Introduction

The Golgi complex is a very dynamic, highly organized and morphologically unique organelle (for reviews see Farquhar and Palade, 1981; Farquhar, 1985; Pfeffer and Rothman, 1987). It is highly organized and morphologically unique because it consists of a number of membrane-enclosed cisternal elements, i.e. “unstacking”, and the effects were analyzed and quantitated by electron microscopy. In control experiments, isolated, intact Golgi stacks were stable at 4°C and 20°C for ≥1 h; however, some unstacking occurred at 32°C. Treatment of intact Golgi stacks with a variety of proteolytic enzymes resulted in a time- and dose-dependent unstacking of the cisternae, although stacks were resistant to various other proteases. Following liberation from the stack, single cisternae remained flattened with dilated rims. The integrity of intact Golgi stacks was unaffected by treatment with various concentrations and combinations of monovalent and divalent cations, or chelators of divalent cations. Electron microscopic observations of tannic acid- or negatively stained Golgi complexes, revealed the presence of highly structured, intercisternal “bridges”. When seen within intact Golgi complexes, these bridges were only consistently found between closely apposed cisternae and were not observed on dilated rims or secretory vesicles. These bridges, on both intact stacks and physically disrupted cisternae, were rectangular, being ~8.5 nm in width, ~11 nm in height. Treatment with proteases under conditions that resulted in the unstacking of intact complexes also removed these bridge structures. These data show that proteinaceous components are responsible for holding Golgi cisternae together into a cohesive, stacked unit, and identify a candidate bridge structure that could serve this purpose.

Adhesion of Golgi cisternae by proteinaceous interactions: intercisternal bridges as putative adhesive structures

EDWARD B. CLUETT and WILLIAM J. BROWN*

Section of Biochemistry, Molecular and Cell Biology, Biotechnology Bldg, Cornell University, Ithaca, NY 14853, USA

*Author for correspondence

Summary

We have investigated the nature of the component(s) responsible for holding the cisternal membranes of the Golgi complex into a stacked unit. Isolated Golgi complexes were treated with a variety of agents to induce the separation of intact Golgi stacks into single cisternal elements, i.e. “unstacking”, and the effects were analyzed and quantitated by electron microscopy. In control experiments, isolated, intact Golgi stacks were stable at 4°C and 20°C for ≥1 h; however, some unstacking occurred at 32°C. Treatment of intact Golgi stacks with a variety of proteolytic enzymes resulted in a time- and dose-dependent unstacking of the cisternae, although stacks were resistant to various other proteases. Following liberation from the stack, single cisternae remained flattened with dilated rims. The integrity of intact Golgi stacks was unaffected by treatment with various concentrations and combinations of monovalent and divalent cations, or chelators of divalent cations. Electron microscopic observations of tannic acid- or negatively stained Golgi complexes, revealed the presence of highly structured, intercisternal “bridges”. When seen within intact Golgi complexes, these bridges were only consistently found between closely apposed cisternae and were not observed on dilated rims or secretory vesicles. These bridges, on both intact stacks and physically disrupted cisternae, were rectangular, being ~8.5 nm in width, ~11 nm in height. Treatment with proteases under conditions that resulted in the unstacking of intact complexes also removed these bridge structures. These data show that proteinaceous components are responsible for holding Golgi cisternae together into a cohesive, stacked unit, and identify a candidate bridge structure that could serve this purpose.

Key words: Golgi, cisternae, proteins.
efficiently handled by the Golgi complex in order to maintain its steady-state volume. The mechanisms for accomplishing this include vesicular transport in the cis to trans direction, exit of membrane vesicles from the trans-Golgi network (TGN) for destination to other compartments and recycling back to the ER via membrane vesicles and/or free phospholipids. It is remarkable that in the face of this tremendous amount of membrane traffic, the Golgi complex maintains its unique architecture.

While the overall ultrastructural morphology of this organelle is well established, the molecular basis for this unique structure is unknown and, consequently, several important aspects of its structure and function cannot be readily explained. For example: what holds the cisternae together? Why are they flat? And, of what consequence is the stacking of the Golgi complex cisternae is maintained by stable, proteinaceous interactions and we have examined the interactions that link individual cisternae together. Although early studies noted the presence of intercisternal material in the Golgi complexes of certain plants and protists, these materials or deposits did not directly contact adjacent cisternae and therefore did not appear to link cisternae together (Amos and Grimstone, 1968; Mollenhauer, 1965). Franke et al. (1971) investigated a variety of closely apposed membranes using electron microscopy and observed what appeared to be bridge-like continuities between these membranes, but these were not characterized further. Morre and colleagues, using crude lysosomal extracts or amylases followed by physical disruption were able to effect the unstacking of isolated Golgi complexes (Ovtracht et al., 1973; Morre et al., 1983), suggesting that some type of molecular interactions hold the cisternae together. To understand how the Golgi complex architecture is maintained, we have examined the interactions that link individual cisternae together into stacked, parallel arrays, using intact Golgi complexes in vitro. These isolated Golgi complexes maintain their integrity in the absence of other possible cellular interactions and are functionally competent under the appropriate conditions (Balch et al., 1984; Bergeron et al., 1982; Leelavathi et al., 1970). We report here that the stacking of the Golgi complex cisternae is maintained by stable, proteinaceous interactions and we identify previously unknown, protease-sensitive, intercisternal bridges that may serve this purpose.

