Assembly and Disassembly of the Golgi Complex: Two Processes Arranged in a *cis-trans* Direction

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Abstract. We have studied the disassembly and assembly of two morphologically and functionally distinct parts of the Golgi complex, the *cis/middle* and *trans* cisterna/*trans* network compartments. For this purpose we have followed the redistribution of three *cis/middle-* (GMP-1, GMP-2, MG 160) and two *trans-* (GMP-1 and GMP-2) Golgi membrane proteins during and after treatment of normal rat kidney (NRK) cells with brefeldin A (BFA). BFA induced complete disassembly of the *cis/middle-* and *trans-*Golgi complex and translocation of GMP-1 and GMP-2 to the ER. Cells treated for short times (3 min) with BFA showed extensive disorganization of both *cis/middle-* and *trans-*Golgi complexes. However, complete disorganization of the *trans* part required much longer incubations with the drug. Upon removal of BFA the Golgi complex was reassembled by a process consisting of three steps: (a) exit of *cis/middle* proteins from the ER and their accumulation into vesicular structures scattered throughout the cytoplasm; (b) gradual relocation and accumulation of the *trans* proteins in the vesicles containing the *cis/middle* proteins; and (c) assembly of the cisternae, and reconstruction of the Golgi complex within an area located in the vicinity of the centrosome from which the ER was excluded. Reconstruction of the *cis/middle*-Golgi complex occurred under temperature conditions inhibitory of the reorganization of the *trans*-Golgi complex, and was dependent on microtubules. Reconstruction of the *trans*-Golgi complex, disrupted with nocodazole after selective fusion of the *cis/middle*-Golgi complex with the ER, occurred after the release of *cis/middle*-Golgi proteins from the ER and the assembly of the *cis/middle* cisternae.

The Golgi complex plays an important role in the post-translational modifications and sorting of proteins transported from the ER, recycling of receptors involved in endocytosis and transport of proteins to organelles, and in the sorting of ligand–receptor complexes internalized by transcytosis (Palade, 1975; Farquhar, 1985; Pfeffer and Rothman, 1987). The organelle is constituted by two morphologically and functionally distinct parts: a stack of cisternae (Morré and Ovtracht, 1977; Farquhar and Palade 1981), and a network of tubules called the *trans* network (Novikoff et al., 1971; Novikoff, 1977; Griffiths and Simons, 1986). A striking feature of the Golgi complex is the functional compartmentation of its components and the arrangement of the compartments (i.e., *cis* cisternae, *trans* network) in a functional order (Dunphy et al., 1981, 1983; Rothman et al., 1984a,b; Orci et al., 1987; Tartakoff, 1983; Kornfeld and Kornfeld, 1985). By acting synchronously, the enzymes contained in different compartments introduce a variety of posttranslational modifications into the proteins transported through the organelle (reviewed in Tartakoff, 1983; Dunphy and Rothman, 1985; Kornfeld and Kornfeld 1985). Transport of molecules to, through, and out of the Golgi complex is probably mediated by specific populations of vesicles (Rothman et al., 1984a,b; Balch et al., 1984a,b; Dunphy and Rothman, 1985; Orci et al., 1986, 1989; Pfeffer and Rothman, 1987). Sorting of the proteins occurs in the *trans*-Golgi network (Novikoff and Novikoff, 1977; Habener et al., 1979; Griffiths et al., 1985, 1989; Snider and Rogers, 1985; Griffiths and Simons, 1986; Sossin et al., 1990).

The Golgi complex is an organelle that through the cell cycle, undergoes dramatic changes in morphology, localization, and function. Throughout interphase the functionally active Golgi displays a characteristic organization in parallel cisternae juxtaposed to the *trans* network (Morré and Ovtracht, 1977; Farquhar and Palade, 1981), and is located in the vicinity of the centrosome (Kupfer et al., 1982; Wehland and Sandoval, 1983; Sandoval et al., 1984; Tassin et al., 1985). Furthermore, Golgi and centrosome show synchronous and superimposed displacements in moving cells (Kupfer et al., 1982). At the onset of mitosis the whole Golgi is disrupted, becomes inactive, and the dispersed fragments are excluded from the area containing the mitotic spindle (Robbins and Gonatas, 1964; Maul and Brinkley, 1970; Zeiligs and Wolman, 1979). Reconstruction of the organelle is initiated during cytokinesis and completed before separation of the two daughter cells (Robbins and Gonatas, 1964; Maul...
and Brinkley, 1970; Zeiligs and Wollman, 1979; Warren et al., 1983, 1984; Luccoq and Warren, 1987; Luccoq et al., 1987; Gaspar et al., 1988).

Recent studies have shown that brefeldin A (BFA), a fungal-antiviral antibiotic (Harri et al., 1963), can be used as a useful tool to study the function and organization of the Golgi complex. Minutes after treatment with BFA, proteins normally transported through the ER-Golgi complex pathway are retained in the ER (Takatsuki and Tamura, 1985; Misumi et al., 1986; Oda et al., 1987; Magnier and Papagiannes, 1988). Furthermore, it has been reported that BFA induces the selective fusion of parts of the Golgi with the ER (Lippincott-Schwartz, 1989; Doms et al., 1989), and promotes the release from the Golgi of β-Coat protein (Donaldson et al., 1990), a protein associated with nonclathrin-coated vesicles (Orci et al., 1986; Malhotra et al., 1989; Serafini et al., 1991; Duden et al., 1991) acting as bulk carriers (Wieland et al., 1987; Orci et al., 1989; Kärrenbauer et al., 1990).

