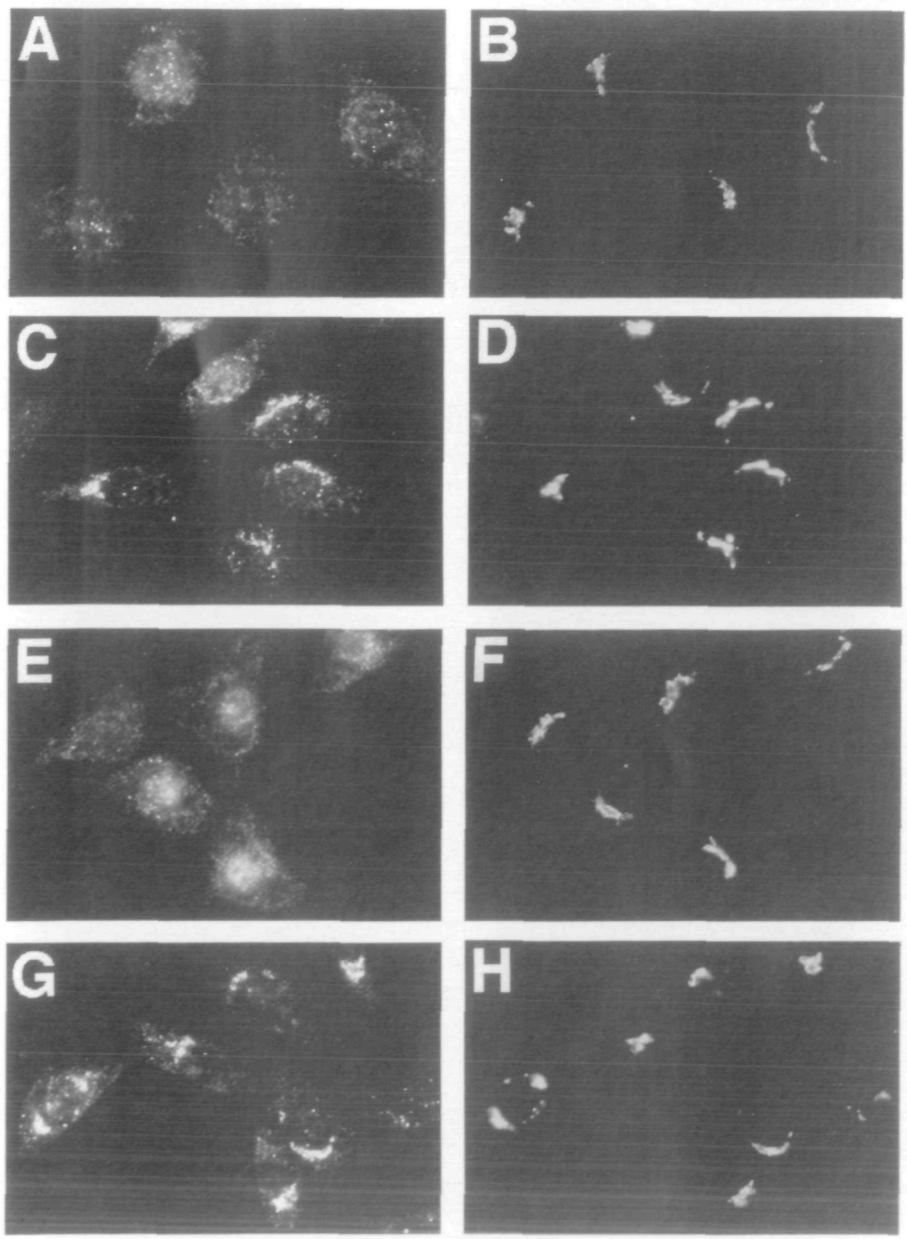

Fig. 6. **NEM-treatment of an ARF-rich fraction inhibits the binding of β-COP to the Golgi apparatus.** NRK cells were permeabilized with digitonin, incubated in the absence of an ATP-regenerating system at 32°C for 20 min (A and B), then incubated at 32°C for 30 min in the presence of 1 μM GTPγS and an ATP-regenerating system with coatomer-rich and ARF-rich fractions (C and D), coatomer-rich and NEM-treated ARF-rich fractions (E and F), or ARF-rich and NEM-treated coatomer-rich fractions (G and H). NEM-treatment was conducted at 1 mM for 10 min at 32°C, and then 2 mM dithiothreitol was added to inactivate the excess NEM. The cells were double-labeled for β-COP (A, C, E, and G) and Man II (B, D, F, and H).

mM NEM in the presence of an ATP-regenerating system and cytosol at 32°C for 30 min, β-COP was released from the Golgi apparatus (Fig. 2E). The release of β-COP was markedly suppressed when the permeabilized cells were incubated with NEM in the presence of 10 μM GTPγS (Fig. 2F). These results suggest that NEM modifies specific protein(s) involved in the association of coatomer.