Materials and methods

Materials

Young, male CD rats (150-200 g) were obtained from Charles River Labs (Wilmington, MA) and fed ad libitum prior to killing. All reagents used were purchased from Sigma Chemical Co. (St. Louis, MO) with the exception of trypsin (Gibco, Grand Island, NY) and dispase (Boehringer Mannheim, Indianapolis, IN).

Isolation of intact Golgi complexes

Intact Golgi complexes were isolated from rat liver by a modification of the method employed by Balch et al. (1984) for cultured cells. Livers excised from freshly killed male Sprague-Dawley rats were minced to a fine puree with a razor blade in several ml of 0.25 M sucrose/10 mM Tris-HCl, pH 7.4 (ST buffer). This and all subsequent procedures were performed at 4°C. Additional ST buffer was added to the puree to make a 20% (w/v) suspension, which was maintained by occasional stirring prior to loading into syringes. The suspension was then passed three times through a Balch/Rothman homogenizer (Balch and Rothman, 1985) equipped with a 0.2460 inch ball bearing, which allowed a calculated chamber clearance of 0.0054 inches. The homogenate was centrifuged at 3000 g for 15 min to pellet nuclei and unbroken cells, the resultant post-nuclear supernatant (PNS) was harvested and an equal volume of 2.3 M sucrose was added to the PNS to make a 1.4 M sucrose final concentration. The PNS (15 ml) was loaded onto the bottom of 38 ml ultracentrifuge tubes (Beckman SW28) and overlaid in succession with 8 ml of 1.2 M sucrose, 8 ml of 1.0 M sucrose, 4 ml of 0.9 M sucrose and ~4 ml of 0.8 M sucrose, all of which contained 10 mM Tris-HCl, pH 7.4. The step gradient is similar to that of Ovtracht et al. (1973). These step gradients were then centrifuged at 90,000 g for 2.5 h in an SW28 rotor. The second band from the top, representing the 0.9/1.0 M interface and containing the highest concentration of intact Golgi complexes (see below), was harvested and used for experiments.

Treatments to isolated, intact Golgi complexes

To investigate the nature of the interactions that hold Golgi cisternae into a cohesive stack, various enzymatic and chemical treatments were applied to samples of isolated, intact Golgi complexes in the hope of finding conditions which would cause the Golgi to “unstack” into individual cisternae. To avoid damage by excessive handling or osmotic fluctuations, reagents were added directly to the freshly harvested Golgi complexes in ~0.95 M sucrose/10 mM Tris-HCl, pH 7.4; this pH was maintained for all experiments.

Various concentrations of proteases were added from 10 mg/ml stock solutions (prepared in 0.9 M sucrose) to 700 µl samples of freshly prepared, intact Golgi complexes and incubated under various conditions of time and temperature. Reactions were stopped by the addition of an equal volume of glutaraldehyde fixative as described below. Protease inhibitors, added in excess to samples, which were then incubated on ice for 15 min prior to glutaraldehyde fixation, did not appear to affect the results and were generally omitted to save time.

Effects of monovalent and divalent cations on the integrity of the intercisternal linkages were also examined. Ca2+ and Mg2+ were added to isolated, intact Golgi complexes to final concentrations of 10 µM or 2 mM in the presence or absence of 1 mM EDTA or 1 mM EGTA for 15 min at 4°C. Golgi complexes were incubated in the presence of EDTA or EGTA under the following conditions: 1-10 mM for 15-60 min at 4°C or 25°C. All of these stock solutions contained 10 mM Tris-HCl, pH 7.4 and were added in samples sufficiently small so as not appreciably to affect the sucrose concentration. NaCl and KCl were added to samples of intact Golgi complexes to various final concentrations (0.01-2.0 M) and incubated at 4°C for 15-60 min. Following the incubations, samples were fixed in glutaraldehyde and processed as described below.

