MEMBRANE INTERACTIONS BETWEEN ADJACENT MUCOUS SECRETION GRANULES

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ABSTRACT

In primate goblet cells, the membranes of adjacent mucous granules form contact areas which appear as extensive pentalaminar fusion sites in thin sections. In freeze-fracture replicas, the same membrane areas are smooth, except for a few 6-8-nm particles which adhere to the E face. These protein-poor membrane interaction sites are relatively long-lived, and it is proposed that further stimulus may be required to trigger membrane fission.

KEY WORDS mucous · secretion · membrane · goblet · fusion

The process of secretion by exocytosis involves the rapid contact and fusion of a secretion granule membrane with the plasma membrane, followed by fission of the fused membranes (24, 25, 27). Comparable short-lived contact and fusion also occurs between the membranes of adjacent intracellular secretion granules in several types of cells which have been induced to rapidly release their stored products in response to specific stimulation (2, 3, 7, 8, 11, 13, 16, 18, 24). In stimulated cells, the interaction and fission of adjacent secretion granule membranes accelerates the process of exocytosis by allowing the contents of multiple secretion granules to be released through a single opening at the cell surface. The transient granule-to-granule contacts of stimulated pancreatic acinar (24), pancreatic islet (3), and mast cells (16, 18) appear in thin sections as pentalaminar structures, 12.5-13.5 nm wide, comprised of two unit membranes whose cytoplasmic leaflets have fused to form a single dense line. A freeze-fracture study of stimulated mast cells reported that intramembrane particles are excluded from the contact areas formed by adjacent granules, and that fission of membranes readily occurs in these smooth areas (18). However, in the absence of stimulation, the secretion granules of all these cell types generally lie separated from one another by cytoplasm (7, 11, 13, 15, 16), and do not form pentalaminar contacts (15, 16, 18, 27).

In contrast, the large secretion granules of mucous cells normally lie so closely packed in the cytoplasm that even in the absence of stimulation or rapid secretion, extensive regions of adjacent granule membranes come into close contact. The membranes involved in these large and relatively long-lived contact areas form pentalaminar structures comparable to those transiently seen in stimulated cells of other types (15, 31). Mucous granule contact areas, like those of stimulated mast cells (18), are labile, and readily fuse or break during fixation (31). However, it is not known whether the close contacts of granule membranes in unstimulated mucous cells are simply a reflection of membrane crowding, or whether they represent specific membrane interactions. The methods of freeze-fracture and conventional electron microscopy were applied to the goblet cells of primate large intestine to investigate the nature of mucous granule contact areas.

MATERIALS AND METHODS

During a study of human rectal glycoprotein secretion (22), biopsies of human rectal mucosa were obtained after receiving informed, written consent from four...
healthy adult volunteers. Mucosal samples, 2-3 mm across, were obtained from a multipurpose biopsy tube (Quinton Instruments, Seattle, Wash.). In addition, mucosal samples from the rectosigmoid junction were obtained from two healthy adult monkeys, *Macaca mulatta*, under nembutal anesthesia.

For conventional electron microscopy, slices of human mucosa were fixed for 1 h at 4°C in a solution of 4% formaldehyde, 5% glutaraldehyde, 0.4% CaCl₂, and 5% sucrose in 0.07 M Na cacodylate buffer, pH 7.4 (14). They were postfixed for 1 h in 1% OsO₄ in 0.1 M cacodylate buffer, and stained en bloc for 1 h in 1% uranyl acetate in maleate buffer (14). Monkey mucosal slices were fixed for 4 h at 23°C in a solution of 2% formaldehyde, 2.5% glutaraldehyde, 0.4% CaCl₂, and 5% sucrose in 0.07 M Na cacodylate buffer at pH 7.4 (14). They were postfixed in 1% OsO₄ as described above, and stained en bloc for 16 h at 4°C with 1% uranyl acetate in Na acetate buffer. All tissues were embedded in Epon-Araldite, and thin sections were stained with uranyl acetate and lead citrate.