Cytosolic Proteins Are NEM-Sensitive—To determine which protein(s) involved in the association of COPI with the Golgi apparatus are sensitive to NEM, we examined the binding of COPI to the Golgi apparatus using a cytosol fraction and permeabilized cells. We first treated NRK cells with 1 mM NEM at 37°C for 10 min to promote the release of COPI, then permeabilized them with digitonin (Fig. 3, A and B). When the NEM-treated cells were incubated with bovine brain cytosol in the presence of an ATP-regenerating system at 32°C for 30 min, a significant amount of β-COP became associated with the Golgi apparatus (Fig. 3, C and D), suggesting that NEM-sensitive factor(s) are not present in membranes, but in the cytosol. To confirm this, we examined the effect of NEM treatment on the cytosol. In this case, cells without NEM treatment were permeabilized, then preincubated in the absence of an ATP-regenerating system at 32°C for 20 min to allow the release of COPI from the Golgi apparatus (Fig. 4, A and B). As shown in Fig. 4, C and D, β-COP was recruited onto the Golgi apparatus when the cells were incubated with bovine brain cytosol in the presence of an ATP-regenerating system at 32°C for 30 min. In contrast, β-COP was not recruited onto the Golgi apparatus when NEM-treated cytosol was used (Fig. 4, E and F).

Similar results were obtained for Golgi membranes isolated from rat liver. When isolated Golgi membranes were incubated with an ATP-regenerating system and bovine brain cytosol in the presence of GTPγS, a significant amount of β-COP bound to the membranes (lane 2, Fig. 5). GTPγS was included in this assay because it is required for the effective binding of COPI (*30-32*). In the absence of GTPγS, the amount of β-COP associated with Golgi membranes upon incubation with ATP and cytosol was low (lane 1, Fig. 5) and comparable to that endogenously associated with rat liver Golgi membranes (data not shown), suggesting that little β-COP is recruited onto isolated Golgi membranes in the absence of GTPγS. Endogenous β-COP was tightly associated with isolated Golgi membranes and not released upon incubation without ATP or cytosol (data not shown), although the reason for this is unclear at present. When Golgi membranes were preincubated with ATP and cytosol in the presence of 1 mM NEM at 37°C for 10 min, no significant association of β-COP with Golgi membranes was detected above the endogenous level (lane 3, Fig. 5).

ARF Is Sensitive to NEM—It is known that coatomer and ARF1 are the only cytoplasmic proteins needed for the assembly of COPI-coated vesicles (*8*). ARF1 promotes the binding of coatomer to the Golgi apparatus by functioning as a complex with coatomer (*6*), an activator of phospholipase D (*16*), or an activator that produces high-affinity binding sites for coatomer (*13*). We therefore prepared ARF-rich and coatomer-rich fractions and examined which fraction contains NEM-sensitive factor(s). As shown in Fig. 6, the NEM-treatment of coatomer did not affect its binding to the Golgi apparatus, whereas the recruitment of coatomer did not occur when ARF was treated with NEM. Similar results were obtained using the purified recombinant ARF1 protein (Fig. 7). Furthermore, significant recruitment of coatomer was observed when purified ARF1 was added back to the NEM-treated cytosol (data not shown). These results imply that ARF1 is an NEM-sensitive factor among cytosolic proteins involved in the association of COPI with the Golgi apparatus.

ARF regulates not only the binding of coatomer to the Golgi apparatus but also the recruitment of the AP-1 complex onto the *trans*-Golgi network (*13, 33, 34*). If ARF is sensitive to NEM, the AP-1 complex should also be released by NEM-treatment. This is indeed the case. ARF