Here we have studied the disassembly and reassembly of the cis/middle and trans (i.e., trans most cisterna and trans network) parts of the Golgi complex in cells treated with BFA by following the redistribution of resident membrane proteins of these compartments. Our results show that both processes occur in an orderly fashion: the cis/middle-precedes the trans-Golgi in the fusion with the ER and in the reassembly that follows the removal of BFA. Moreover, the cis/middle-Golgi is reconstructed under conditions preventing the reorganization of the trans-Golgi. In contrast conditions inhibiting the reconstruction of the cis/middle-Golgi also block the reorganization of the trans-Golgi. The significance of these results is discussed within the context of the anterograde and retrograde mechanisms involved in the transport of molecules between the ER and compartments of the Golgi complex, and of the mechanisms operating in the disruption and reconstruction of the Golgi complex during the cell cycle.

Materials and Methods

Cell Culture

Normal rat kidney (NRK) cells were grown on plastic bottles or glass coverslips, in 90% DME, 10% FCS, 10 mM morpholinoethane-sulfonic acid, 2 mM glutamine, penicillin (50U/ml), streptomycin (50 μg/ml) (normal medium) at 37°C, or 23°C, in an atmosphere of 93% air, 7% CO2, and 85% humidity.

Antibodies

All the mAbs against rat Golgi complex and lysosomal membrane proteins were raised in mice. Monoclonal antibodies IgG 15C8 and IgM 20.1 reacted with membrane proteins of 150 kD (Golgi integral membrane protein [GMP]; GMP -2) (Yuan et al., 1987) and 180 kD (GMP -2), respectively, contained in the cis- and middle-Golgi cisternae. Monoclonal antibodies IgG 18B11 and IgM 21.1 reacted with proteins of 100 kD (GMP -1) (Yuan et al., 1987) and 200 kD (GMP -2), respectively, contained in the trans-most Golgi cisternae and trans network. Polyonal antibodies against GMP -1 and GMP -2 were raised by injecting the protein purified by monoclonal antibody affinity chromatography into rabbits. Monoclonal antibodies IgG 38C7 and 29C10 reacted with lysosomal integral membrane protein (LIMP) III and LIMP II, two LIMPs of 100 kD and 74 kD, respectively (Barriocanal et al., 1986). Other antibodies used were the rat antibody YL 1/2 (Kilmartin et al., 1982; Wehland et al., 1983b), the rabbit polyclonal antibody against rat protein disulfide isomerase (PDI), and the anti-MG-160 antibody, which recognizes a 160-kD protein located in the cis-middle-Golgi (Croul et al., 1990; Gonatas et al., 1989). Fluorescein-, rhodamine-, and peroxidase-conjugated antibodies were from Coopers-Biomedical Inc. (Malvern, PA). Peroxidase conjugated protein A was from Boehringer Mannheim Diagnostics, Inc. (Houston, TX).

Light Microscopy

The morphological changes of the Golgi complex and the redistribution of GMPs in cells treated with BFA were studied by immunofluorescence and immunoperoxidase microscopy. The strong signal produced by the accumulation of the product of the peroxidase reaction made the staining of cells with peroxidase the method of choice to study the morphology of depleted Golgi cisternae, and to detect the presence of GMPs in the ER. Cells studied by immunoperoxidase staining were prepared by the modification of the PLP/saponin procedure (McLean and Nakane, 1974) described before (Yuan et al., 1987). The cellular distribution of individual Golgi membrane proteins was studied by simple immunofluorescence microscopy as described (Yuan et al., 1987). Double-immunofluorescence microscopy was performed by the four-step procedure described before (Barriocanal et al., 1986).

Other Methods

Metabolic labeling, immunoprecipitation, treatment with neuraminidase, and analysis of LIMPs by two-dimensional IEF/SDS-PAGE were performed as described (Barriocanal et al., 1986).

Results

Disassembly of the cis/middle- and trans-Golgi Network after BFA Treatment

To study the disassembly of the Golgi complex we have analyzed the model of NRK cells treated with BFA.

The drug promotes the rapid disassembly of the Golgi complex (Fujiwara et al., 1988), and quick translocation of the cis-middle-Golgi marker, mannosidase II, and middle/trans marker, β-galactosyltransferase (Lippincott-Schwartz et al., 1989, 1990) to the ER. However, from studies performed with the markers of the trans-most cisterna and trans-Golgi network, antigen GMP -1 (Lippincott-Schwartz et al., 1989), and sialyltransferase (Chege and Pfeffer, 1990), it has been concluded that the trans-most Golgi cisternae is not fused with the ER.

