Reconstitution of Vesiculated Golgi Membranes into Stacks of Cisternae: Requirement of NSF in Stack Formation

Usha Acharya, J. Michael McCaffery,* Richard Jacobs,‡ and Vivek Malhotra

Department of Biology, *Division of Cellular and Molecular Medicine, University of California, San Diego, La Jolla, California 92093-0347; and ‡Salk Institute, Molecular and Neurobiology Laboratories, La Jolla, California 92093

Abstract. We have developed an in vitro system to study the biochemical events in the fusion of ilimaquinone (IQ) induced vesiculated Golgi membranes (VGMs) into stacks of cisternae. The Golgi complex in intact normal rat kidney cells (NRK) is vesiculated by treatment with IQ. The cells are washed to remove the drug and then permeabilized by a rapid freeze–thaw procedure. VGMs of 60 nm average diameter assemble into stacks of Golgi cisternae by a process that is temperature dependent, requires ATP and a high speed supernatant from cell extract (cytosol), as revealed by immunofluorescence and electron microscopy. The newly assembled stacks are functionally active in vesicular protein transport and contain processing enzymes that carry out Golgi specific modifications of glycoproteins. The fusion of VGMs requires NSF, a protein known to promote fusion of transport vesicles with the target membrane in the exocytic and endocytic pathways. Immunoelectron microscopy using Golgi specific anti-mannosidase II antibody reveals that VGMs undergo sequential changes in their morphology, whereby they first fuse to form larger vesicles of 200–300-nm average diameter which subsequently extend into tubular elements and finally assemble into stacks of cisternae.

THE Golgi apparatus is perhaps one of the most complex of intracellular organelles. Its basic unit is a disc-shaped cisterna containing a specific set of enzymes. In mammalian cells four to eight cisternae are stacked in a polarized fashion and numerous such stacks are held together in the pericentriolar region by association with the microtubule organizing center or MTOC. Microtubules are required for holding the stacks together and for their retention in the pericentriolar region (Rogalski and Singer, 1984; Cooper et al., 1990; Kreis, 1990; Corthesy-Theulaz et al., 1992). What is missing from the picture, however, is an understanding of how cisternae are built, what holds the cisternae together in a stack, and finally whether there are components in addition to microtubules that may confer a pericentriolar localization.

One approach to address these questions is to first dismantle the entire organelle into its smallest unit (vesicles) and then follow the stepwise fusion and assembly of these units into Golgi stacks. This is in fact observed after cytokinesis in the cytoplasm of newly divided mammalian cells. At the onset of mitosis the Golgi complex undergoes extensive vesiculation; the vesicles are partitioned into daughter cells where after cytokinesis they fuse and assemble into a Golgi complex (Lucocq et al., 1987; Lucocq et al., 1989; Warren, 1993). This is of course a highly simplified picture. It is not evident how many kinds of vesicles are produced from each cisterna, and whether there is a complete vesiculation of the Golgi complex or if remnants of the Golgi complex remain to provide nucleation site(s) during the reassembly process. The fusion and assembly of vesicles into stacks of Golgi cisternae has not been reconstituted in vitro. Pharmacological agents such as nocodazole, okadaic acid (OA)[1] and BFA affect Golgi morphology but their effects are either indirect or too pleiotropic. For example, OA inhibits protein phosphatase 2A and promotes entry into mitosis. The Golgi complex, expectedly, breaks down but the recovery, which is observed only in 15–20% of cells, takes 7–8 h at 37°C (Lucocq et al., 1991). Nocodazole treatment and conversion of the Golgi complex into a mitotic form is again a corollary of ensuing mitosis which is prolonged by the drug's inhibitory effect on the mitotic spindle (Jordan et al., 1992). BFA causes the earlier Golgi compartments, cis, medial and trans to fuse with the ER while the TGN fuses with the endosomal compartment (Lippincott-Schwartz et al., 1990). Although the effect of BFA on the overall structural organization of the Golgi is quite extensive, these alterations are distinct from the mitotic phenotype and have not been reconstituted in

Address all correspondence to V. Malhotra, Department of Biology, University of California, San Diego, Pacific Hall 0347, La Jolla, CA 92093-0347. Tel.: (619) 534-8910. Fax: (619) 534-0555.

1. *Abbreviations used in this paper*: IF, immunofluorescence microscopy; IQ, ilimaquinone; Man II, mannosidase II; OA, okadaic acid; SNAP, soluble NSF attachment protein; SNARE, SNAP receptors; VGM, vesiculated Golgi membrane; VSV, vesicular stomatitis virus.

vitro. The Golgi membranes do not undergo any change in morphology during mitosis in *Saccharomyces cerevisiae.* (Preuss et al., 1992). Therefore, unlike advances made in our understanding of vesicular trafficking from mutants defective in secretion, a similar scheme for identifying mutants defective in vesiculation and assembly of the Golgi has not been successful. These points highlight the difficulty in developing a cell-free system for Golgi breakdown and assembly and therefore a lack of understanding of the mechanisms underlying the structural organization of the Golgi complex.

We have recently shown that ilimaquinone (IQ) causes Golgi membranes to vesiculate into numerous small structures (VGMs for vesiculated Golgi membranes) in vivo (Takizawa et al., 1993; Veit et al., 1993). Upon removal of IQ, the VGMs first assemble into Golgi stacks by a microtubule-independent process which is then followed by the congregation of stacks into the pericentriolar region by a process that requires microtubules (Veit et al., 1993). The breakdown and assembly of Golgi stacks in association with IQ treatment does not require new protein synthesis, indicating that these processes are regulated by reversible modification of proteins (Takizawa et al., 1993; Veit et al., 1993). The effect of IQ on the Golgi membranes is summarized in Fig. 1. VGMs appear dispersed throughout the cytoplasm following IQ treatment while stacks appear as large discrete aggregates by immunofluorescence microscopy (IF) in intact cells after removal of IQ. We have used the ability to distinguish VGMs from stacks by light microscopy to reconstitute the process of Golgi stack formation in permeabilized cells. This reconstitution was possible because of two major reasons; firstly, the effects of IQ appear specific for the Golgi (and the microtubules) and secondly, the effects are rapidly reversible. The biochemical requirements of the fusion and assembly of VGMs into stacks of Golgi cisternae are discussed further.

Materials and Methods

Reagents, Antibodies, and Cells

IQ was made up as a stock solution of 5 mg/ml in DMSO and stored at −20°C. Taxol was purchased from Sigma Chemical Co. (St. Louis, MO) and stored at 10 mg/ml in DMSO at −20°C. Pronectin F was obtained from Protein Polymer Tech (San Diego, CA). Endo H was purchased from New England Biolabs (Beverly, MA). Formaldehyde (methanol free) was obtained from Polysciences Inc. (Warrington, PA).

Rabbit anti-rat mannosidase II (Man II) was a gift from Dr. M. Farquhar (University of California, San Diego, CA). Mouse anti-β-COP antibody (CM1A10) was a gift from Dr. James Rothman (Sloan-Kettering, New York, NY). The anti-NSF antibodies and purified NSF were gifts from Dr. Peggy Weidman (St. Louis University, St. Louis, MO) and Dr. James Rothman (Sloan-Kettering, New York, NY). The anti-VSV-G antibody was a gift from Dr. William Balch (Scripps Research Institute, La Jolla, CA). Rhodamine-labeled goat anti-rabbit and fluorescein-labeled goat anti-mouse were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Rabbit anti-mouse antibody was purchased from Cappel Laboratories (West Chester, PA). Fab fragments of goat anti-rabbit IgG conjugated to HRP were from Biosys (Compiegne, France).

All tissue culture cells were grown in complete medium consisting of DME (GIBCO BRL, Gaithersburg, MD) with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (tissue culture medium) at 37°C in a 5% CO_2 cell incubator.

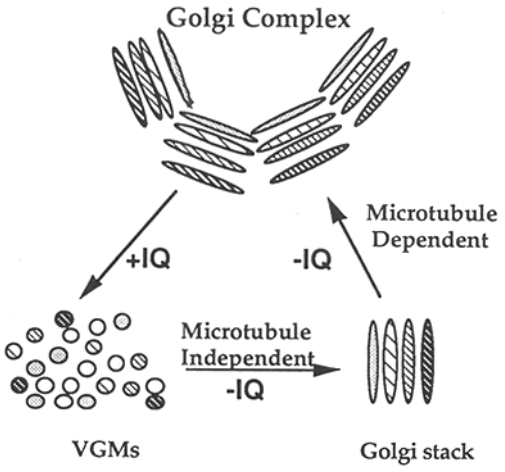

Figure 1. The effects of IQ on the Golgi complex. Based on the data from previous studies (Takizawa et al., 1993; Veit et al., 1993).

Cell Permeabilization

NRK cells were grown to ~70% confluency on Pronectin F-coated coverslips. Pronectin F helps increase the attachment of cells to glass coverslips. The cells were pretreated with taxol (final concentration 5 μg/ml) in complete medium with 25 mM Hepes, pH 7.4, for 30 min at 37°C. IQ depolymerizes cytoplasmic microtubules and although upon removal of IQ, tubulin repolymerizes into microtubules in intact cells, in the permeabilized cells (freeze-thawed) the repolymerization is impaired. We therefore pretreat cells with taxol to stabilize microtubules which are used to monitor the permeabilization of cells by using anti-tubulin antibody. Pretreatment of cells with taxol has no effect on the IQ-mediated vesiculation of Golgi membranes nor does it affect the assembly of VGM into large structures, upon removal of IQ, in intact or permeabilized cells. The cells were subsequently incubated with taxol and IQ (final concentration 30 μM) for 45 min at 37°C. To prepare cells for permeabilization, they were washed with KHM buffer (25 mM Hepes-KOH, pH 7.0, 125 mM potassium acetate, 2.5 mM magnesium acetate, 1 mM DTT, and 1 mg/ml glucose). The cells were air dried leaving a thin film of liquid over the wells. The cells were frozen by placing the plate on a metal block that had been cooled on dry ice. (Robinson and Kreis, 1992). After thawing the cells at room temperature for 4 min, the cells were washed with cold KHM buffer on ice to enhance cytosol dependence. The coverslips were inverted on 50 μl of reaction mix in a petri dish and incubated at 32°C in a water bath. The reaction mix contained KHM buffer, ATP-regeneration system (final concentration 5 mM creatine phosphate, 0.25 mM UTP, 0.05 mM ATP, 12 IU/ml creatine kinase) and cytosol (final concentration 4 mg/ml). Bovine brain cytosol was prepared according to Malhotra et al. (1989) and the estimated protein concentration was ~15 mg/ml.