Electron microscopy (EM)

For standard EM observations, samples containing 500-700 µl of treated Golgi complexes were fixed by the dropwise addition of an equal volume of freshly prepared 5% glutaraldehyde/100 mM sodium cacodylate (pH 7.4)/0.25 M sucrose and then incubated on ice for 1 h. Intact and disrupted Golgi elements were harvested by centrifugation at 25,500 g for 30 min, and the resultant pellets were post-fixed in 1% OsO2/100 mM sodium cacodylate (pH 7.4) for 1 h, progressively dehydrated in EtOH and embedded in Spurr’s resin. After curing, each pellet was split in half and re-
Photographic negatives were taken through the entire depth of pellets at room temperature. This stock fixative solution also contained 0.25 M Millonig’s phosphate buffer (pH 7.4) (Millonig, 1961) for 30 min at room temperature. This stock fixative solution also contained 0.25 M sucrose. After formation of a pellet by centrifugation at 25,500 g for 30 min, the samples were incubated overnight at room temperature with 1% tannic acid/1% glutaraldehyde/0.1 M Millonig’s phosphate buffer (pH 7.4). The following day, the samples were post-fixed in OsO₄ as above, washed in distilled water, stained with 1% aqueous uranyl acetate, dehydrated in EtOH and embedded in Spurr’s resin.

Negatively stained Golgi complexes were performed as follows: approximately 10 pl of a sample containing Golgi elements was placed on a 400-mesh copper grid coated with a carbon support film and incubated at room temperature for 15 min. Prior to staining, samples were post-fixed in 10% aqueous phosphotungstic acid (PTA) (pH 7.4). The droplet of 2% aqueous phosphotungstic acid (PTA) (pH 7.4) was placed on the grid for 15-30 s. Again, the excess was removed and the grid was rinsed three times in 2% PTA, with blotting either after each rinse or only after the last one. All EM samples were examined and photographed on a Philips 301 electron microscope.

Quantitation of intact and unstacked Golgi complexes

EM was used to assay the “unstacking” of intact Golgi complexes. Photographic negatives were taken through the entire depth of pellets at ×8900, prints were made to a final enlargement of ×14750 magnification and the photographs were visually examined. In rat liver, the average Golgi complex consists of 3-5 flattened and closely apposed cisternae (Bruni and Porter, 1965). Therefore, in these studies, any isolated Golgi complex or set of cisternal elements was considered to be “intact” if it contained ≥ 3 flattened, closely apposed cisternae. We defined closely apposed as follows. In our preparation of intact Golgi complexes, which were fixed immediately upon isolation, the distance between adjacent cisternae in a stack was estimated to be 10 ± 5 nm (± 1 s.d.). This was determined by measuring the distance between outer leaflets of apposed cisternae taken at 102 random sites on 10 different Golgi stacks from micrographs at magnifications ranging from ×13000 to ×75000. The intercisternal distances measured here agree well with those determined for Golgi complexes in situ (Amos and Grimstone, 1968; Dalton and Felix, 1954). For the purposes of determining the “intactness” of isolated Golgi complexes it was not practical to measure the intercisternal spacing of all possible cisternal pairs; therefore, cisternae were judged to be closely apposed if they exhibited an intercisternal spacing of ≤30 nm along at least half the length of the cross-sectional profile; cisternae with a spacing of ≥30 nm along more than half the length the cross-sectional profile were judged to be not closely apposed and therefore not a part of an intact complex.

In many of our experiments, the results will be expressed as the “% Intact Golgi Stacks (IGS)”. Simply put, % IGS is the proportion of total Golgi cisternae counted that are contained within intact stacks, i.e. within stacks containing ≥3 closely apposed cisternae. In practice, the % IGS was determined as follows. All cross-sectional images of Golgi cisternae on micrographs were scored and the total number of cisternae was determined. We also counted the number of stacks with ≥3 closely apposed cisternae (no. IGS). For any one micrograph (or set of micrographs from one experimental condition), the maximum number of IGS (Max. no. IGS) that could be constructed from the total individual cisternae actually counted will be the total number cisternae divided by the average number of cisternae per intact stack. The average number of cisternae per stack was determined by counting the number of cisternae within numerous isolated stacks and the sum of all these cisternae was divided by the number of stacks counted. This number was found to be 3.7 cisternae per intact stack (n = 196 intact Golgi stacks counted). For example, if on one micrograph we counted 200 total cisternae and 27 intact stacks, then the Max. no. IGS will be:

\[
\text{Max. no. IGS} = \frac{200}{3.7} \approx 54
\]

and, therefore, the percentage of intact Golgi stacks found on micrographs will be:

\[
\% \text{ IGS} = \frac{\text{no. IGS}}{\text{Max. no. IGS}} \times 100
\]

In some cases, quantitative comparisons between treatments performed on different Golgi preparations was desirable; however, the % IGS of control samples varied somewhat between preparations. In these cases, the results will be expressed as intact Golgi stacks as a percentage of controls “IGS, % control”, which was determined by the following equation:

\[
\text{IGS, % control} = \frac{\text{no. of IGS (experimental)}}{\text{no. of IGS (control)}} \times 100
\]