A study of both the disassembly and assembly of the Golgi complex in cells treated with BFA requires an exact definition of which parts of the Golgi complex fuse with the ER in response to the drug. For this purpose, we have reexamined the effects of the drug on the distribution of three membrane proteins resident in the cis/middle-Golgi, antigens GMP -1, GMP -2, and MG 160 (Yuan et al., 1987; Gonatas et al., 1989), and two located in the trans-most cisterna and trans-Golgi network, GMP -1, and GMP -2 (Yuan et al., 1987). The results are shown in Fig. 1. In contrast to previous studies we observe that cells treated with 1–10 μg/ml BFA and stained for GMP -1 with antibody 18B11, displayed marked changes in the morphology of the trans-Golgi. Identical changes were observed when the cells were stained for GMP -2 with antibody 21.1. The changes were already observed 3 min after addition of the drug (Fig. 1, C and D), and consisted in extensive disorderization of the
Figure 1. Effects of BFA on the morphology and organization of the Golgi complex. NRK cells were incubated with normal medium (A and B), or 10 μg/ml BFA for 3 min (C and D), 15 min (E and F), or 60 min (G and H). The distribution of GMP₁ was studied by light microscopy using the monoclonal antibody 18B11, and fluoresceine-(A, C, and H), or peroxidase-(B, D-G, and I) conjugated goat anti-mouse antibodies. Comparable results were obtained with the anti-GMP₁ antibody 21.1. Note the rapid disorganization of the trans-Golgi complex in response to BFA. Bars, 15 μm.

trans-Golgi, which acquired a necklace morphology (i.e., strings of beads connected by fibers) already described for the cis/middle-Golgi complex (Lippincott-Schwartz et al., 1989). Longer incubations resulted in progressive loss of the reticular structure (Fig. 1, E–G), its substitution by small vesicles clustered in the vicinity of the nucleus (Fig. 1 H), and the appearance of faintly stained fibers extended throughout the cytoplasm (Fig. 1 H). Staining with peroxidase revealed that the fibers corresponded to a polygonal network of tubules extending throughout the cytoplasm (Fig. 1 I). The tubular network was identified as the ER by its staining with DiOC₆ (2), and with peroxidase using an antibody
Figure 2. Translocation of enzymes involved in the sialylation of N-linked carbohydrates from the trans-Golgi complex to the endoplasmic reticulum in BFA-treated cells. LIMP III (A and B) immunoprecipitated from NRK cells treated for 2 h with 10 μg/ml BFA and then labeled for 3 h with [35S]methionine in the presence of 10 μg/ml BFA. LIMP III (C-F) and LIMP II (E and F) immunoprecipitated from NRK cells pulse-labeled for 15 min with [35S]methionine, and then incubated for 2 h with 100 μM cycloheximide and 10 μg/ml BFA. The immunoprecipitated proteins were incubated without (A, C, and E) and with (B, D, and F) neuraminidase, and resolved by two-dimensional SDS-PAGE to analyze their content in sialic acid.

against the ER resident enzyme, PDI (not shown). Finally, after 75 min incubation with BFA, the vesicles disappeared and the staining was spread throughout the cytoplasm (Fig. 4 B). These results strongly suggested that BFA promoted the translocation of proteins resident in the trans most cisternae and trans-Golgi network to the ER.

To further test that suggestion, we studied whether the drug promoted the translocation from the trans most cisternae and trans-Golgi network (Bennett and O'Shaughnessy, 1981; Roth et al., 1985) to the ER of the sialyltransferases involved in transferring sialic acid to N-linked carbohydrates. For this purpose, we studied the acquisition of sialic acid by the lysosomal membrane proteins LIMP II and LIMP III (Barriocanal et al., 1986). Both proteins were shown to contain sialylated N-linked oligosaccharides and to lack O-linked carbohydrates (Barriocanal et al., 1986). As shown in Fig. 2 A, LIMP III retained for 3 h in the ER of cells pretreated with BFA displayed four major forms with PI between 8.2 and 9.2. Treatment of the protein with neuraminidase (Fig. 2 B) produced three forms with PI between 9.0 and 9.5. The shift in PI strongly suggested that the molecules of LIMP III retained in the ER acquired sialic acid. To exclude the possibility that the protein was sialylated by sialyltransferases synthesized and retained in the ER during the 3-h period of protein labeling, LIMP III was pulse labeled for 15 min (a lapse of time during which the protein synthesized in normal NRK cells remained in the ER and displayed only high mannose carbohydrates) (Morales et al., 1989) in the presence of 100 μM cycloheximide (concentration of antibiotic that caused an immediate inhibition of protein synthesis but had no effect on the BFA induced fusion of the trans-Golgi with the ER [data not shown]). Under the later
conditions LIMP III showed the four major forms with pI between 8.0 and 9.2 observed after the 3-h labeling in the absence of antibiotic (compare Fig. 2, A with C). Furthermore, the shift in pI showed by the protein, after treatment with neuraminidase (compare Fig. 2, C with D) indicated that it acquired sialic acid in the absence of new synthesis of sialyltransferase. Similar results were obtained with LIMP II (Fig. 2, E and F). These results agreed with those of recent studies on the processing of alkaline phosphatase (Takami et al., 1990).

Taken together, the translocation of GMP-1, GMP-2, and sialyltransferase activity to the ER of cells treated with BFA indicated that the drug promoted the fusion of the trans-Golgi and trans-Golgi network with the ER.