Immunofluorescence Microscopy

The cells after appropriate incubations were fixed for 15 min at room temperature with 4% formaldehyde (methanol-free) and washed with PBS (150 mM NaCl, 10 mM KCl, 1.8 mM NaH_2PO_4, 8.4 mM Na_2HPO_4). The cells were blocked in a non-detergent block (PBS containing 2.5% fetal bovine serum) for 30 min at room temperature. The cells were incubated for 1 h at room temperature with anti-tubulin antibody diluted in blocking buffer. The cells were washed with PBS and subsequently incubated with a detergent containing buffer (PBS containing 2.5% FBS and 0.1% Triton X-100) for 30 min followed by anti-Man II antibody for 1 h. The cells were washed and incubated with a mixture of secondary antibody conjugated to fluorescein and rhodamine for 1 h. Cells were washed, mounted onto glass slides in 10 mg/ml *p*-phenylenediamine in 90% glycerol, and visualized with a Nikon microscope through a 100× oil lens.

Electron Microscopy

The in vitro assembly of Golgi membranes was carried out as described earlier. Duplicate coverslips were processed for immunofluorescence and electron microscopy. Cells on coverslips were fixed for 6 h at 4°C in 2% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, rinsed briefly in buffer, post-fixed for 1 h at 4°C in 1% OsO_4 in buffer, rinsed in buffer, and dehydrated through a graded series of ethanol. The samples were stained en bloc in 0.5% uranyl acetate in 95% ethanol, further dehydrated in 100% ethanol and propylene oxide and embedded in EMbed 812. The

coverslips were separated from the polymerized epon blocks by alternately plunging them into water at 60°C and liquid nitrogen. Thin sections (60 nm) cut from these blocks were mounted on formvar-coated slot grids, stained with uranyl acetate and lead citrate and examined in a JEOL electron microscope at 60 kV.

Immunoelectron Microscopy

For immunoperoxidase labeling, cells on coverslips were fixed in 100 mM phosphate buffer containing 75 mM lysine, 10 mM periodate, and 2% formaldehyde, pH 6.2, for 4 h at room temperature. Fixed cells were then permeabilized with 0.1% saponin in PBS/1% BSA and sequentially labeled with primary antibodies to Man II, followed by HRP-conjugated secondary antibodies diluted in PBS/1% BSA containing 0.1% saponin. The cells were washed in 100 mM cacodylate-HCl buffer, pH 7.4, followed by postfixation in 4% glutaraldehyde in the same buffer for 1 h. This was followed by incubation in diaminobenzidine and postfixation in cacodylate buffer containing 1% OsO_4/1% KFeCN as previously described (Brown and Farquhar, 1989). Cells were dehydrated and embedded with the cover slips mounted on beem capsules. Coverslips were subsequently removed by immersion of beem capsules/coverslips in liquid nitrogen. Ultrathin sections were cut parallel to the monolayer on a Reichert Ultracut E microtome, stained with lead citrate, observed, and photographed on a Philips CM 10 or JEOL 1200 EX-II electron microscope.

Infection with tsO45 and Labeling of Cells

The infection and labeling of cells were done as described previously for intact cells (Takizawa et al., 1993). Cells were washed after labeling in KHM buffer, permeabilized by freeze-thaw, washed on ice, and incubated with buffer containing cytosol and ATP-generating system at 32°C for 1 h. Cells were lysed and subjected to immunoprecipitation and endo H digestion as described before (Takizawa et al., 1993).

Incubation of Permeabilized Cells with anti-NSF Antibodies

The anti-NSF antibody 4A6 (final concentration 0.08 mg/ml) and 7G11 (final concentration 0.08 mg/ml) were added to IQ-treated semi-permeable cells for 25 min on ice in the absence of an ATP-regenerating system. This was followed by incubation of the antibodies in the complete reaction mix for 45 min at 32°C. The KHM buffer used here included 50 mM KCl in addition to the components mentioned earlier. Purified NSF (final concentration 200 ng in a 50 μl reaction mix) was preincubated on ice with the 4A6 antibody for 30 min and then added to the permeabilized cells.

Results

Assembly of VGMs in a Permeabilized Cell System

NRK cells grown on glass coverslips were pretreated with taxol for 30 min at 37°C to stabilize the microtubules, followed by treatment with IQ for 45 min at 37°C. IQ treatment did not affect the taxol stabilized microtubules but caused Golgi membranes to break down into VGMs as judged by immunofluorescence microscopy using *cis*, medial, and TGN specific antibodies. The cells were washed in buffer, subjected to a rapid freeze–thaw procedure for permeabilization (Robinson and Kreis, 1992), and then incubated at 32°C for 1 h in buffer containing an ATP regeneration system. Permeabilization and access to the cell interior was assayed by incubating the cells with an anti-tubulin antibody following fixation in the absence of a detergent. Since all the incubations up to this stage were carried out without a detergent, only permeabilized cells show staining with the anti-tubulin antibody. The cells were subsequently treated with a detergent containing buffer to access the lumenally exposed epitope of the Golgi-specific Man II protein (Saraste et al., 1988). Under conditions used, the freeze-thaw method resulted in the permeabilization of the plasma membrane while the Golgi membrane remained intact. We routinely observed permeabilization of greater than 90% of the cells by this method. The cells were incubated with a mixture of two secondary antibodies conjugated to different fluorochromes (fluorescein and rhodamine) to reveal the distribution of tubulin and Man II by fluorescence microscopy. Fig. 2 shows the sequential stages of the assay in permeabilized cells. After removal of IQ, VGMs assembled into large structures distributed in the cytoplasm (Fig. 2 *G*). This change in morphology, i.e., Man II remaining completely dispersed (Fig. 2 *C*) or assembled into large aggregates (Fig. 2 *G*) is observed in 90% of the cells. This is determined by counting 200 cells on duplicate coverslips. This criterion is maintained throughout the study to score for assembled (aggregated Man II) and unassembled (dispersed) Man II. The assembly of VGMs into these aggregates was inhibited at 4°C (Fig. 3 *A*) or by depletion of the ATP pool using hexokinase and glucose (Fig. 3 *B*).

The Assembly of VGMs into Discrete Golgi Structures In Vitro Is Cytosol Dependent

As shown in Fig. 2 *G*, VGMs can assemble into large aggregates in permeabilized cells indicating that the activities required for the assembly of VGMs are retained in these cell preparations. However, when cells were permeabilized and washed extensively with buffer on ice, VGMs failed to assemble in greater than 90% of the cells in the presence of an ATP-regenerating system (Fig. 3 *C*). Addition of cytosol from bovine brain and an ATP-regenerating system to the buffer reconstituted the formation of the large structures from VGMs in greater than 90% of the cells (Fig. 3 *D*). A high speed supernatant from CHO cells could also support the formation of large structures from VGMs (data not shown). Addition of either BSA or purified tubulin at protein concentrations equivalent to cytosol did not support the assembly of VGMs (data not shown). These findings demonstrated that VGMs assembled into large aggregates in a process that had an obligate requirement for cytosolic proteins. The large aggregates contained coatomers (Duden et al., 1991; Waters et al., 1991) as revealed in cells double stained with antibodies to Man II (Fig. 3 *E*) and coatomers (Fig. 3 *F*). These aggregates also contained membranes from the TGN (Luzio et al., 1990) as revealed by the presence of TGN-38 (data not shown).

The Large Aggregates of Golgi Membranes Are Stacks of Cisternae

The large structures that assembled from VGMs in semi-intact cells under conditions described above could be clusters of VGMs or they could have fused to form stacks of Golgi cisternae. In order to morphologically characterize the large aggregates, permeabilized cells as described above (Fig. 3 *D*) were fixed with glutaraldehyde and processed for electron microscopy on cover slips. As shown in Fig. 4, the presence of stacks of Golgi cisternae (G_1 and G_2) were evident in the cells. Vesicular structures about 60–90 nm in diameter were also seen in the immediate vicinity of these stacks. It is not possible to distinguish, at present, whether these vesicular structures are VGMs participating in the process of stack formation or Golgi derived transport vesicles. The newly assembled stacks were on an average 0.5 μm in length. In addition, the same cells showed small clusters of vesicles which again could be VGMs en route to fusion (Fig. 4, *asterisk*).