Results

Isolation of intact Golgi complexes

Golgi complexes were isolated from rat liver by a modification of a procedure that yields structures that are morphologically comparable to in vivo structures. In preliminary experiments, assays of marker enzymes showed the 0.9/1.0 interface to be highly enriched in galactosyl transferase (29% of total recovered activity), whereas only low levels (<2% of total activity) of glucose 6-phosphatase (rough endoplasmic reticulum), 5'-nucleotidase (plasma membrane) and α-glucosidase (lysosomes) were present. EM of this material revealed that the preparation was dominated by intact Golgi complexes (Fig. 1A). A typical isolated intact Golgi stack when viewed in cross-sectional profile contained 3-5 closely apposed cisternae, with dilated rims and associated tubules and vesicles. Because the intact Golgi complexes are randomly oriented in these preparations, not all will be seen in cross-sectional profile. However, since Golgi cisternae were most reliably identified in cross-sectional profile, only these images of intact and disrupted Golgi elements were considered for quantitation. From micrographs like those shown in Fig. 1A, the percentage of Golgi cisternae contained within intact Golgi stacks (% IGS) was determined by scoring all identifiable Golgi cisternae. In this figure, a variety of Golgi profiles were easily recognizable, scored and used to determine the % IGS as described in the figure legend.

Some of the contaminating membranes in these preparations were probably derived from the plasma membrane, smooth endoplasmic reticulum and endosomes. Although
Proteolytic unstacking of intact Golgi complexes into single, flattened cisternae. (A) Control preparation and (B) Golgi complexes after treatment with proteinase K. In this typical experiment, isolated rat liver Golgi preparations were harvested, divided into aliquots and proteinase K was added (to 0.1 mg/ml final concentration) (B) or not (A). Both samples were incubated for 1 h at 4°C and then fixed and prepared for electron microscopy. The control sample consisted of numerous intact Golgi stacks, each containing ≥ 3 closely apposed cisternae (see arrows). In addition to the intact Golgi stacks, several large, smooth-surfaced membranes are seen as circular profiles. It is apparent that many of these membranes are not contaminants, but are, in fact, swollen regions of Golgi cisternae (see arrowheads). In the intact preparation, very little “unstacking”, i.e. the presence of single cisternae or stacks of 2 cisternae, is evident. By way of an example, quantitation of the Golgi cisternae in A revealed that 76% of the total number of cisternae (n = 83) were contained within intact Golgi stacks (IGS), i.e. those containing ≥ 3 closely apposed cisternae (see arrow 1). Only 14% were found as single cisternae (see arrow 1) and 9% as stacks of 2 cisternae (see arrow 2). Following treatment with proteinase K (B), very few intact stacks were observed and, instead, numerous single cisternae had been liberated. Quantitation of this micrograph revealed that of the total cisternae counted (n = 73), 90% were found as single cisternae, 8% in stacks of 2 cisternae and ~1% as IGS. Bars, 1 mm.

Fig. 1

the 1.0/1.2 interface of our gradients contained significant GT activity, we determined by EM that the highest number of intact Golgi complexes was found at the 0.9/1.0 interface. The 1.0/1.2 interface primarily contained rough ER and some individual Golgi cisternae and fragments of intact stacks (data not shown). We also found that the Balch/Rothman ball-bearing homogenizer was particularly useful for isolating intact Golgi complexes from rat liver and was superior to conventional homogenization methods that we tried.

Effects of temperature on stability of isolated intact Golgi complexes

When Golgi complexes were isolated and kept at 4°C, >95% remained intact (containing ≥ 3 closely apposed cisternae) after 1 h (Fig. 2). Even at 25°C, ~90% of the Golgi complexes remained intact. However, if isolated Golgi complexes were incubated at 32°C, there was a progressive loss of intact stacks, so that by 1 h only 60% remained intact. Therefore, most of the subsequent experiments were carried out at 4°C or 25°C.

Effects of hydrolytic enzymes on Golgi stacking

We applied various proteases to isolated, intact Golgi complexes to examine the potential role of proteins in the maintenance of Golgi stacking. As stated above, the vast majority of Golgi cisternae in control samples were contained within intact stacks (Fig. 1A). However, incubation of Golgi complexes with 0.1 mg/ml of proteinase K for 1 h at 4°C produced a marked separation or unstacking of the cisternae (Fig. 1B). Even those cisternae not completely disjoined were no longer closely apposed, but noticeably separated from the others in the stack. These single cisternae retained their characteristic flattened morphology with dilated rims, indicating that they must have been liberated from intact

Fig. 2

Effects of temperature on the stability of intact Golgi complexes. Isolated Golgi complexes were divided into aliquots, one was fixed immediately to serve as a control, and the others were incubated for 1 h at the temperatures shown. The extent of Golgi stacking was determined from electron micrographs as described in Materials and methods. Data are expressed as the number of intact Golgi stacks (IGS), as a percentage of controls, and each bar represents an average of 752 cisternae counted. Controls in this case were fixed immediately upon isolation.