With respect to the effects of BFA on the organization of the cis/middle-Golgi stained with anti-GMP antibodies, our results were identical to those described using antibodies against the cis/middle marker mannosidase II and the itiner-

Exit of cis/middle- and trans-Golgi Proteins from the ER and Reconstruction of the Golgi Complex

Previous studies have demonstrated the reversibility of the effects of BFA on the Golgi complex (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989; Doms et al., 1989; Lippincott-Schwartz et al., 1990). This provided us with a system to study the assembly of the cis/middle and trans parts of the organelle. The studies were performed by double immunofluorescence microscopy. 10 min after removal of BFA the distribution of the cis/middle-Golgi markers, GMP-1, GMP-2, and MG 160 were dramatically changed. The proteins were found in discrete vesicular structures (Fig. 4, C and D). By comparison, the relocation of the trans-Golgi proteins from the ER proceeded significantly more slowly, and no change in their distribution was observed 10-12 min after removal of the drug (Figs. 3 B and 4 E). It was only after 15 min that half of the cells began to show redistribution of the trans proteins (Figs. 3 B and 4 G). Interestingly, the redistributed trans proteins were found to colocalize with the cis/middle proteins in the same structures (Fig. 4, F and G). The process of colocalization increased with time and was complete 15 min after the exit of the first trans molecules from the ER (Fig. 4, H and I). Finally, the reassembly of the Golgi complex (i.e., cisternae) (Fig. 4, J and K) was slow as compared to the rapidity with which the Golgi proteins exit the ER (Fig. 3, compare B with C).

Further analysis of the process of Golgi assembly revealed that the organelle was reconstructed in an area from which the ER marker PDI was excluded and that remained visible after complete disassembly of the Golgi complex (Fig. 5, A and B). The vesicular structures that became loaded with GMPS shortly after removal of the drug were found scattered throughout the cytoplasm (Fig. 5, C and D). Later the GMPS residing at tubular structures were concentrated at the periphery of the area of ER exclusion (Fig. 5, E and F), before entering (Fig. 5, G and H) and forming the reticular structure characteristic of the Golgi complex (Fig. 5, I and J).

In 90% of the cells studied, the centrosome (organelle) that regulates the assembly and organization of the cytoplasmic microtubules and whose function has been associated with the assembly of the Golgi complex (Kupfer et al., 1982; Wheland and Sandoval, 1983) was located at the edge of the area of ER exclusion closer to the nucleus (Fig. 5, K and L). In the remaining 10% of the cells, the centrosome was found within that area.
Figure 4. Exit of cis/middle- and trans-proteins from the ER and reconstruction of the Golgi complex. NRK cells were treated for 75 min with 10 µg/ml BFA (A and B) and then incubated in drug-free medium for 10 min (C, D, and E), 15 min (F and G), 30 min (H and I), and 1 h (J and K). The distribution of cis/middle- and trans-Golgi proteins was studied by double immunofluorescence microscopy using the mouse monoclonal anti-GMPc antibody 15C8 (fluorescein channel; A, C, F, H, and J) and a rabbit polyclonal anti-GMPc antibody (rhodamine channel; B, E, G, I, and K). The distribution of GMPc between the endoplasmic reticulum and vesicles after removal of BFA was studied with peroxidase staining (D). Note the faster exclusion of GMPc as compared with GMPc from the ER, and the gradual incorporation of the trans protein into vesicular structures loaded with the cis/middle protein. Similar results were obtained with cells stained with anti-GMPc and anti-GMPc. Bars, 15 µm.
Reconstruction of the Golgi complex occurs in an area devoid of ER located in the vicinity of the centrosome. NRK cells treated 60 min with 10 μg/ml BFA and simultaneously stained with a rabbit polyclonal antibody against the ER marker PDI (A; rhodamine channel) and the anti-GMP \(_{c_{-},f_{-}}\) antibody (B; fluoresceine channel). Note that the PDI negative area remains intact after the disassembly of the cis-Golgi complex. Identical results were obtained in studies of the trans-Golgi. Cells treated 60 min with 10 μg/ml BFA and incubated for 10 min (C and D), 30 min (E and F), 45 min (G and H), and 75 min (I and J) in drug-free medium, simultaneously stained with the anti-PDI (C, E, G, and I; rhodamine channel) and the mouse monoclonal anti-GMP \(_{c_{-},f_{-}}\) (D, F, H, and J; fluorescein channel) antibodies. Note the dotty fluorescence throughout the cytoplasm 10 min after removal of BFA, and later the increasing formation of tubular structures, first around and then inside the area of ER exclusion, before formation of the reticular structure characteristic of the Golgi complex. Similar results were obtained with the anti-GMP \(_{c_{-},f_{-}}\) antibody. Cells treated for 45 min with 20 μM nocodazole stained for PDI (K; rhodamine channel) and for microtubules with the anti-tubulin antibody YL 1/2 (L; fluorescein channel). Observe the location of the centrosome at the edge of the area of ER exclusion and in the vicinity of the nucleus. Bars, 15 μm.