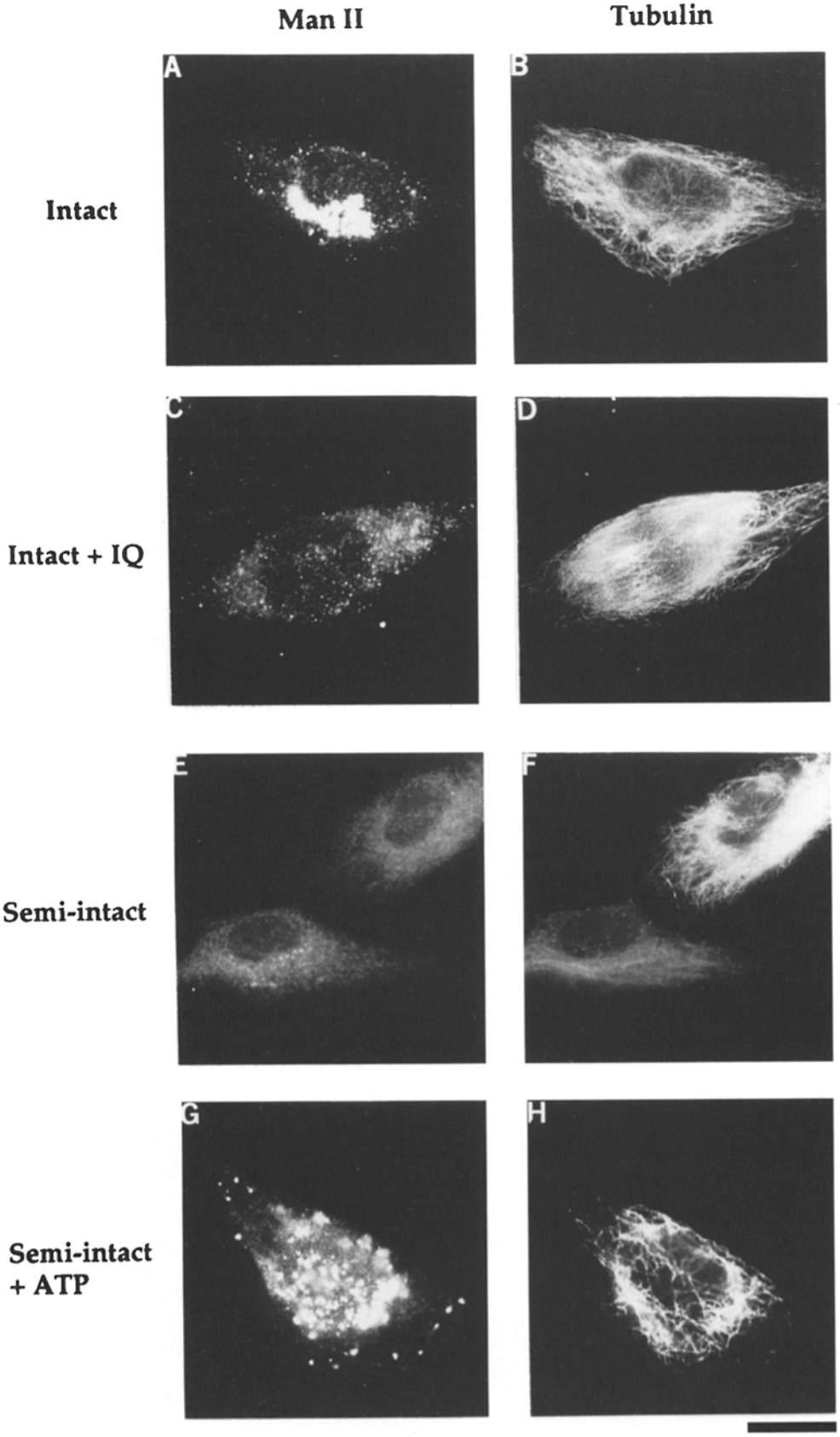

Figure 2. Steps involved in the assembly of IQ derived VGMs into discrete Golgi structures. NRK cells grown on coverslips were incubated with taxol followed by IQ at 37°C. The cells were washed to remove IQ, permeabilized by freeze–thaw, and then incubated with an ATP-regenerating system at 32°C. The cells were fixed for indirect immunofluorescence using an antibody to tubulin and a Golgi-specific antibody (*Man II*). The rabbit polyclonal antibody against Man II and mouse monoclonal antibody to tubulin were colocalized by using goat anti–rabbit conjugated to rhodamine and goat anti–mouse conjugated to fluorescein. The sequential stages from the vesiculation of Golgi complex with IQ to the assembly of VGMs into large aggregates are shown. Untreated NRK cells (*A* and *B*), IQ-treated cells (*C* and *D*). Cells permeabilized after removal of IQ (*E* and *F*) and permeabilized cells incubated with ATP-regenerating system at 32°C (*G* and *H*). The Golgi complex vesiculates with IQ and reassembles into large aggregates on removal of IQ in a permeabilized system. Bar, 10 μm.

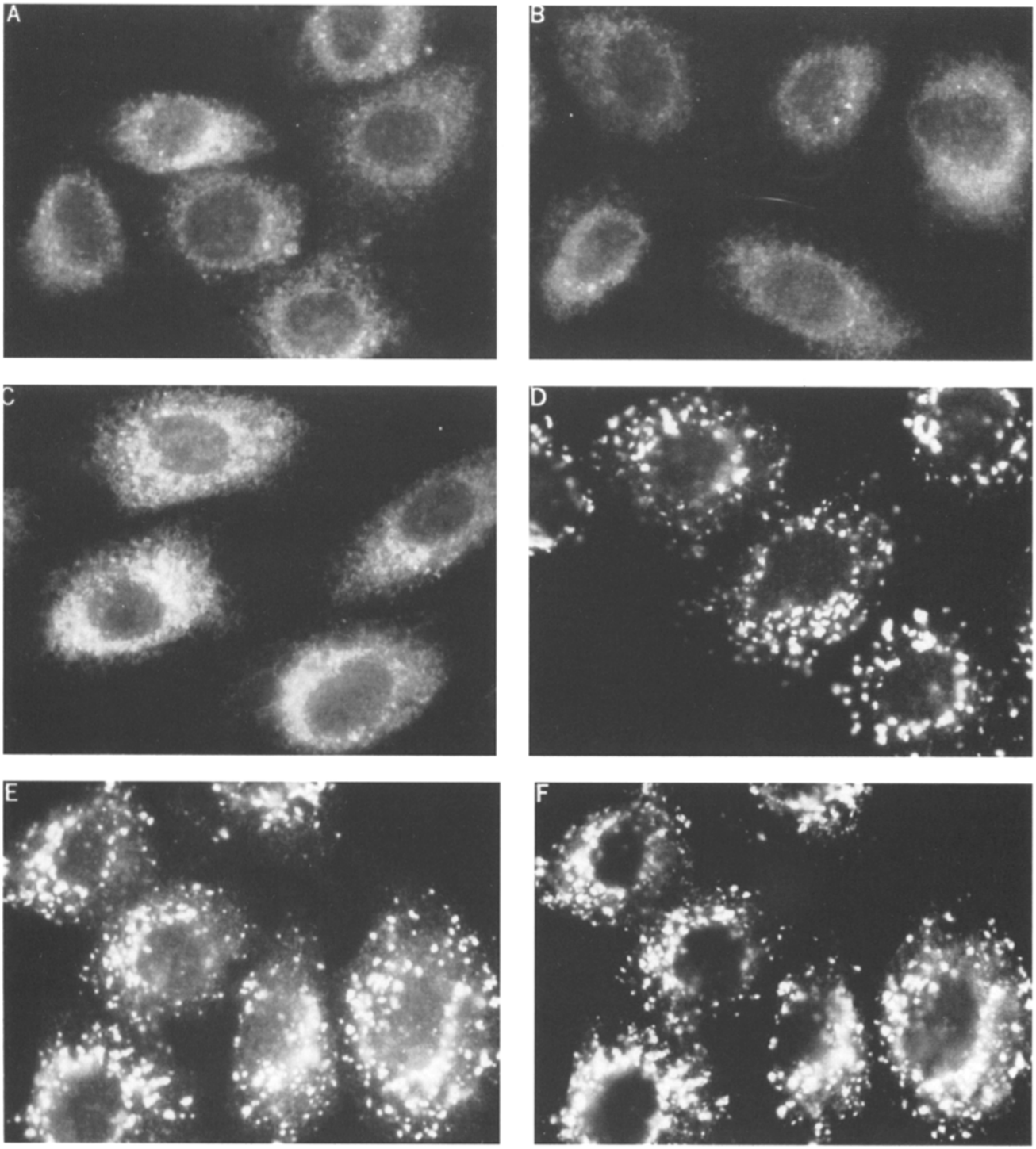

Figure 3. The assembly of VGMs into large aggregates is temperature, energy, and cytosol dependent. NRK cells were incubated with IQ, permeabilized after removal of the drug as described in the legend to Fig. 2 and washed with cold KHM buffer on ice. One set of coverslips was maintained at 4°C, a second set was treated with 2 mg/ml hexokinase, 5 mM D-glucose and incubated in buffer, a third set of coverslips was incubated in KHM buffer and an ATP-regenerating system, while a fourth set was incubated with bovine brain cytosol in addition to buffer and ATP. The cells were processed for immunofluorescence microscopy using antibodies to Man II. Incubation at 4°C for 2 h (*A*) and depletion of ATP (*B*) completely abolished the ability of VGMs to form large structures. In the absence of cytosol (*C*) VGMs remain completely dispersed while in the presence of cytosol (*D*) they assembled into large structures. Cells that were incubated with buffer containing ATP and cytosol were also double stained with antibodies to Man II (*E*) and coatomer (*F*) to show colocalization of Man II and coatomer in the large aggregates. Bar, 10 μm.