Golgi stacks. In addition, it also appears that the tubular and vesicular structures associated with intact Golgi complexes were separated from flattened cisternae following protease treatment (cf. Fig. 1A, B). The loss of intact Golgi complexes and concomitant increase in the number of single cisternae produced by protease treatment was qualitatively obvious in this figure, a fact borne out when the % IGS was quantitatively determined and plotted in Fig. 3. Other proteases such as dispase, subtilisin, elastase and chymotrypsin all produced marked unstacking of the cisternae compared to untreated samples. Conversely, a variety of other proteases were found to have no effect on the intact Golgi complexes under the conditions employed (Fig. 3). However, assays of protease activity employing azocoll as a substrate revealed that all proteases were active under these incubation conditions (data not shown). Furthermore, it is unlikely that the proteases which do not unstack Golgi cisternae cannot gain access to the region between cisternae, since the dimensions of most of the proteases are significantly smaller than the distance between cisternae. Of the serine proteases, chymotrypsin and elastase unstack cisternae while trypsin does not (Fig. 3).

This disruption of intercisternal linkages by various proteases occurred in a time- and concentration-dependent manner (Fig. 4). Unstacking took place fairly rapidly with a t1/2~25 minutes for proteinase K, chymotrypsin, elastase and subtilisin, at final protease concentrations of 0.1 mg/ml. Unstacking in all cases was essentially complete by 1 h at 4°C using these concentrations of protease while control Golgi complexes retained >90% of their integrity. Varying the concentrations of either chymotrypsin, which has a narrow specificity, or protease K, with a broader specificity, revealed that the amount of protease necessary to achieve half-maximal unstacking in 1 h was ~0.001–0.01 mg/ml for each (Fig. 5). From these results, it appears that a protein-mediated linkage is involved in holding cisternae together.

In addition to proteases, we attempted to reproduce the
original observation that amylase could affect the unstacking of intact Golgi complexes in vitro (Morre et al., 1983; Ovtchark et al., 1973). Using several different sources of amylase (only one of which is shown), we were unable to find any effect on the integrity of isolated Golgi stacks (Fig. 3). It should be noted that in our studies no additional mechanical disruption was employed.

It is conceivable that the population of single Golgi cisternae observed following protease treatment, as shown in Fig. 1B, result not from the unstacking of cisternae, but from the complete degradation of all but one Golgi cisterna (for example, loss of all but the cis-most cisterna from each stack). To resolve this issue, isolated Golgi complexes were assayed before and after proteolytic unstacking to determine the relative amounts of two antigens found in different cisternal compartments of the Golgi. One marker, a luminal antigen recognized by monoclonal antibody 10E6, is only detected in cis cisternae (Wood et al., 1991); the other is the mannose 6-phosphate receptor (M6PR) which is concentrated in the TGN but can also be found within cisternal stacks (Wood et al., 1984, 1988). Degradation of all but one particular cisterna should result in a disproportionate loss of at least one of the above markers from these pelleted Golgi membranes. Analysis by immunoblotting of Golgi samples showed that, following treatment with proteinase K (0.1 mg/ml) for 60 min at 4°C, conditions which cause substantial unstacking (Figs 1B,3), there were only 33% and 18% losses of 10E6 and M6PR, respectively, from pelleted Golgi membranes. We conclude that the proteolysis experiments are most consistent with an unstacking and liberation of all cisternae which remain morphologically intact as single, separated elements.

Stacking of Golgi cisternae is independent of divalent cations

In some instances, divalent cations are required for the protein-mediated attachment or apposition of biological membranes. For example, the stacking of thylakoid membranes is cation-sensitive (Izawa and Good, 1966). Consequently, it is conceivable that the population of single Golgi cisternae observed following protease treatment, as shown in Fig. 1B, result not from the unstacking of cisternae, but from the complete degradation of all but one Golgi cisterna (for example, loss of all but the cis-most cisterna from each stack). To resolve this issue, isolated Golgi complexes were assayed before and after proteolytic unstacking to determine the relative amounts of two antigens found in different cisternal compartments of the Golgi. One marker, a luminal antigen recognized by monoclonal antibody 10E6, is only detected in cis cisternae (Wood et al., 1991); the other is the mannose 6-phosphate receptor (M6PR) which is concentrated in the TGN but can also be found within cisternal stacks (Wood et al., 1984, 1988). Degradation of all but one particular cisterna should result in a disproportionate loss of at least one of the above markers from these pelleted Golgi membranes. Analysis by immunoblotting of Golgi samples showed that, following treatment with proteinase K (0.1 mg/ml) for 60 min at 4°C, conditions which cause substantial unstacking (Figs 1B,3), there were only 33% and 18% losses of 10E6 and M6PR, respectively, from pelleted Golgi membranes. We conclude that the proteolysis experiments are most consistent with an unstacking and liberation of all cisternae which remain morphologically intact as single, separated elements.