For freeze-fracture, both human and monkey mucosal slices were fixed for 30-45 min at 23°C in the 2% formaldehyde, 2.5% glutaraldehyde solution described above, and were subsequently trimmed and equilibrated with 20% glycerol in 0.1 M cacodylate buffer for 1-2 h. Tissue blocks were mounted on gold disks, rapidly frozen in the liquid phase of partially solidified Freon 22, (Virginia Chemicals, Inc., Portsmouth, Va.) and stored in liquid nitrogen. Specimens were fractured in a Balzers freeze-etch device (model BA 360, Balzers AG, Balzers, Principality of Liechtenstein) at a stage temperature of −115°C, replicated with platinum-carbon without etching, cleaned with methanol and bleach, and mounted on either 300-mesh copper grids or Formvar-coated single-slot grids. Replicas and sections were examined with a JEOL 100 B electron microscope. Illustrations of freeze-fractured specimens are presented with the shadow direction approximately from bottom to top.

The biopsy procedures did not induce rapid release of mucus by goblet cells because the great majority of cells were filled with intact mucous granules at the time of fixation (Fig. 1). Furthermore, goblet cells in the human biopsy samples, adjacent to those described here, were pulse-labeled in organ culture with [³H]glucosamine, and by use of autoradiography, they were shown to slowly and continuously transport and secrete mucus (22).

**RESULTS**

Large intestinal goblet cells, similar to mucous cells throughout the body, are characterized by a large accumulation of closely packed mucous granules whose maximum diameter (~3 μm) is relatively uniform throughout the cell. 1-μm Epon section, periodic acid-Schiff-iron hematoxylin stain. × 1,000.

Large accumulation of closely packed mucous granules extending from the supranuclear region to the luminal surface of the cell (Fig. 1). The apical granules are usually separated from the luminal plasma membrane by a thin layer of fibrillar cytoplasm which is interrupted where individual granules or groups of granules are in contact or fusing with the plasma membrane in the course of exocytosis (23, 24, 32). Peripheral mucous granules are consistently separated from the lateral

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**FIGURE 1** Epithelium lining a human rectal crypt. Goblet cells are filled with intact, closely packed mucous granules whose maximum diameter (~3 μm) is relatively uniform throughout the cell. 1-μm Epon section, periodic acid-Schiff-iron hematoxylin stain. × 1,000.

**FIGURE 2** Electron micrograph of the lateral aspect of a goblet cell near the midpoint of the theca. A portion of a columnar cell is seen at right. The curved membranes of peripheral mucous granules (MG) are separated from the lateral plasma membrane (PM) by a layer of cytoplasm (cyto) rich in microfilaments. Medially, (to the left), granule membranes are flattened where they make direct contact with the membranes of neighboring granules. (Bar, 0.1 μm. × 58,500).

**FIGURE 3** Freeze-fracture appearance of a goblet cell, near the midpoint of the theca. At the right, portions of the P face of the goblet cell plasma membrane (GC-P), and the E face of a columnar cell plasma membrane (CC-E) are exposed. The peripheral cytoplasm (cyto) of the goblet cell is cross-fractured, and the fracture plane has followed the curved membranes of peripheral mucous granules (MG-E) × 47,400.
plasma membrane by an uninterrupted layer of
cytoplasm (the "theca" of classical histologists)
which is rich in microfilaments, and which also
contains cisternae of rough endoplasmic reticu-
lum, free ribosomes, and an occasional mitochon-
drion (Fig. 2). The gently curved membranes of
peripheral mucous granules are separated from
these organelles by intervening cytoplasmic ma-
trix. More centrally, mucous granule membranes
are flattened where they are pressed together, and
intervening cytoplasm is limited to the angular
interstices of three or more granules (Figs. 2, 4).
Therefore, although noncontact regions of a
mucous granule membrane abut either peripheral
or intergranular cytoplasm, contact regions di-
rectly abut the membrane of an adjacent mucous
granule. In thin sections, the areas of close apposi-
tion of mucous granule membranes in primate
intestinal goblet cells most often appear as penta-
laminar structures ~13.5 nm thick (Fig. 4). In
some cases, contact areas are trilaminar, appear-
ing as a single unit membrane shared by two
adjacent granules, and in other cases, the mem-
branous barrier between granules is discontin-
uous, fragmented, or absent. However, in optim-
ally fixed cells, the majority of contact areas are
intact and are pentalaminar.