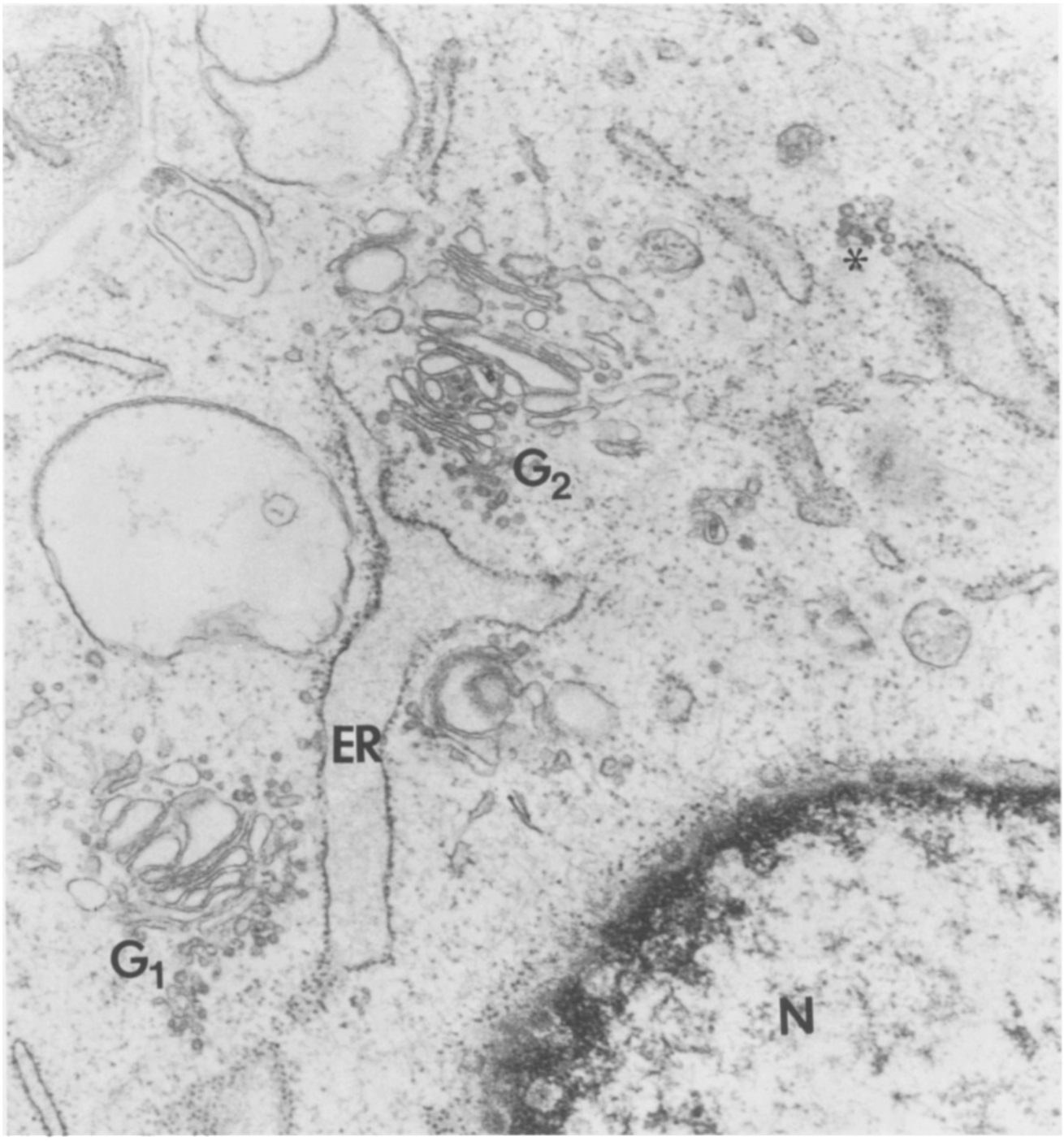

Figure 4. Ultrastructural analysis of the large aggregates of VGMs. VGMs were assembled into large structures as shown in Fig. 3 *D*. The cells on coverslips were fixed in parallel for immunofluorescence and electron microscopy. Thin sections of the cells were visualized by electron microscopy. As is evident from the micrograph, under conditions described in the text, VGMs have assembled into stacks of Golgi cisternae (G_1 and G_2). Small clusters of vesicles (*) may be intermediates in the process of Golgi stack formation. The small vesicles in the vicinity of the Golgi stacks 1 and 2 may be VGMs en route to assembling into stacks or transport vesicles. *ER*, endoplasmic reticulum; *N*, nucleus; G_1, Golgi stack 1; G_2, Golgi stack 2; *, cluster of vesicles. Bar, 0.5 μm.

Morphological Analysis of Protein Transport in the Permeabilized Cells

To test whether the newly formed stacks of Golgi are capable of receiving transport vesicles from the ER, we carried out the following experiment. NRK cells grown on coverslips were infected with the vesicular stomatitis virus (VSV) mutant strain tsO45 that expresses a form of VSV-G protein with a temperature-sensitive defect in export from the ER (Lafay, 1974; Balch et al., 1986). Infected cells were incubated at

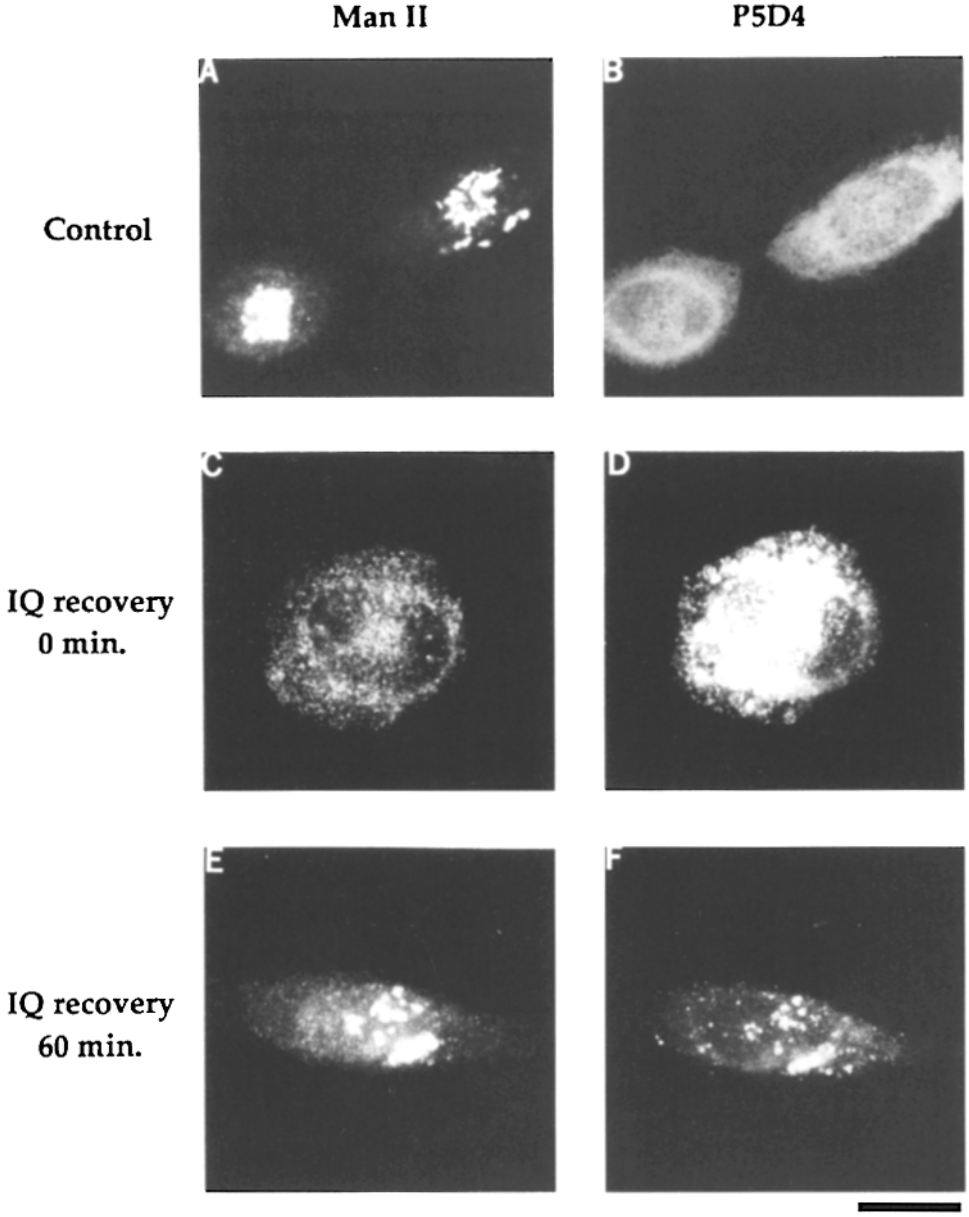

Figure 5. The newly formed Golgi stacks are functionally active in receiving proteins from the ER. NRK cells grown on coverslips were infected with the tsO45 strain of VSV. Infected cells were incubated with IQ at 40°C for 25 min and then washed to remove IQ. These cells were permeabilized and incubated at 32°C for 0 min (*C* and *D*) and 60 min (*E* and *F*) in buffer containing cytosol and an ATP-regenerating system. Untreated infected cells at 40°C double stained with Man II and P5D4 are shown in *A* and *B*. The P5D4 antibody (without detergent), which recognizes a cytoplasmic epitope of VSV-G protein was used to monitor cell permeabilization. VSV-G protein after 60 min of incubation at 32°C colocalizes with the newly assembled Man II-positive structures indicating transport of the G-protein from the ER to the Golgi stacks. Bar, 10 μm.

the restrictive temperature (40°C). The cells were treated with IQ at 40°C for 25 min to vesiculate the Golgi membranes and washed to remove IQ. These cells were subjected to freeze–thaw, washed, and transferred to 32°C (permissive temperature) for 60 min with buffer containing cytosol and an ATP-regenerating system. The cells were processed for IF in the absence of a detergent with an antibody P5D4 (Kreis, 1986) against the cytoplasmic tail of the VSV-G protein (instead of the anti-tubulin antibody used in the earlier experiments to score for semi-intact cells). This was followed by detergent treatment and staining with the Golgi specific Man II antibody. These double stained cells showed that at 40°C, VSV-G protein was retained in the ER as expected (Fig. 5 *B*). Upon shifting cells to 32°C, at the 0 min time point, VSV-G appeared located in the ER (Fig. 5 *D*) and Man II was diffusely distributed throughout the cytoplasm (Fig. 5 *C*). After 60 min of incubation, Man II appeared in large aggregates (Fig. 5 *E*) and VSV-G protein colocalized with these structures as revealed by double labeling of the cells (Fig. 5 *F*). These results indicate that the large structures that assemble from VGMs are capable of receiving newly synthesized proteins from the ER. Additionally the newly formed stacks breakdown upon IQ or BFA treatment (data not shown), indicating that the stacks of Golgi cisternae assembled from VGMs contain appropriate protein components necessary for BFA- and IQ-mediated breakdown of the Golgi.