High-magnification observations of intercisternal linkages

The above studies indicate that proteinaceous links are responsible for holding Golgi cisternae into a stacked unit. When we attempted to visualize such linkages by high-magnification EM in samples prepared by standard procedures, i.e. glutaraldehyde fixation followed by osmication, the intercisternal spaces seemed largely devoid of material. To visualize better membranes and possible structural details, isolated, intact Golgi complexes were fixed with glutaraldehyde in the presence of tannic acid prior to osmication. These studies revealed a surprising array of small, discrete intercisternal “bridges” of uniform size, which extended from the cytoplasmic side of one cisternal membrane to the adjacent partner (Fig. 7A and B). Moreover, these bridges were clearly not membranous and often appeared to be regularly spaced along much of the length.
Adhesion of Golgi cisternae

of two closely apposed cisternae. These bridges could easily and consistently be seen only between closely aligned Golgi membranes and were not frequently found on any dilated rims, associated vesicles or anastomosing tubules. Nor were they seen on any contaminating membranes, even if these membranes were joined.

We then sought to visualize these structures in vivo using cultured cells. As in vitro, when Golgi complexes were processed by regular EM procedures in vivo, very little intercisternal structure was discernible, especially in the presence of the cytoplasmic ground substance. However, when clone 9 hepatocytes were processed with tannic acid as above, we observed similar distinct, rectangular bridges which nearly always connected two apposed cisternae (Fig. 8). This material was localized to the same regions of the Golgi membranes as in isolated organelles and it did not appear to run parallel to the cisternae. These bridges seem to be a general feature of Golgi structure, since they were also seen in bovine testicular cells and MDBK cells (data not shown). Furthermore, these bridges could be visualized both in vivo and in vitro, whether tannic acid was employed prior to or after osmication, or if phosphate or cacodylate buffer was substituted for Millonig’s phosphate buffer (data not shown).

To obtain an alternative view of these bridge structures, isolated, intact Golgi complexes were physically disrupted (by rigorous centrifugation, freeze-thawing or vigorous pipetting) to produce vesicles and tubules. On some of these fragmented Golgi membranes, there was a striking collection of rectangular nodules extending from the membrane surface (Fig. 7C, D). These nodules were identical in both size and shape to the bridges seen between adjacent cisternae of intact stacks. In some cases, bridges and nodules could be found both between apposed membranes and on the continuous free surfaces of cisternae that were only partially adherent (Fig. 7D).

To confirm that these were indeed real structures and not fixation artifacts, bridges were visualized using negative staining techniques (Fig. 7E-G). Visualization of the bridges in intact Golgi preparations proved difficult, since the complexes were deposited en face and collapsed on the grids, obscuring any nodules from side view. Therefore, it was necessary to disrupt the stacks physically to expose the adjoining faces and increase the possibility of visualizing the bridges protruding into the field. Again, this procedure produced single cisternae and small vesicles; however, the negative stain also revealed discrete rectangular nodules, which could be seen extending from the surface of the membrane into the field. These nodules were less frequently seen, as might be expected, since the disrupted membranes would have to fall onto the grid in just the right orientation.

We measured the dimensions of both the tannic-acid-stained bridges and the PTA-stained nodules in order to compare the structures seen by the two methods. Measurements were made of the dimensions of bridges between two apposed membranes in both tannic-acid and PTA-stained preparations and of the nodules that were found to extend free from a membrane surface. As seen in Fig. 9, the dimensions of the bridges and nodules compare favorably in the two preparations with a mean height (distance extending from the membrane) of 11 ± 2.8 nm and width of 8.5 ± 2.7 nm. Histograms of the measurements show little variation in dimensions of the items in the various samples, although the width measurements were more variable. Although we have lumped the measurements of bridges and nodules together, there were small differences between the two. The mean width and height of bridges were 10.7 nm × 8.4 nm.
Adhesion of Golgi cisternae

...Adhesion of Golgi cisternae... whereas those of the nodules were 12.6 nm × 9.3 nm. These small differences probably result from the fact that it was easier to measure nodules, because of the lack of apposed membranes and unidentified cellular material.