**Figure 5.** Reconstruction of the Golgi complex occurs in an area devoid of ER located in the vicinity of the centrosome. NRK cells treated 60 min with 10 μg/ml BFA and simultaneously stained with a rabbit polyclonal antibody against the ER marker PDI (A; rhodamine channel) and the anti-GMP \(_{c_{-},f_{-}}\) antibody (B; fluoresceine channel). Note that the PDI negative area remains intact after the disassembly of the cis-Golgi complex. Identical results were obtained in studies of the trans-Golgi. Cells treated 60 min with 10 μg/ml BFA and incubated for 10 min (C and D), 30 min (E and F), 45 min (G and H), and 75 min (I and J) in drug-free medium, simultaneously stained with the anti-PDI (C, E, G, and I; rhodamine channel) and the mouse monoclonal anti-GMP \(_{c_{-},f_{-}}\) (D, F, H, and J; fluorescein channel) antibodies. Note the dotty fluorescence throughout the cytoplasm 10 min after removal of BFA, and later the increasing formation of tubular structures, first around and then inside the area of ER exclusion, before formation of the reticular structure characteristic of the Golgi complex. Similar results were obtained with the anti-GMP \(_{c_{-},f_{-}}\) antibody. Cells treated for 45 min with 20 μM nocodazole stained for PDI (K; rhodamine channel) and for microtubules with the anti-tubulin antibody YL 1/2 (L; fluorescein channel). Observe the location of the centrosome at the edge of the area of ER exclusion and in the vicinity of the nucleus. Bars, 15 μm.

**Back Transport of cis/middle- and trans-Proteins from Vesicles to the ER**

The accumulation of GMPs in vesicular structures upon removal of BFA raised the question whether they were part of the ER or constituted a separate compartment. To answer that question the following experiment was performed: cells were incubated for 75 min with 100 μg/ml BFA to induce the complete fusion of the Golgi with the ER, then for 20 or 40 min in the absence of the drug to release the GMPs from the ER, and again with 10 μg/ml BFA for periods of time between 10 and 60 min to induce their relocation into the ER. It was reasoned that if the vesicles loaded with GMPs were part of the ER, upon addition of BFA the cis/middle and trans proteins would diffuse with the same rates through the ER cisternae. On the contrary, accumulation of GMPs into vesicles independent from the ER would result in a pattern of ordered transport similar to that observed in the transport of GMPs from the Golgi complex. The results are shown in Fig. 6. It can be observed that the cis/middle-Golgi proteins entered the ER faster than the trans-Golgi proteins. Furthermore, the translocation of GMPs from the vesicles to the ER occurred considerably faster than from the Golgi complex (compare Fig. 3 with Fig. 6), suggesting that they were in a compartment close to the ER. It was noteworthy that during the process of transport, we never noted the fibrous struc-
prompted us to study whether NEM could inhibit the disassembly and reassembly of the Golgi complex. Low temperatures below 17°C were compatible with two models of Golgi reorganization:

- **Inhibition of the Disassembly and Assembly of the Golgi Complex by N-ethylmaleimide (NEM) and Low Temperatures**

The fusion of the Golgi complex with the ER upon addition of BFA and the substitution of cisternae for vesiculotubular elements after removal of the drug strongly suggested the occurrence of fusion events mediated by vesicles. The recent discovery that an NEM-sensitive fusion protein, or fusogen, is involved in the traffic of vesicles moving from the ER to and through the Golgi complex (Malhotra et al., 1989; Wilson et al., 1989; Beckers et al., 1989; Díaz et al., 1989), prompted us to study whether NEM could inhibit the disassembly and reassembly of the Golgi complex. The studies were performed under conditions that caused no cell mortality (see Fig. 7, A and B). It was noted that incubation of cells with 200 μM NEM for 1 min resulted in immediate inhibition of the BFA-induced disassembly of the Golgi complex (Fig. 7, C–F). The rapidity of the inhibition permitted the observation that swelling and disruption of the Golgi cisternae into vesicles were events preceding the fusion of the Golgi membranes with the ER. Moreover, NEM also caused the immediate inhibition of the transport of Golgi proteins from the ER (in Fig. 7, compare G with H).

In separate experiments we also studied the effects of low temperatures on the disassembly and assembly of the Golgi complex. Briefly, only temperatures below 17°C inhibited completely the disassembly of the Golgi complex induced by BFA. Transport of Golgi proteins from the ER was completely blocked at 17°C and markedly slowed down at 23°C, a temperature that also decreased the rate of assembly of the cisternae. As described below, the use of low temperatures was a strong tool to study the separate assembly of Golgi compartments.

**Assembly of the cis/middle-Golgi Complex under Conditions Inhibiting the Reconstruction of the trans-Golgi Complex**

To gain further insight into the reconstruction of the Golgi complex we examined if the cis/middle and trans parts of the organelle could be assembled separately. For this purpose, cells were incubated at 37°C for 90 min with 10 μg/ml BFA to induce the complete resorption of the whole Golgi complex into the ER; then, for 10-12 min at 37°C with drug-free medium to allow the selective transport of cis/middle proteins from the ER; and finally, at 23°C for 40–60 min to study the reconstruction of the cis/middle-Golgi complex under conditions inhibiting the transport of trans proteins out from the ER. The results are shown in Fig. 8. 90% of the cells double stained for trans- (A) and cis/middle-Golgi (B) proteins showed the diffuse staining of fibers characteristic of the ER and strong staining of a reticular organelle forming a cap near the nucleus, respectively. Only five percent of the cells showed a few GMP-positive vesicles scattered throughout the cytoplasm and incorporation of GMP, Golgi proteins into the retiform organelle. A study of the organelle stained with anti-cis antibodies using peroxidase produced the following results. In 70% of the cells the trans proteins were located in elements displaying a beaded or tubular shape, arranged in two or three rows of strings juxtaposed to the nucleus (Fig. 8, E–G). The structure of the organelle housing the cis proteins was comparable to that of the cis-Golgi complex in normal cells incubated for 40 min at 23°C (Fig. 8, C and D), with the difference that the elements constituting the later were significantly longer. The remaining 30% of the cells showed lower forms of organization, often consisting in independent vesicular elements clustered in the vicinity of the nucleus (Fig. 8 H). The staining patterns suggested that the cis/middle-Golgi complex was assembled under conditions preventing the reconstruction of the trans-Golgi complex.