In replicas of freeze-fracture mucous granule
membranes, the arrangement and density of intra-
membrane particles on the fracture faces of con-
tact areas are distinctly different from those of
noncontact areas (Figs. 3, 5). In noncontact areas,
both membrane faces are characterized by the
presence of 6.0–12.5-nm particles which are usu-
ally distributed at random. The majority of these
particles is associated with the E face (Fig. 6). The
sizes and number of E-face particles are compara-
table to those of the E face of the plasma membrane
(Fig. 3). The sparse P-face particles are usually
randomly arranged (Figs. 5, 6), but occasionally,
loose clusters of 10–12-nm particles are present
(Fig. 7).

In contrast, contact areas appear as facets on
the spherical granules. They are large, round, flat
regions whose P face is consistently smooth and
particle-free (Figs. 5–7). The E face of contact
areas contains a few small 6–8-nm particles, but
the larger particles, typical of the E face of non-
contact areas, are absent (Figs. 6, 7). In a single
contact area, the fracture plane often shifts from
the interior of one granule membrane to the in-
terior of its closely apposed neighbor, exposing the
P face of one membrane and the E face of the
other (Fig. 7). The majority of granules observed
in freeze-fracture replicas of well-fixed cells are
intact, membrane-bounded spheres whose contact
areas appear to consist of two tightly apposed
membranes (26).

The maximum diameter of the mature mucous
granules that fill the theca is about 3 μm (Fig. 1).
By ultrastructural and freeze-fracture criteria,
contact areas are as frequent and extensive among
mature granules near the base of the theca as they
are among those near the apical, secretory surface
of the cell. However, close membrane apposition
is not limited to mature granules. Groups of
smaller granules, comparable in size to those asso-
ciated with the supranuclear Golgi complex, are
found congregated just within the layer of micro-
filaments that defines the lower border of the
theca. They consistently form pentalaminar con-
tacts with one another, and may coalesce here to
form larger granules.

DISCUSSION
The pentalaminar contact regions, observed be-
tween adjacent mucous granules within the pri-
mate goblet cells described here, are indistinguish-
able from those previously described in thin sec-
tions of mucous cells of the rat sublingual (15) and
the cat submandibular glands (31). In stimulated
serous exocrine (24), endocrine (3), and mast cells
(16, 18), such regions are thought to represent a
brief stage in a rapid sequence of membrane con-

FIGURE 4 Thin section of mucous granules in the central region of a goblet cell. Cytoplasm (cyto) is
excluded and adjacent cytoplasmic leaflets appear to be fused (arrows, and inset) at the point where
adjacent granule membranes come into close contact. The resulting pentalaminar structures are ~13.5 nm
wide. (Bar, 0.1 μm. × 75,600; inset, × 137,200.)

FIGURE 5 Freeze-fracture replica of mucous granules in a region comparable to that shown in Fig. 4.
Flat, disk-shaped contact areas (C) are easily distinguished from noncontact areas. Small triangular areas
of cross-fractured cytoplasm (cyto) lie in the angles between granules. × 41,100.
Figure 6 A large mucous granule has been stripped away by the fracture process, leaving behind part of the cytoplasmic leaflet (P face) of its limiting membrane which adheres to the membranes of two underlying granules. The E faces of the underlying granule membranes are exposed. Three flat contact areas (C) are visible. The P face of contacting membranes (left and center) are essentially particle-free, but the E face (right) contains a few small particles. The membranes of noncontact areas (NC) abutting cytoplasm (cyto) contain randomly distributed 6-12-nm particles, most of which are associated with the E face. × 66,600.