Fig. 7. Visualization of intercisternal bridges within isolated, intact Golgi complexes and on membranes of disrupted stacks. The samples (A-D) were all stained by the tannic acid procedure and then thin sectioned. (A, B) Isolated, intact stacks stained with tannic acid reveal in even greater detail the nature of intercisternal bridges (arrows), which were often found in groups and many times appeared to be regularly spaced. (C) Golgi stacks were disrupted by physical means (three cycles of freeze-thawing in C and F) or low concentrations of detergent (0.01% CHAPS in D) and then stained with tannic acid to reveal electron-dense "nodules" (arrowheads) on single cisternae and bridges (arrows) on still-attached cisternae. The nodules seen in C and D are structurally identical to the bridges seen between cisternae of intact stacks. They are rectangular and extend outward, perpendicular to the membrane. In (D) discrete bridges and nodules can be found on the same membrane, with the bridges still present between the two apposed membranes (arrows) and the nodules on the free surface (arrowheads). (E-G) Disrupted Golgi membranes visualized by negative staining using PTA to show the presence of discrete rectangular nodules identical to those seen by tannic acid staining and thin section EM. Bars, 100 nm.

Protease treatment removes the intercisternal bridges

To investigate the nature of these intercisternal bridges, intact Golgi complexes were treated with various proteases under conditions that cause unstacking and then tannic acid stained. Following treatment with proteinase K for 1 h at 4℃, conditions which result in >90% unstacking, the liberated cisternae appeared smooth and largely devoid of bridges or nodules (Fig. 10). Occasionally, a single cisterna could be found which contained one or a few bridge structures that apparently had not been completely removed. Treatment with proteases that did not affect unstacking had no effect on the intercisternal bridges (Fig. 10D).

Discussion

The Golgi complex is perhaps the most morphologically unique organelle found in all eucaryotic cells, consisting of a stack of flattened membranous cisternae which are held together as a cohesive unit. One major conclusion of this
study is that individual Golgi cisternae are joined together into an intact stack by proteins that form intercisternal linkages. Over the years, electron-microscopic observations of fixed Golgi complexes, both in vitro and in vivo, have occasionally revealed the presence of membranous continuities between the lipid bilayers of adjacent cisternae (Bracker et al., 1971; Tanaka et al., 1986), suggesting that Golgi cisternae may be held together by these membrane bilayer connections. We believe that our experiments render this unlikely, since such bilayer linkages should be resistant to mild proteolytic treatments - conditions which effectively cause the unstacking of the Golgi complex. Although the stability of these connections is difficult to ascertain, these membrane continuities are infrequently seen, making it more unlikely that they are the primary adhesive forces. Early studies by Morre and coworkers (1983) demonstrated that the Golgi complex could be unstacked by a combination of treatment with amylase and mechanical disruption. Using a variety of amylases, but no physical force, we were unable to produce any unstacking (Morre et al., 1983). Thus, it is unlikely that the early amylase-induced unstacking was due to amylase itself but was perhaps the result of contaminating proteases as Morre suggested.

Following their proteolytic release from the stack, the individual cisternae maintained their flat, plate-like morphology and remained so for long periods of time at 4°C (≥90 min). In our experiments, we did find that prolonged protease treatment (>2 h) would eventually lead to a loss of recognizable cisternal morphology (unpublished data). It is worth reiterating that in our studies, “unstacking” was strictly defined as the liberation of flattened cisternae from an intact Golgi stack. When these cisternae became separated from the stack, they remained flattened and were thus easily recognized as originating from an intact Golgi complex as shown in the original studies of mechanically disrupted stacks (Morre et al. 1983; Ovtracht et al., 1973). Any treatment that altered the morphology of single cisternae; for example, those that caused vesicle or vacuole formation, were considered uninterpretable. Therefore, we view the liberation of single flattened cisternae as a necessary first step before making any conclusions about Golgi complex unstacking in vitro. The persistence of the flattened morphology of liberated cisternae suggests that each individual cisterna has its own inherent structural framework, perhaps located within the lumen where it is protected from exogenously added proteases. Thus, we conclude that the architecture of an individual Golgi stack is maintained by at least two different mechanisms: (1) those that hold indi-

---

**Fig. 10.** Proteolytic removal of intercisternal bridges and membrane nodules from Golgi membranes. Intact Golgi complexes were isolated, treated with proteinase K (A-C) or V-8 protease (D) exactly as for Fig. 3 (0.1 mg/ml protease for 1 h at 4°C) and then processed for tannic acid staining. Whereas physically disrupted Golgi membranes exhibited numerous rectangular appendages (Fig. 8), proteolytically unstacked Golgi membranes were very smooth and essentially devoid of rectangular bridges and nodules (A-C). Occasionally, a remaining nodule can be seen on these membranes (C, black arrowhead). However, bridges can clearly be seen after treatment with V-8 protease, which does not unstack Golgi cisternae (D). White arrows indicate several of these intercisternal bridges. Bar, 100 nm.
individual cisternae as flattened, plate-like structures and (2) those that bind flattened cisternae together into a stack.