When the 37°C and 23°C incubations were done in the presence of 20 μM nocodazol, it was observed that disruption of microtubules by nocodazol (Fig. 8 I) produced the scattering of vesicles loaded with cis proteins throughout the cytoplasm and prevented the assembly of the cis/middle-Golgi complex in the vicinity of the nucleus (Fig. 8 J). This result suggested that the network of cytoplasmic microtubules played an important role in the reorganization and relocation of the cis/middle-Golgi complex (see Discussion).

**Reconstruction of the trans-Golgi Complex Is Preceded by the Assembly of the cis/middle-Golgi Complex**

The reassembly of the cis/middle-Golgi complex under conditions preventing the assembly of the trans-Golgi complex was compatible with two models of Golgi reorganization:
first, a reorganization preceded by the independent assembly of the different compartments constituting the organelle; second, a reorganization based on the early assembly of the cis/middle compartments. To distinguish between these two possibilities the reconstruction of the trans-Golgi complex disrupted with nocodazol under conditions interfering with the reorganization of the cis/middle-Golgi complex previously fused with the ER was studied. Two different protocols were used. In both, the cells were incubated for 10 min with 1 μg/ml BFA to induce the fusion of the cis/middle-Golgi complex with the ER and to limit the effects of the drug on the trans-Golgi complex, and then for 40 min at 23°C with 20 μM nocodazol and 0.1 μg/ml BFA to disrupt the trans-Golgi located in the cytoplasm and to slow the release of cis/middle proteins from the ER (Fig. 9, A and B). In the first protocol, the reconstruction of the trans- and cis-middle-Golgi was studied after incubation of the cells without drugs for periods between 1 and 2 h at 23°C. In the second, the study was performed after incubating the cells at 37°C for 40 min in the presence of 0.1 μg/ml BFA. Both treatments
slowed drastically the transport of cis/middle proteins out from the ER, and permitted the reorganization of the cytoplasmic network of microtubules disrupted by nocodazol. A significant difference between them was that cells incubated at 37°C with 0.1 µg/ml BFA showed more extensive incorporation of trans-Golgi proteins into the ER. Study of the cells by double immunofluorescence microscopy, using anti-GMP, and anti-GMP, antibodies, revealed that 80-90% of them displayed no reorganization of the Golgi compartments. This population of cells showed large trans-Golgi-derived vesicles clustered in the vicinity of the centrosome (Fig. 9 B), many small vesicles loaded with cis/middle proteins translocated from the ER (Fig. 9 A), and significant amounts of cis/middle proteins in the ER, as shown by the diffuse staining of the cytoplasm (Fig. 9 A). The rest of the cells displayed Golgi complexes in different stages of reorganization, from the very poorly to the completely restructured. Further analysis revealed that the trans-Golgi was never reorganized in cells showing no reconstruction of the cis/middle-Golgi complex. Moreover, study of partly organized Golgi complexes showed that in the process of reorganization the assembly of the cis/middle-Golgi complex preceded the assembly of the trans-Golgi complex (Fig. 9, C–J) (see Discussion).

Discussion

In this study we have examined the disassembly and assembly of the cis/middle and trans parts (i.e., trans-most cisterna and trans network) of the Golgi complex in NRK cells treated with BFA. The approach to examine these problems has been to analyze in parallel the changes in localization undergone by cis/middle- and trans-Golgi membrane proteins in response to BFA. We have assumed that these changes are the result of changes in the organization and integrity of the Golgi compartments in which the proteins reside.