Modification of Oligosaccharides by the Enzymes of the Newly Assembled Golgi Stacks

We then addressed whether the processing enzymes in these newly formed stacks from VGMs are functionally active. We monitored the maturation of newly synthesized VSV-G protein as it arrived from the ER and was transported through the successive cisternae of the newly formed stacks. VSV-G protein acquires high mannose oligosaccharides during co-

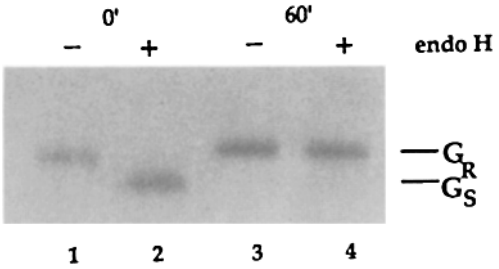

Figure 6. The newly assembled Golgi stacks carry out specific post-translational processing of proteins in transit through the Golgi. NRK cells were infected with the Indiana strain of VSV and incubated with IQ at 37°C for 30 min. The cells were labeled with [^{35}S]methionine, permeabilized, washed and incubated in the presence of an ATP-regenerating system and cytosol. At time points depicted during the chase, cells were lysed by extraction with detergent, and the VSV-G protein immunoprecipitated. One half of each precipitate was digested with endo H, and both halves subjected to SDS-PAGE followed by autoradiography. G_S represents the endo H-sensitive form and G_R represents the resistant form.

translational insertion into the ER and in transit through the Golgi, N-acetyl glucosaminosyl transferase and resident mannosidases convert the oligosaccharides of the G protein from the high mannose to the complex type (Kornfeld and Kornfeld, 1985). Sensitivity to endo H, which only cleaves high mannose oligosaccharides can thus be used to monitor the transport and modification of ER derived proteins through the medial Golgi complex. NRK cells were infected with the wild type Indiana strain of VSV for 2 h and then treated with IQ for 30 min at 37°C. The cells were pulse labeled with [^{35}S]methionine for 8 min. The cells were washed, permeabilized, and incubated in buffer containing cytosol, ATP-regenerating system in the absence of IQ. VSV-G protein was immunoprecipitated at time 0 and at the end of 60 min of incubation. One half of each immunoprecipitate was digested with endo H and the samples were subjected to SDS-PAGE followed by autoradiography. As shown in Fig. 6, at the onset of incubation, VSV-G protein was sensitive to endo H digestion (G_S) while after 60 min of incubation, the protein was endo H resistant (G_R). These results demonstrate that the newly assembled Golgi membranes contain functionally active processing enzymes and are transport competent.

NSF Is Required for the Fusion of VGMs

NSF is a homotrimer of 70-kD subunits that was first identified as a factor necessary for the fusion of Golgi derived transport vesicles with the target Golgi cisternae (Glick et al., 1987; Block et al., 1988; Malhotra et al., 1988). Is NSF also required for the fusion of VGMs during stack for-

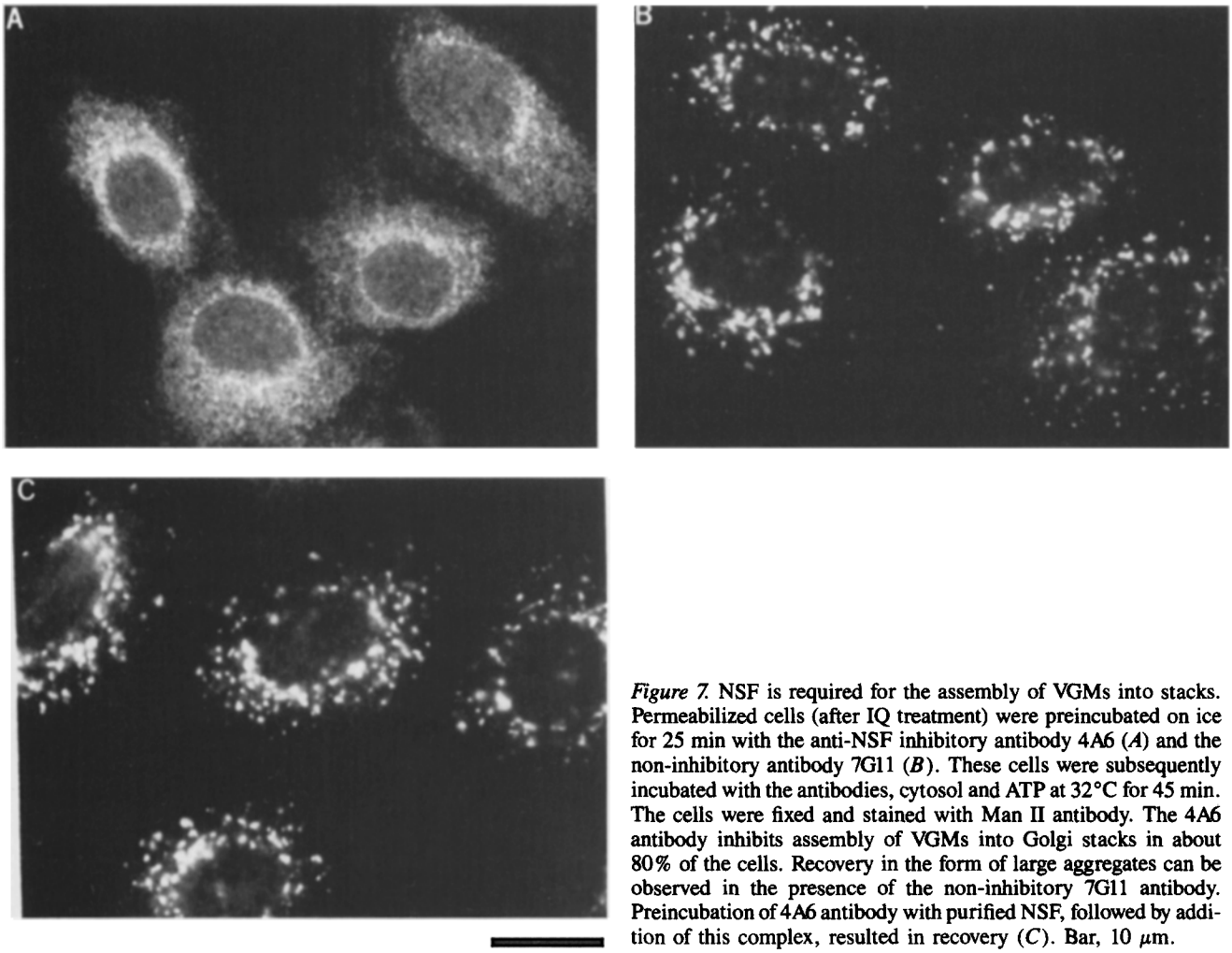

Figure 7. NSF is required for the assembly of VGMs into stacks. Permeabilized cells (after IQ treatment) were preincubated on ice for 25 min with the anti-NSF inhibitory antibody 4A6 (*A*) and the non-inhibitory antibody 7G11 (*B*). These cells were subsequently incubated with the antibodies, cytosol and ATP at 32°C for 45 min. The cells were fixed and stained with Man II antibody. The 4A6 antibody inhibits assembly of VGMs into Golgi stacks in about 80% of the cells. Recovery in the form of large aggregates can be observed in the presence of the non-inhibitory 7G11 antibody. Preincubation of 4A6 antibody with purified NSF, followed by addition of this complex, resulted in recovery (*C*). Bar, 10 μm.

mation? The procedure used for the preparation of the cytosol fraction from bovine brain inactivates NSF (Block et al., 1988). The only source of NSF in the reaction described here is that associated with the membranes in semi-intact cells. To test the involvement of NSF, we have used the monoclonal anti-NSF inhibitory antibody 4A6 that has been used in the past to demonstrate NSF requirement in transport between Golgi cisternae, ER to Golgi and between endosomes (Block et al., 1988; Beckers et al., 1989; Diaz et al., 1989). Permeabilized cells were preincubated with the anti-NSF monoclonal antibody 4A6 and then the cells were incubated with the antibody along with the assembly buffer, cytosol, and ATP for 45 min at 32°C. In parallel, cells were incubated with the non-inhibitory anti-NSF antibody 7G11 as a control. It is evident from Fig. 7 *B*, that the large aggregates are formed in cells treated with 7G11 antibody, while the 4A6 antibody inhibited the assembly reaction in ~80% of cells (Fig. 7 *A*). About 20% of cells treated with 4A6 antibody showed very small aggregates of Man II which may correspond to early stages of assembly. It is not clear whether this early recovery represents clusters of vesicles that have docked but failed to fuse or these cells have passed the stage of NSF requirement in the fusion and assembly process. To confirm the specificity of antibody inhibition, the 4A6 antibody was preincubated with purified NSF. When this complex was added to IQ-treated permeabilized cells, recovery was observed in >90% of the cells (Fig. 7 *C*). This suggests that the antibody inhibition is not a consequence of steric hindrance but due to inactivation of NSF on the membranes by the antibody.