Since divalent cations have been shown to affect membrane interactions (Poste and Allison, 1973), they may also play a role in the maintenance of cisternal stacking. However, we found that the presence or absence of divalent cations had little effect on Golgi stacking in vitro. Furthermore, the isolated Golgi complexes withstood a wide variation in ionic strength, ranging from ≤15 mM to 1 M KCl or NaCl. A clear interpretation of these results is difficult, since proteins, membranes, and the interactions among these components can be influenced by many factors, but in conducting these experiments we were struck by how difficult it was to affect the unstacking of intact Golgi complexes in vitro.

In an investigation of structural components that could account for the proteinase sensitivity of stacked Golgi cisternae, we were able to visualize structural elements that were strikingly rectangular in shape and fairly uniform in size. Almost exclusively found between the cisternae within intact stacks, these structures formed bridges which spanned from one membrane to the adjacent partner. When seen in cross-section or negative stain on disrupted Golgi membranes, the structures formed distinct nodules. Neither the bridges nor nodules were readily visible using standard preparation methods for transmission EM of thin sections, but required tannic acid enhancement. We believe that these bridge structures are real and not artifacts, for the following reasons: (1) bridges were found between Golgi cisternae in vivo and in vitro; (2) they were found in both positive staining of thin sections and negative staining of isolated Golgi membranes; (3) the dimensions of the bridges seen by tannic acid staining between intact Golgi membranes were identical to those nodules seen on disrupted Golgi membranes by both positive and negative staining; (4) the bridges were removed following protease treatment under conditions that result in Golgi unstacking; and (5) the heights of the nodules and bridges were exactly the same as the distance between adjacent cisterna, i.e. ~11 nm. We conclude that the bridges seen between cisternae of intact stacks are the same structures as the nodules seen protruding from membranes of physically disrupted Golgi stacks.

It should be noted that these intercisternal nodules can be clearly distinguished from both clathrin- and non-clathrin-coated structures. The intercisternal nodules are significantly larger and especially wider, than the bristles seen on clathrin-coated membranes (Pearse and Robinson, 1990). Furthermore, they are found almost exclusively on the planar faces of cisternae, even if the cisternae are fragmented, rather than on the more curved ends where coated vesicles appear to form. The nodules are also more widely spaced along membranes compared to the more compact, regularly spaced bristles of clathrin coats.

Little previous work has been done to identify structures that could serve as adhesive elements between Golgi cisternae. However, Franke et al. (1971) surveyed the intermembrane cross-bridges between a number of different organelles including Golgi cisternae. Interestingly, a reexamination of those Golgi micrographs clearly reveals the presence of rectangular inter-cisternal structures, which appear to be identical to the bridges reported here (see Fig. 5, of Franke et al., 1971). To our knowledge, no subsequent studies were devoted to these structures.

At present, we do not know the molecular nature of these intercisternal Golgi bridges. However, a variety of membrane-to-membrane connections between various organelles have been observed by EM. For example, light harvesting complex II (LHC II) links thylakoid membranes together (for review, see Murphy, 1986). Bridge-like structures have been observed which appear to link secretory granules to the plasma membrane (Morimoto et al., 1990). However, we were particularly struck by biochemical and morphological similarities of these Golgi “bridges” to the calcium-release channel (CRC) of skeletal muscle and the inositol 1,4,5-trisphosphate (IP$_3$) receptor of smooth and non-muscle cells, molecules which mediate the release of Ca$^{2+}$ from the sarcoplasmic reticulum (SR) and the ER, respectively (Hymel et al., 1988; Ferris et al., 1989). Of particular interest to us, is the finding that the CRCs form the “foot” processes which join the terminal ends of the SR to the central transverse-tubule membrane to form the muscle triad (Mitchell et al., 1983). There is very strong biochemical and morphological evidence that the CRC links these membranes together (Mitchell et al., 1983; Ferguson et al., 1984; Saito et al., 1984; Chadwick et al., 1988) and the structure that is formed is not unlike that of a Golgi stack with its bridges. For example, the distance between the cytoplasmic surface of the SR membrane and the apposed transverse-tubule membrane and the height of the CRC, is ~12 nm. In our studies we found the distance between adjacent Golgi cisternae and the height of the bridge structures to be 11-12 nm. In addition, the visualization of both the CRC of triads and the bridges of Golgi cisternae is greatly enhanced by tannic acid staining (Saito et al., 1984).

Whether the intercisternal Golgi bridges identified here are related at all to the CRC remains to be seen. It is also possible that structures other than the bridges link Golgi cisternae together. However, the results reported here suggest an obvious line of investigation for identifying the adhesive proteins responsible for the unique architecture of the Golgi complex.

The authors thank Margot Szalay and Marian Strang for excellent technical help with the enormous amount of thin sectioning required for this work. This work was supported by N.I.H. grant AM 37249 (to W. J. B.).

**References**


Bergmann, J. E. and Singer, S. J. (1983). Immunoelectron microscopic studies of the intracellular transport of the membrane glycoprotein (G) of...


(Received 6 May 1992 - Accepted 4 August 1992)