Disorganization of the Golgi Complex and Vesicle-mediated Retrograde Transport

The effects of BFA on the cis- and middle-Golgi are well established (Lippincott-Schwartz et al., 1989; Dom, et al., 1989). In contrast, studies on the response of the trans parts (i.e., trans cisternae and trans network) of the Golgi to the drug have produced contradictory results (Lippincott-Schwartz et al., 1989, 1990; Dom, et al., 1989; Nuchtern et al., 1989; Ulmer et al., 1989; Chege and Pfeffer, 1990). The later studies were performed by examining the effects of the drug on the morphology of the trans compartments and on the relocation of galactosyltransferase and sialyltransferases resident in them. From immunofluorescence microscopy studies of NRK cells treated with BFA and stained either with the monoclonal anti-GMP, antibody or wheat germ lectin, it was concluded that the trans-Golgi was insensitive to the drug (Lippincott-Schwartz et al., 1989). This is in contrast to the results of our studies of NRK cells studied with the antibodies against GMP, and GMP,; we observed a dramatic and rapid effect of BFA on the organization of the trans-Golgi complex (3 min) and its complete disassembly after long incubations (75–90 min) with the drug. The reason for the discrepancy between the results of Lippincott-Schwartz et al. (1989) and ours is not clear. Our results are in agreement with the observations made by Ulmer and Palade (1989) who noted the complete disappearance of the cisternae stack in transformed erythroblastic cells treated with BFA. Also a recent study performed with an antibody against the middle/trans-resident enzyme β-galactosyltransferase (Roth et al. 1986) in human M, cells has shown that BFA promotes the disorganization of the cisternae containing the enzyme (Lippincott-Schwartz et al., 1990). Transfer of sialyltransferase to the ER has been also observed in rat liver H4-II-E-C3 cells treated with BFA and studied by immunofluorescence microscopy (Berger, E. G., personal communication). Biochemical studies on the transport of enzymes from the trans-Golgi to the ER in cells treated with BFA have produced contradictory results. Analysis of the oligosaccharide chains of the α subunit of the T-cell receptor (Lippincott-Schwartz et al., 1989) and the G protein of vesicular stomatitis virus (Doms et al., 1989), extracted from cells treated with BFA did not reveal the presence of sialic acid. In the same line of results the N-linked carbohydrates of the CI-M6P retained into the ER of cells incubated with BFA were found to acquire galactose but only trace amounts of sialic acid (Chege and Pfeffer, 1990). In contrast, analysis of the carbohydrates of glycoporins and of alkaline phosphatase in cells treated with BFA showed high levels of sialic acid (Ulmer and Palade 1989; Takami, et al., 1990). The finding that LIMPs II and III are sialylated while retained in the ER of cells treated with BFA is in agreement with the later results. The discrepant results obtained in the analysis of the translocation of sialyltransferase to the ER could be explained by disparities in the processing of different substrates under the abnormal and different conditions that may exist in the ER of the different cell lines studied. The effect of ER conditions on the activity of sialyltransferases is clearly shown by the differences in pl observed between the forms of LIMP III isolated from normal (pl 4.5) (Barrioconical et al., 1986) and BFA-treated (pl 7.9–8.8) NRK.
Figure 9. Assembly of the *trans*-Golgi complex follows the reconstruction of the *cis/middle*-Golgi complex. Cells were incubated at 37°C for 10 min with 1 μg/ml BFA, and then at 23°C for 40 min with 20 μM nocodazol and 0.1 μg/ml BFA (A and B) before incubation for 90 min at 23°C in drug-free medium (C-J). The cells were stained with the mouse monoclonal anti-GMP<sub>1</sub> antibody (A, C, E, G, and I) and rabbit polyclonal anti-GMP<sub>2</sub> antibody (B, D, F, H, and J) and studied by double-immunofluorescence microscopy. It can be seen that before the removal of nocodazol and BFA, GMP<sub>1</sub> was located to small vesicles scattered throughout the cytoplasm and to the ER (diffuse fluorescence), whereas GMP<sub>2</sub> was found in comparatively large vesicles retained in the vicinity of the nucleus. Removal of both drugs resulted in slow reconstruction of the Golgi complex, as shown by the presence of partially reorganized Golgi complexes in 10-20% of the cells (C-J). It is important that inhibition of the reconstruction of the *cis/middle*-Golgi complex (cells marked with white arrows in C and I) resulted in failure of the cells to organize the *trans* part. The examination of incompletely reconstructed Golgi complexes reveals that in the process of reconstruction the assembly of the *cis/middle*-Golgi complex always precedes the assembly of the *trans*-Golgi complex (E-J). Bars, 15 μm.

Cells (see Fig. 2). In conclusion, so far most of the results from morphological and biochemical studies indicate that BFA promotes the fusion of the *trans*-most cisternae and *trans* network with the ER, and therefore the drug can not act selectively on the *cis/middle* parts of the Golgi complex (Lippincott-Schwartz et al., 1989, 1991; Doms et al., 1989; Chege and Pfeffer, 1990).

The need for a system of retrograde transport that could recover the membranes of the vesicles involved in the transport of molecules from the ER to the Golgi complex, and
help to maintain constant the difference between the surface areas of both organelles, was postulated several years ago (Warren, 1985). More recently, the observation made by Pelham (1988) that soluble ER proteins escaped from the ER to the Golgi complex are recovered from the cis-Golgi, suggests that the system of retrograde transport could be involved in the recovery of proteins. Lippincott-Schwartz et al. (1989) have recently suggested that both the shuttling of transporting vesicles between the cis-Golgi and the ER, and the prevalence of the retrograde transport in cells treated with BFA, might explain the fusion of the cis-Golgi with the ER in these cells. However, the involvement of vesicles in the anterograde transport of proteins and lipids through the entire Golgi complex (Rothman et al., 1984a,b; Balch et al., 1984a,b; Dunphy and Rothman, 1985; Orci et al., 1986, 1989; Pfeffer and Rothman, 1987) extends the recovering problem to the middle and trans parts of the Golgi complex. The existence of a vesicle-mediated, or as recently proposed tubular-mediated (Orci et al., 1991) transport of molecules from the trans- and through the middle- to the cis-Golgi could solve the problem. The existence of such transport could explain the presence of sialic acid in the carbohydrate of GMP<sub>r</sub>, (Yuan et al., 1987) and MG 160 (Gonatas et al., 1989). Moreover, it could also explain the observation that in cells treated with BFA the cis/middle-Golgi is disorganized and fuses with the ER faster than the trans-Golgi. In the presence of BFA the fusion of the trans-Golgi with the ER could be carried out by vesicles or tubules specialized in transporting molecules from the trans- to the middle-Golgi. This fusion could require the expression on the surface of the ER of medial Golgi proteins specialized in the recognition of such vesicles or tubules. According to this model, the fusion of the Golgi complex with the ER would proceed in a cis-trans direction.