Ultrastructural Analysis of the Stack Formation from VGMs

The question we want to address is how small vesicular structures such as VGMs upon fusion and assembly acquire the morphology of stacks of cisternae, i.e., what are the structural changes that VGMs go through en route to assembling into stacks of cisternae. For this we have used immuno-electron microscopy using the *cis*/medial Golgi-specific protein Man II. The cells were fixed at various stages of the assembly process and thin sections were visualized by immuno-electron microscopy (Figs. 8 and 9) using anti-Man II antibody. Coatomers (visualized by double labeling cells with the coatomer specific antibody CM1A10) co-localize with Man II staining at different time points by immunofluorescence microscopy (data not shown). This indicates that both Man II containing membranes and coatomers assemble under these conditions with similar kinetics. Immunoelectron microscopy of the samples reveals Man II at the onset of the assembly process in small 60–90 nm size vesicles. Very few of these vesicles are found in a given section of the sample and the vesicles are largely distributed randomly throughout the cell (Fig. 8, *A* and *B*). At twenty minutes of the assembly process, Man II is found in larger vesicular structures of distinct sizes that are clustered together. The large vesicles in these clusters have an average diameter of ~200–300 nm (Fig. 8, *C–E*). 45 min after the onset of the assembly reaction, Man II is found mostly in tubular elements and very small cisternae that are stacked (Fig. 9, *A–C*, stacks of cisternae are labeled as Gc and tubular elements as Gt). At this stage of the assembly process, smaller stacks of cisternal elements reveal Man II throughout the stack. At 60 min, Man II is found predominantly in stacks of cisternae (Fig. 9, *D–F*). Interestingly, however, the distribution of Man II containing cisternae in these stacks varies. While there are stacks of cisternae in which Man II is restricted to one or two cisternae (Fig. 9, *D* and *E*) the same sections also show stacks where Man II is present in more than two cisternae (Fig. 9 *F*). Could there be two different states of enzymatic compartmentation within the newly formed stacks?

Discussion

The Golgi complex, which is composed of stacks of cisternae is a very dynamic organelle. There is an extensive flux of membranes in the form of transport vesicles both to and from each of the Golgi cisterna. Additionally, these membranes undergo extensive vesiculation followed by reassembly into the native structure coincident with the cell cycle in mammalian cells (Warren, 1993). Thus, not only is there a need to maintain the structural organization of stacks in interphase cells but these stacks have to be rebuilt from their vesiculated form after mitosis in the cytoplasm of daughter cells. How then is this structural organization of the Golgi complex established and regulated? We have focused on one aspect of this dynamic behavior, namely the assembly of vesiculated Golgi membranes (VGMs) into stacks of cisternae, and provide a first attempt to characterize the biochemical requirements for the stack assembly process.

The stack formation requires ATP and cytosol. While assembly of stacks is observed at 37°C, we have found 32°C to be optimum as incubation of semi-intact cells at 37°C results in >50% of the cells sloughing from the cover slips. Since the procedure of freeze–thaw disrupts the organization of cytoplasmic microtubules, it is not surprising that the newly assembled stacks remain dispersed and do not congregate in the pericentriolar region. The structural and spatial organization (with reference to the nucleus) of the newly assembled stacks is similar to those observed in mammalian cells lacking cytoplasmic microtubules, for example cells treated with microtubule depolymerizing agents such as nocodazole (Rogalski and Singer, 1984; Ho et al., 1989; Turner and Tartakoff, 1989; Kreis, 1990). The dispersed Golgi stacks in nocodazole-treated cells were fully functional in protein secretion and modification (Iida and Shibata, 1991). Similarly, the stacks that assemble from VGMs are functionally active as these membranes are competent in receiving and processing newly synthesized proteins made in the ER that are en route to the cell surface. The stacks formed in vitro contain a full complement of cisternae as revealed by the presence of *cis*/medial-, and TGN-specific polypeptides. The newly formed stacks contain Golgi-specific peripheral proteins such as coatomers and are susceptible to pharmacological agents, BFA and IQ. These findings substantiate our proposal that the polypeptide composition, function and structural organization of the newly formed stacks in vitro are highly similar to that observed in vivo.

The Fusion of VGMs in the Process of Cisternal Biogenesis Is NSF Dependent

The process of transport vesicle docking and fusion with the target membrane in addition to NSF, requires soluble NSF attachment proteins (SNAPs) and SNAP receptors

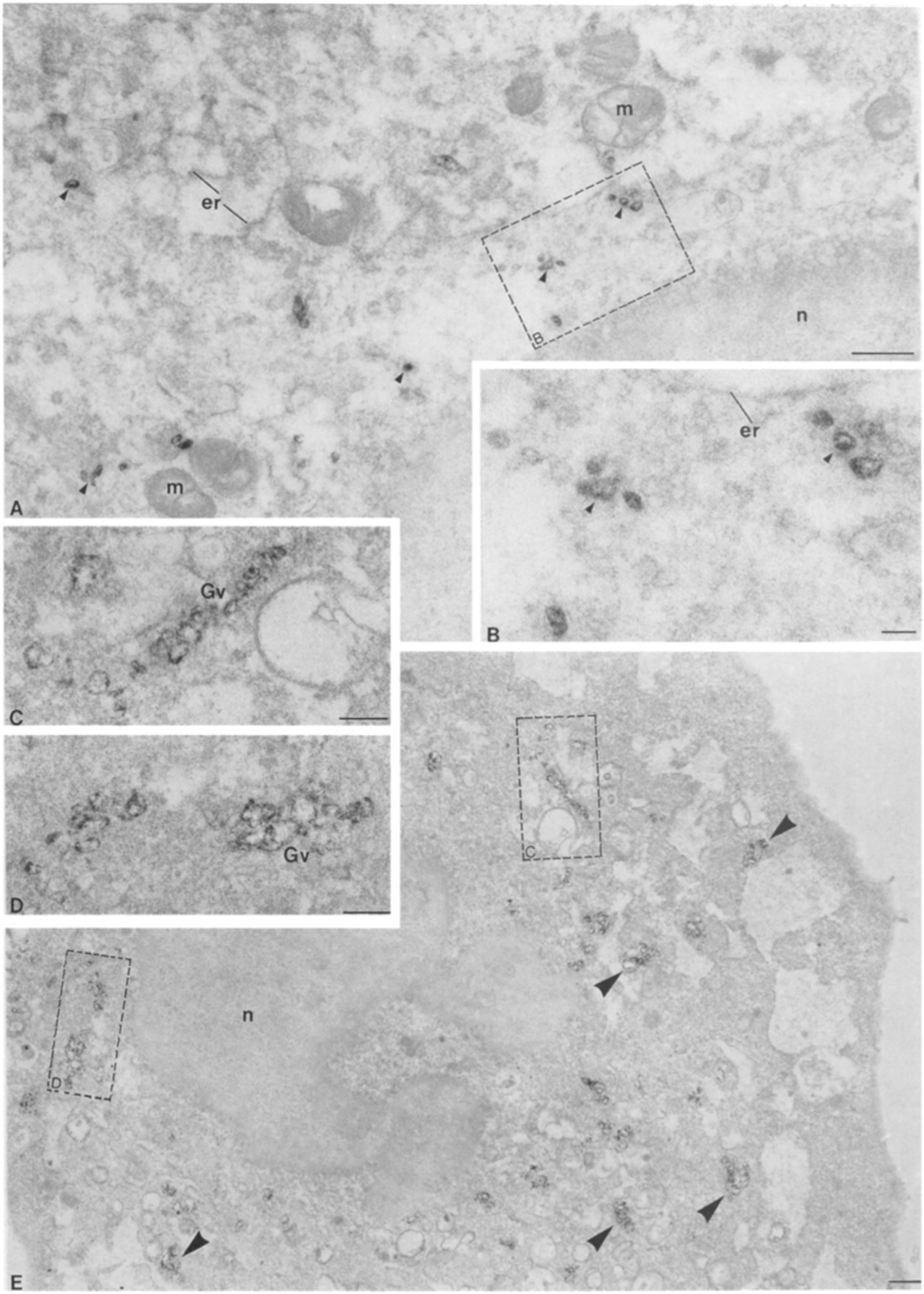

Figure 8. Ultrastructural analysis of the intermediate stages in the fusion and assembly process. Permeabilized cells on coverslips, recovered after IQ treatment were fixed for immunoelectron microscopy. Progressive changes in the morphology of the Man II containing structures are shown at 0 min (*A* and *B*) and 20 min (*C–E*) of the assembly process. Bars: (*B*) 0.1 μm, (*A*, *C*, and *D*) 0.5 μm, and (*E*) 1.0 μm.