Figure 7 A large contact area (C) and portions of two others are visible. In the central one, the E face of the underlying granule membrane is seen through a "window" in the smooth P face of the overlying membrane. The E face of this area, as well as the extensive E face at right, contains scattered 6-8-nm particles. In noncontact regions (NC), some P-face particles form loose aggregates (arrows). × 73,300.
contact, fusion, and fission (1, 27). In stimulated mast cells pentalaminar granule-to-granule fusion sites are smooth and particle-free in freeze-fracture replicas (18), and therefore they are presumed to be areas of lipid membrane from which membrane proteins have been excluded (4, 6, 33). Similarly, freeze-fracture replicas of isolated chromaffin granules, induced by calcium ions to aggregate in vitro, show particle-free contact areas which correspond to pentalaminar contacts in thin sections (29). Granule-to-granule contacts, as seen either in freeze-fracture replicas or in thin sections, are comparable to the particle-free, pentalaminar contact sites formed by a secretion granule membrane with the plasma membrane in a wide variety of cells engaged in exocytosis (5, 7, 16, 18, 24, 25, 27, 28, 31).

Contact, fusion, and fission of adjacent secretion granules in serous cells of pancreas (13), parotid (2), von Ebner's (11) and submaxillary glands (15), as well as pancreatic islet (3), adrenal chromaffin (8), and mast cells (5, 16, 18), seem to occur only after a granule membrane has fused with the plasma membrane, and has somehow acquired the ability to fuse with the membrane of an underlying granule. However, the membrane interactions in goblet cells occur throughout the cell, and may not require the fusion of apical granule membranes with the plasma membrane as a prerequisite.

It has been postulated that a calcium-mediated flight or lateral displacement of membrane proteins occurs as membranes which are capable of fusion approach each other (1, 5, 8, 18, 27–29). The intramembrane particle arrays which may predetermine and delimit fusion sites in plasma and mucocyst membranes of Tetrahymena (28) have not been observed during exocytosis in the mammalian secretory cells (7, 18, 26). In mammalian cells, pentalaminar contacts of interacting membranes have been reported to be completely particle-free, and in the mast cell, they are even devoid of antibody or lectin receptors (18). On this basis, it has been suggested that most, if not all, membrane proteins may be displaced from contact areas before the occurrence of fusion of cytoplasmic leaflets (18). In goblet cells, although the pentalaminar contact areas formed by adjacent secretion granule membranes are devoid of intramembrane P-face particles, a subpopulation of 6–8-nm particles is consistently retained on the E-face. This finding should ideally be confirmed in goblet cells prepared by the rapid-freeze method (12). However, we tentatively conclude that some, but certainly not all, membrane proteins are displaced from mucous granule contact areas.

It is thought that closely apposed, protein-depleted lipid bilayers may readily interact, and may then spontaneously undergo molecular rearrangements that lead to the breakdown of the fused bilayers (1, 18, 27). Indeed, in serous, endocrine, and mast cells, granule-to-granule fusion sites have proven to be difficult to study because they are very short-lived (27). In contrast, the extensive interactions which occur between adjacent mucous granules are relatively long-lived. Although particles are displaced, and cytoplasmic leaflets appear to be fused, fission of the membranes does not immediately follow; the process seems to be arrested in the contact or fusion stage. This suggests that translocation of certain intramembrane proteins may be necessary for membrane fusion, but not sufficient for membrane fusion to occur. Intermingling and breakdown of fused membranes may require additional events at the cytoplasmic aspect of the interacting membranes.

It is known that in the absence of stimulation, intestinal goblet cells release mucus at a low rate. In the rat, an individual granule spends at least 4 h en route from the Golgi region to the lumen (23), and in the human, a longer time is required (22). Nevertheless, a variety of substances, including neurotransmitters (9, 19), bile salts (20), bacterial toxins (21, 30), and immune complexes (34), are known to induce very rapid mucus release. Some of these may be mediated by the formation of cyclic nucleotides (9, 17). Our results suggest that goblet cells may be primed for secretion by the preformation of multiple and extensive membrane fusion sites. Further stimulation could trigger a cell-wide fission of membranes and rapid release of mucus without the rupture of the cell apex and loss of cytoplasm that has been previously postulated (10, 15).

However, it is possible that many of these particle-poor contact areas never reach the fission stage at all, and that the events involved in their formation are reversible. Individual mucous granules may move at different speeds toward the apical cell membrane (22), and therefore, they may repeatedly lose and reform contact sites en route.

It is clear that the large and stable membrane contacts of mucous secretion granules may pro-
vide a valuable model for the further study of membrane interactions in the process of exocytosis, whether they are reversible or not.

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