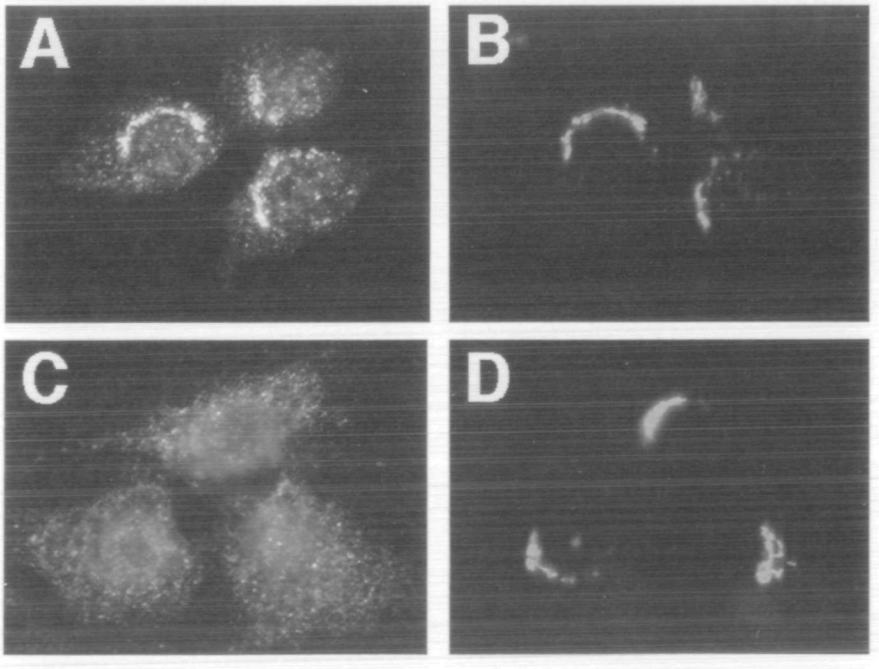

Fig. 7. **ARF1is NEM-sensitive.** NRK cells were permeabilized, incubated in the absence of an ATP-regenerating system, then incubated at 32°C for 30 min in the presence of 1 μM GTPγS, a coatomer-rich fraction, and an ATP-regenerating system with purified ARF1 (A and B) or NEM-treated ARF1 (C and D). NEM-treatment was conducted at 1 mM for 10 min at 32°C, then 2 mM dithiothreitol was added to inactivate the excess NEM. The cells were double-labeled for β-COP (A and C) and Man II (B and D).

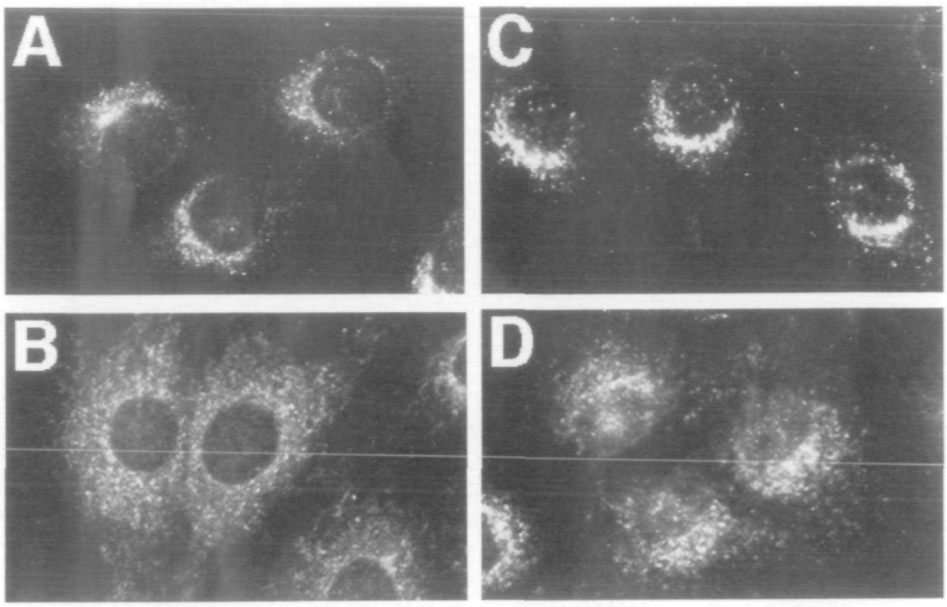

Fig. 8. **Release of γ-adaptin and ARF from the Golgi apparatus.** NRK cells were incubated without (A and C) or with (B and D) 1 mM NEM at 37°C for 10 min, then labeled for ARF1 (A and B) and γ-adaptin (C and D).

and γ-adaptin, a component of the AP-1 complex, both of which are located in the Golgi region in intact cells (Fig. 8, A and C), were released and dispersed throughout the cytoplasm when cells were treated with 1 mM NEM at 37°C for 10 min (Fig. 8, B and D).

Cys-159 Is the Only Cysteine Residue in ARF—To confirm that ARF is modified by NEM, the sulfhydryl content of ARF was determined before and after NEM-treatment. Before NEM-treatment, the sulfhydryl content of ARF was 0.91 ± 0.04 mol/mol protein. Concomitant with NEM treatment, the sulfhydryl content decreased to 0.17 ± 0.02 mol/mol protein (the mean $\pm$ SD of duplicate samples). Since the ARF family contains only one cysteine residue, at position 159 (*35*), this result implies that NEM modified Cys-159.

Cys-159, which is located in the CAT motif in loop λ10, and is conserved not only in the ARF family (*36*) but also in heterotrimeric GTP-binding proteins (*37*), participates in the formation of the roof of the guanine pocket (*38*). This cysteine residue is replaced by a homologous amino acid, a serine residue, in small GTP-binding proteins such as Ras-p21 and Rab proteins, and this serine residue forms a hydrogen bond with the side chain of a highly conserved aspartic acid residue, which determines the specificity for guanine of GTP-binding proteins (*37*). The presence of Cys-159 in the guanine pocket of ARF1 explains why AlF_4^- and GTPγS block the release of β-COP from the Golgi apparatus induced by NEM. Probably, GDP-AlF_4^- or GTPγS occupies the guanine pocket so that NEM cannot gain access to the side chain of Cys-159.

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