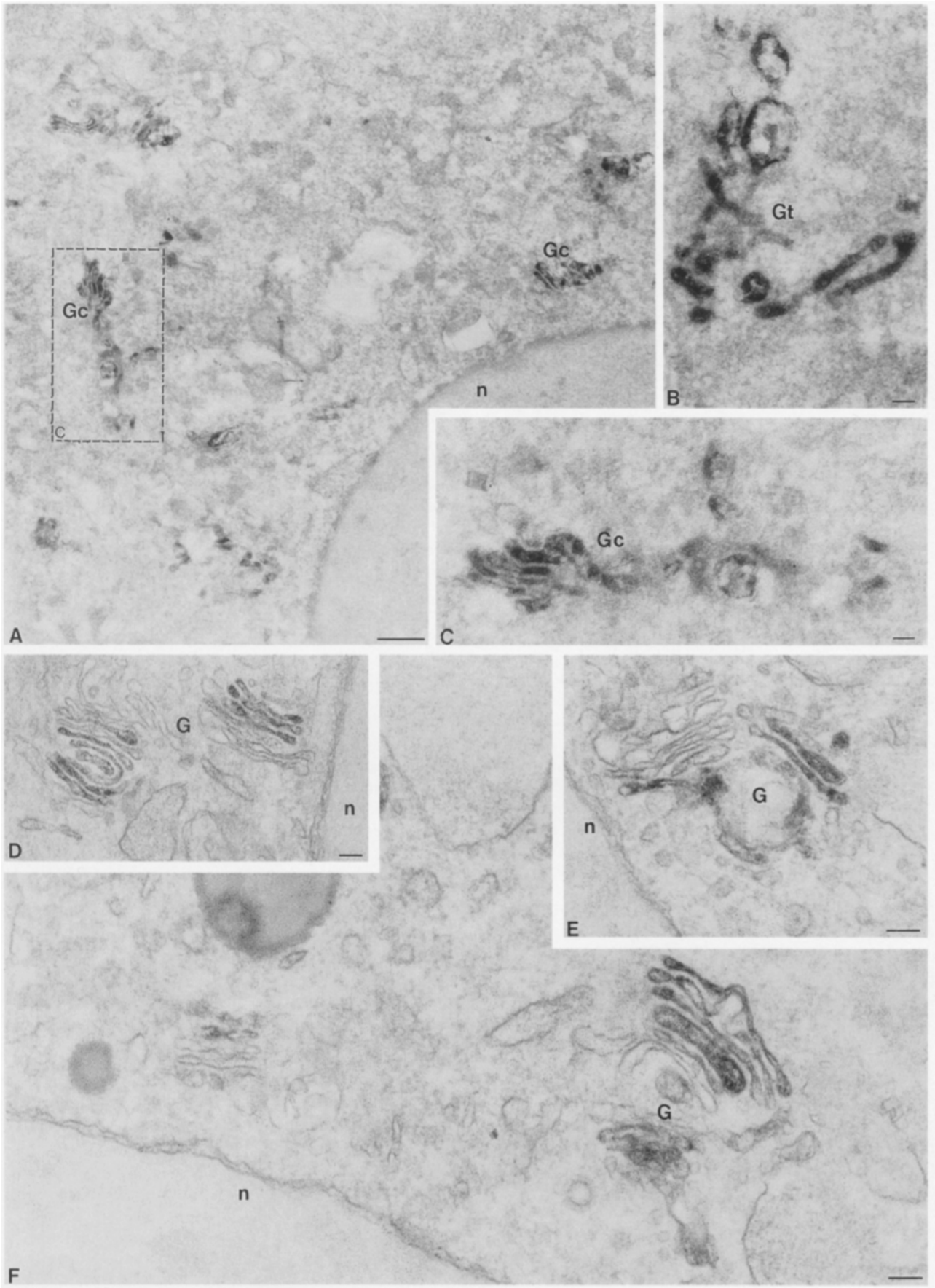

Figure 9. Immunoelectron microscopy of samples at 45 and 60 min of the assembly process. At the end of 45 min, many tubular elements (*Gt*) and short stacks of cisternae (*Gc*) are seen (*A–C*). Golgi stacks (*G*) are the predominant products at 60 min of the assembly process (*D–F*). Bars: (*A*) 0.5 μm; and (*B–F*) 0.1 μm.

(SNAREs) (Wilson et al., 1992; Clary et al., 1990; Sollner et al., 1993; Whiteheart et al., 1993). Rothman and colleagues (Sollner et al., 1993) have proposed a general hypothesis for vesicle docking and fusion based on studies monitoring vesicular transport between two membrane-bound compartments. In this scheme, or the "SNARE hypothesis," SNAPs bind to receptors on vesicles (v-SNARE) and the target membrane (t-SNARE). NSF then binds to this complex via its association with SNAPs and subsequent events, catalyzed by ATP hydrolysis, disassemble the complex in vitro. These findings are translated into steps corresponding to the docking and fusion of transport vesicles with the target membrane (Sollner et al., 1993). It has been proposed that similar proteins (or their isoforms) are involved in all intracellular homotypic and heterotypic membrane fusion events (Rothman and Warren, 1994). While membrane fusion events in the exocytic protein transport pathway and endosome fusion require NSF (Block et al., 1988; Malhotra et al., 1988; Beckers et al., 1989; Diaz et al., 1989) exceptions do exist, the most recent being the finding that fusion between ER membranes from *Saccharomyces cerevisiae* does not require NSF (Latterich and Schekman, 1994). Why some membrane fusion events involve such an elaborate interplay between the cytoplasmic and membrane proteins and others do not is not obvious. Schekman and colleagues have suggested that fusion with target membranes (as observed during protein transport) may need cytosolic components to ensure tight membrane attachment. The fusion of large membranes such as the ER could occur over a large surface area and may therefore not need accessory proteins to potentiate contact between apposing membranes (Latterich and Schekman, 1994). Regardless, our finding that NSF is required for VGM fusion suggests that other proteins that interact with NSF in promoting membrane docking/fusion are also likely to be involved. In this regard it is particularly interesting that Golgi associated isoforms of both SNAPs and SNAREs have been identified (Clary et al., 1990; Bennett et al., 1993). Whether these isoforms confer specificity to NSF-dependent membrane fusion events, e.g., stack formation during Golgi assembly versus vesicular transport can now be tested in this assay.

The Assembly of Compartmentalized Stacks of Golgi Cisternae

The assay described here not only monitors the fusion between VGMs but also documents the assembly of the dispersed VGMs into stacks of flattened Golgi cisternae. While the understanding of the mechanism of fusion of VGMs is of fundamental importance, the biggest advantage of this assay is the ability to score for components regulating the assembly of VGMs into Golgi stacks. There are three major questions that need to be addressed.

The first question pertains to the shape of the structure that develops from the fusion of VGMs. In general, we would expect the fusion of VGMs to progressively form larger vesicles. Instead, the end product of fusion and assembly are flattened cisternae that are morphologically distinct from the starting material. How is this achieved? We reasoned that VGMs may indeed first fuse to form large vesicular structures that condense or flatten during the stacking process. To test this working hypothesis we monitored the kinetics of the assembly process by using anti-Man II antibody of the *cis*/medial Golgi cisternae. Our results revealed that the VGMs undergo a progressive increase in size, to first form large vesicular structures that are clustered together. These large vesicles then undergo a change and become more tubular. This raises an interesting question; does this process involve another round of membrane fusion or is it mediated by specialized proteins (molecular motors and cytoskeletal elements) which pull large vesicles into more tubular elements? If another round of fusion is involved at this step of the assembly process is it mediated by NSF and accessory proteins or does it take place independent of NSF as is the case for the fusion of larger ER vesicles with each other during ER assembly (Latterich and Schekman, 1993)?

The second question that follows is how are cisternae held together in a stack? Brown and colleagues have shown that Golgi stacks can be separated from each other by proteolytic treatment in vitro (Cluett and Brown, 1992). Such unstacking by proteolysis correlates with a loss of proteinaceous bridges on the cytoplasmic side of the cisternae as seen by electron microscopy. The cisternae released as a result, however, retain their flattened appearance. The polypeptides that compose these inter-cisternal bridges are not known. Proteins that may fulfill this functional requirement are giantin (Linstedt and Hauri, 1993), actin binding proteins such as comitin (Weiner et al., 1993), an isoform of β-erythroid spectrin (Beck et al., 1994) and a detergent insoluble pool of proteins identified from Golgi enriched membrane fractions (Slusarewicz et al., 1994). The list of potential candidates for the stacking of cisternae at present is quite diverse, and it remains to be seen which of these are the actual players.

The third question that needs to be addressed is how are Golgi cisternae stacked in a polarized fashion such that the cis and the TGN are on the opposite face of the stack and how are these stacks oriented such that the *cis* is proximal to the nucleus. In order to address this question we first need to know whether the fusion of VGMs is homotypic (between vesicles of the same type) or heterotypic (i.e., VGMs from different cisternae fuse randomly with each other). If the fusion between VGMs is between vesicles of the same type, then according to this scheme the ensuing cisternae will have to be sorted during the stack assembly process such that the cis is followed by the medial, trans and the TGN. The second possibility is that the VGMs fuse at random with each other (heterotypic fusion) to form stacks of cisternae where the resident enzymes are not compartmentalized. Once the stacks have been established, the resident enzymes could then be relocated to their respective cisternae by protein transport and sorting. In this context it is interesting to note that at the time point in our assembly assay when stacks of cisternae are evident both by conventional and immunoelectron microscopy, Man II shows a hybrid distribution—from being predominantly confined to one or two cisternae in a stack, to being present in numerous and in some cases all cisternae. It is, therefore, tempting to speculate that the initial fusion between VGMs may be indiscriminate, and the enzymatic compartmentation may take place, post-stacking, by protein transport and sorting, in the anterograde and retrograde direction. It is, however, also possible that our assay does not reconstitute the retention mechanism within the Golgi cisternae. The newly formed stacks, therefore, may be highly compartmentalized to begin with, but because of a

faulty retention mechanism, the resident enzymes are transported to other cisternae of the stack by the transport process. Since little is known about the general protein machinery involved in retention, the problem of establishing compartmentation within the Golgi stack is beyond our scope at present.

These points highlight the complexity of the organization of Golgi stacks and also acknowledge a lack of our understanding of the processes by which the stacks are established. The assay reconstituting stack formation from its building blocks "VGMs" as described here has already revealed that this process shares some similarities with the events controlling fusion of transport vesicles with target membranes. Further studies will undoubtedly reveal the underlying biochemistry of the organization of the stacks and its regulation during mitosis.

We thank Jairaj Acharya and Yukiko Goda for helpful suggestions and critical reading of the manuscript. Jen Yucel is gratefully acknowledged for valuable input throughout this study and for assistance with photography. We thank Pete Takizawa for helpful suggestions, Kristi Elkins for excellent technical assistance, Lance Washington for help with EM photography, Chuck Stevens and Marilyn Farquhar for the generous use of their EM facilities, and Scott Emr and Mike Yaffe for comments on the manuscript.