Characterizing the Pathway Followed by the Golgi Membrane Proteins in the Reconstruction of the Golgi Complex

The study by double immunofluorescence microscopy of the redistribution of cis/middle and trans proteins occurring after removal of BFA, had the limitation that the organelles involved in the putative transport of Golgi proteins as well as the Golgi precursors could not be characterized. Such characterization should await double label-gold immuno-EM experiments using antibodies against GMPs and markers of the organelles suspected of being involved in those processes. However, the study has disclosed some interesting features of the process of Golgi reconstruction (Fig. 3). The reassembly of the Golgi complex appears to proceed in at least two steps. The first consists in the accumulation of the Golgi proteins into distinct vesicular structures scattered throughout the cytoplasm. During the second step the vesicles are replaced by tubular structures, the long cisternae are assembled, and the Golgi complex is organized. With respect to the first step it is clear that the cis/middle proteins contained in the ER are more rapidly mobilized than the trans proteins after removal of BFA. Furthermore, the ordered back transport of GMPs from the vesicles to the ER suggests that the former are entities distinct from the ER. The earliest vesicles could be transporting vesicles (i.e., transitional) (Palade, 1975; Beckers et al., 1987, 1989) or belong to the intermediate compartment that mediates the transport of vesicles between the ER and Golgi complex (Saraste and Kuismanen, 1984; Schweizer et al., 1988, 1990). Whether cis/middle and trans proteins are transported in the same or in separate closely juxtaposed vesicles, organized in clusters as observed in mitosis (Lucocq and Warren, 1987), or kept in register, as seen in cells treated with taxol (Wehland et al., 1983a), will have to be determined by EM.

With respect to the assembly of the Golgi cisternae, we have noted that it occurs together with a parallel decrease in the number of vesicles containing the GMPs. Whether the latter fuse directly with each other to produce the cisternae we do not know. The compartments forming the Golgi complex (i.e., cisternae, trans network) are probably highly dynamic structures (Griffiths et al., 1989; Kreis et al., 1990) formed and stabilized by the simultaneous operation of segregating mechanisms (involved in separating resident from itinerant molecules and components of neighboring Golgi compartments as well as functionally linked compartments (i.e., ER, intermediate compartment, PLC)), and salvage devices specialized in recovering the molecules mis-sorted during the processes of segregation (Warren, 1985; Pelham, 1988). With respect to this it is possible that the vesicle-mediated systems of anterograde and retrograde transport that function throughout the ER and Golgi pathway might play an important role in the ordered and gradual (see below) formation of Golgi compartments.

An interesting aspect of the reconstruction of the Golgi complex is that the assembly and reorganization of the cisternae always occurs in a discrete area located in the vicinity of the nucleus. The more relevant characteristics of the assembly area are the almost complete exclusion of the ER, as judged by the absence of PDI and the presence of the centrosome. This area probably corresponds to the “zone of exclusion” described by Morré (reviewed in Morré and Ovtracht, 1978) and the one stained with antibodies against the KDEL binding protein (Vaux et al., 1990). The observation that the assembly area remains intact after the complete disassembly of the Golgi complex with the ER raises the possibility that it is permanently occupied by a structural scaffold or by organelles functionally related to the Golgi complex (i.e., prelysosomal compartment). The presence of the centrosome in the vicinity of the assembly area is consistent with evidence that microtubules play an important role in maintaining the location and organization of the Golgi complex (Kupfer et al., 1982, 1983; Wehland and Sandoval, 1983; Sandoval et al., 1984; Turner and Tartakoff, 1989), and of the cis/middle-Golgi complex when assembled under conditions inhibiting the reorganization of the trans elements.

Mechanisms that May Operate in Assembling the Golgi Compartments in a Functional Order

The Golgi complex disrupted at the onset of mitosis is reassembled during cytokinesis. How the organelle is organized with the different compartments arranged in a functional order (Dunphy and Rothman, 1985; Kornfeld and Kornfeld, 1985; Griffiths and Simons, 1986) is an important question that we are still far from understanding. We do not know if formation and arrangement of the compartments occur simultaneously. In either case the compartments could be formed from the same or separate (Lucocq and Warren, 1987; Lucocq et al., 1987) precursors. The arrangement of the compartments in a functional order might require that the
precursor(s), or one of the mature compartments, acts as a primer and template, and that the casting role is assumed in an ordered fashion by the rest of the precursors, or compartments, until the organelle is completed. The mechanism operating in this process could be based on the mutual and specific recognition between molecules expressed on the surface of contiguous compartments. Our results indicate that the cis and middle compartments can be assembled under conditions in which the trans-Golgi complex is inhibited. They also suggest that the reorganization of the trans-Golgi complex requires the previous reconstruction of the cis/middle-Golgi complex. We would like to speculate that the cis, or less likely the middle compartment, could play the role of primer and first template in the process of organization. Implicit in this model is that the assembly of the Golgi complex would proceed in a cis to trans direction.

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