J. M. McCaffery was supported by the National Institutes of Health grant (CA58689) to Marilyn G. Farquhar. This work was supported by a Basil O'Connor starters scholar's award from the March of Dimes Birth Defect Foundation and NIH grant GM 46224 to V. Malhotra.

Received for publication 23 December 1994 and in revised form 26 February 1995.

References

Balch, W. E., M. M. Elliot, and D. S. Keller. 1986. ATP-coupled transport of VSV-G protein between the endoplasmic reticulum and the Golgi. *J. Biol. Chem.* 261:14681–14689.

Beck, K. A., J. A. Buchanan, V. Malhotra, and W. J. Nelson. 1994. Golgi spectrin: identification of an erythroid β-spectrin homolog associated with the Golgi complex. *J. Cell Biol.* 127:707–723.

Beckers, C. J. M., M. R. Block, B. S. Glick, J. E. Rothman, and W. E. Balch. 1989. Vesicular transport between the endoplasmic reticulum and the Golgi stack requires the NEM-sensitive fusion protein. *Nature (Lond.).* 339: 397–398.

Bennett, M. K., J. E. Garcia-Arraras, L. A. Elferink, K. Peterson, A. M. Fleming, C. D. Hazuka, and R. H. Scheller. 1993. The syntaxin family of vesicular transport receptors. *Cell.* 74:863–873.

Block, M. R., B. S. Glick, C. A. Wilcox, F. T. Wieland, and J. E. Rothman. 1988. Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport. *Proc. Natl. Acad. Sci. USA.* 85:7852–7856.

Brown, W. J., and M. G. Farquhar. 1989. Immunoperoxidase methods for the localization of antigens in cultured cells and tissue sections by electron microscopy. *Methods Cell Biol.* 31:553–569.

Clary, D. O., I. C. Griff, and J. E. Rothman. 1990. SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. *Cell.* 61:709–721.

Cooper, M. S., A. H. Cornell-Bell, A. Chernjavsky, W. J. Dani, and S. J. Smith. 1990. Tubulovesicular processes emerge from the trans-Golgi cisternae, extend along microtubules and interlink adjacent trans-Golgi elements into a reticulum. *Cell.* 61:135–145.

Corthésy-Theulaz, I., A. Pauloin, and S. R. Pfeffer. 1992. Cytoplasmic dynein participates in the centrosomal localization of the Golgi complex. *J. Cell Biol.* 118:1333–1345.

Cluett, E. B., and W. J. Brown. 1992. Adhesion of Golgi cisternae by proteinaceous interactions: intercisternal bridges as putative adhesive structures. *J. Cell Sci.* 103:773–784.

Diaz, R., L. S. Mayorga, P. J. Weidman, J. E. Rothman, and P. D. Stahl. 1989. Vesicle fusion following receptor-mediated endocytosis requires a protein active in Golgi transport. *Nature (Lond.).* 339:398–400.

Duden, R., G. Griffiths, R. Frank, P. Argos, and T. E. Kreis. 1991. β-COP, a 110 kD protein associated with non-clathrin coated vesicles and the Golgi complex, shows homology to β-adaptin. *Cell.* 64:649–665.

Glick, B. S., and J. E. Rothman. 1987. Possible role for fatty acyl-coenzyme A in intracellular protein transport. *Nature (Lond.).* 326:309–312.

Ho, W. C., V. J. Allan, G. vanMeer, T. E. Berger, and T. E. Kreis. 1989. Reclustering of scattered Golgi elements occurs along microtubules. *Eur. J. Cell. Biol.* 48:250–263.

Iida, H., and Y. Shibata. 1991. Functional Golgi units in microtubule-disrupted cultured atrial myocytes. *J. Histochem. Cytochem.* 39:1349–1355.

Jordan, M. A., D. Thrower, and L. Wilson. 1992. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles—implications for the role of microtubule dynamics in mitosis. *J. Cell Sci.* 102:401–416.

Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* 54:631–664.

Kreis, T. E. 1986. Microinjected antibodies against the cytoplasmic domain of Vesicular Stomatitis Virus glycoprotein block its transport to the cell surface. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:931–941.

Kreis, T. E. 1990. Role of microtubules in the organization of the Golgi apparatus. *Cell Motil. Cytoskeleton.* 15:67–70.

Lafay, F. 1974. Envelope proteins of vesicular stomatitis virus: effect of temperature-sensitive mutations in complementation groups III and V. *J. Virol.* 14:1220–1228.

Latterich, M., and R. Schekman. 1994. The karyogamy gene Kar2 and novel proteins are required for ER membrane fusion. *Cell.* 78:87–98.

Linstedt, A. D., and H.-P. Hauri. 1993. Giantin, a novel conserved Golgi membrane protein containing a cytoplasmic domain of at least 350 kDa. *Mol. Biol. Cell.* 4:679–693.

Lippincott-Schwartz, J., J. G. Donaldson, A. Schweizer, E. G. Berger, H.-P. Hauri, L. C. Yuan, and R. D. Klausner. 1990. Microtubule-dependent retrograde transport of proteins into the ER in the preence of brefeldin A suggests an ER recycling pathway. *Cell.* 60:821–836.

Lucocq, J. M., J. G. Pryde, E. G. Berger, and G. Warren. 1987. A mitotic form of the Golgi apparatus in Hela cells. *J. Cell Biol.* 104:865–874.

Lucocq, J. M., E. G. Berger, and G. Warren. 1989. Mitotic Golgi fragments in Hela cells and their role in the reassembly pathway. *J. Cell Biol.* 109:463–474.

Lucocq, J. M., G. Warren, and J. Pryde. 1991. Okadaic acid induces Golgi apparatus fragmentation and arrest of intracellular transport. *J. Cell Sci.* 100:753–759.

Luzio, J. P., B. Brake, G. Banting, K. E. Howell, P. Braghetta, and K. K. Stanley. 1990. Identification, sequencing and expression of an integral membrane protein of the trans-Golgi network (TGN38). *Biochem. J.* 270:97–102.

Malhotra, V., L. Orci, B. S. Glick, M. R. Block, and J. E. Rothman. 1988. Role of N-ethylmaleimide-sensitive transport component in promoting fusion of transport vesicles with cisternae of the Golgi stack. *Cell.* 54:221–227.

Malhotra, V., T. Serafini, L. Orci, J. C. Shepherd, and J. E. Rothman. 1989. Purification of a novel class of coated vesicles mediating biosynthetic protein transport through the Golgi stack. *Cell.* 58:329–336.

Preuss, D., J. Mulholland, A. Franzusoff, N. Segev, and D. Botstein. 1992. Characterization of the *Saccharomyces* Golgi complex through the cell cycle by immunoelectron microscopy. *Mol. Biol. Cell.* 3:789–803.

Robinson, M. S., and T. E. Kreis. 1992. Recruitment of coat proteins onto Golgi membranes in intact and permeabilized cells: effects of brefeldin A and G protein activators. *Cell.* 69:129–138.

Rogalski, A. A., and S. J. Singer. 1984. Association of the elements of the Golgi apparatus with microtubules. *J. Cell Biol.* 99:1092–1100.

Rothman, J. E., and G. Warren. 1994. Implications of the SNARE hypothesis for intracellular membrane topology and dynamics. *Curr. Biol.* 4:220–233.

Saraste, J., M. Bronson, G. E. Palade, and M. G. Farquhar. 1988. Organelle-specific antibodies; production of antibodies to Golgi subcompartments. *Prog. Clin. Biol. Res.* 270:129–139.

Slusarewicz, P., T. Nilsson, N. Hui, R. Watson, and G. Warren. 1994. Isolation of a matrix that binds medial Golgi enzymes. *J. Cell Biol.* 124:405–413.

Sollner, T., S. W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geromanos, P. Tempst, and J. E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature (Lond.).* 362:318–324.

Takizawa, P. A., J. K. Yucel, B. Veit, J. D. Faulkner, T. Deerinck, G. Soto, M. Ellisman, and V. Malhotra. 1993. Complete vesiculation of Golgi membranes and inhibition of protein transport by a novel sea sponge metabolite ilimaquinone. *Cell.* 73:1079–1090.

Turner, J. R., and A. M. Tartakoff. 1989. The response of the Golgi complex to microtubule alterations: the roles of metabolic energy and membrane traffic in Golgi complex organization. *J. Cell Biol.* 109:2081–2088.

Veit, B., J. K. Yucel, and V. Malhotra. 1993. Microtubule independent vesiculation of Golgi membranes and the reassembly of vesicles into Golgi stacks. *J. Cell Biol.* 122:1197–1206.

Warren, G. 1993. Membrane partitioning during cell division. *Annu. Rev. Biochem.* 62:323–348.

Waters, M. G., T. Serafini, and J. E. Rothman. 1991. "Coatomer": a cytosolic protein complex containing subunits of non-clathrin-coated Golgi transport vesicles. *Nature. (Lond.).* 349:248–251.

Weiner, O. H., J. Murphy, G. Griffiths, M. Schleicher, and A. A. Noegel. 1993. The actin-binding protein comitin(p24) is a component of the Golgi apparatus. *J. Cell Biol.* 123:23–34.

Whiteheart, S. W., I. C. Griff, M. Brunner, D. O. Clary, T. Mayer, S. A. Buhrow, and J. E. Rothman. 1993. SNAP family of NSF attachment proteins includes a brain-specific isoform. *Nature (Lond.).* 362:353–355.

Wilson, D. W., S. W. Whiteheart, M. Weidmann, M. Brunner, and J. E. Rothman. 1992. A multi subunit particle implicated in membrane fusion. *J. Cell Biol.* 117:355–359.