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Intracellular pathway of a mucin-type membrane glycoprotein in mouse mammary tumor cells*,†

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ABSTRACT

Epiglycanin, a mucin-type glycoprotein, was found by immunoelectron microscopy to be located in cytoplasmic compartments, as well as at the surface of the TA3-Ha mammary carcinoma ascites cell. The glycoprotein was identified by means of gold-labeled secondary antibody bound to a primary anti-epiglycanin monoclonal antibody or by lectins specific for carbohydrate structures in epiglycanin. The primary antibody recognized a glycopeptide component containing a β-D-(1→3)-D-GalNAc chain attached to a serine or threonine residue. Two routes to the cell surface from epiglycanins's first-recognized location in the *trans*-Golgi reticulum were suggested. Its presence in vesicles, which fuse with the cell surface, would explain the presence of epiglycanin as an integral membrane protein. Some of these observed vesicles, however, may be endocytotic in character. Epiglycanin was also found in large multivesiculate sacs which were observed on occasion to be open to the extracellular milieu. This finding, as well as the observed fusion of small vesicles from the *trans*-Golgi network with the sacs, strongly suggested exocytotic migration for the large sacs. Endocytotic migration may also be possible, although incubation of viable cells with gold-labeled antiepiglycanin antibody resulted in minimal uptake within the intracellular sacs, and incubation with [^{125}I]-epiglycanin under metabolic conditions resulted in no detectable uptake of radiolabel by the cells.

INTRODUCTION

Many types of cancer cells have been found to possess high concentrations of mucin-type glycoproteins at their surfaces[1]. These glycoproteins, characterized by the presence of multiple *O*-glycosylally-linked carbohydrate chains and extended rod-like conformations, have been identified in human colorectal[2], ovarian[3], breast[4], laryngeal[5], and pancreatic[6] carcinoma cells, as well as in cells of rat mammary carcinoma[7], hepatoma[8], and neuroblastoma[9], and in cells of a mouse mammary carcinoma[10,11]. This mouse glycoprotein, epiglycanin, was first reported[12] by our laboratory in 1972, and

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evidence for its probable role in tumor cell protection was first presented[13] in 1973. We have suggested that the capability of the TA3-Ha and TA3-MM[14] mouse ascites cells to proliferate in foreign mouse strains[15] and in certain foreign species[16] was due to the masking of cell surface histocompatibility (H-2) antigens by epiglycanin molecules[15]. This function is consistent with biochemical[17] and immunological[15] data. More recently, evidence suggesting that mucin-type glycoproteins in other tumors may also play important roles in hindering immune surveillance mechanisms, such as blocking the action of host immune effectors[18], and shielding tumor cells from harmful environmental factors, such as intolerable changes[5] in pH, has been suggested. Important roles for mucin-type glycoproteins in the metastatic process have also been suggested[19]. Much current interest in mucin-type glycoproteins as tumor markers[20,21] stems from the possible use of their monoclonal antibodies in diagnostic tests, tumor imaging, and immunotherapy in human cancer.

Labeled precursors[8] and gold-labeled lectins[22] have been used in studies of the biosynthesis and location of mucin-type glycoproteins within the cell. In this report, by the use of gold-labeled monoclonal antibodies specific for epiglycanin, we demonstrate the intense surface labeling for the protein surrounding the entire periphery on some cells, though on other cells a more patchy, albeit locally, intense label was apparent. We demonstrate, by immunocytochemistry, two possible pathways taken by epiglycanin to the cell surface from the *trans*-Golgi network where it is first observed.

EXPERIMENTAL

Cells. — The origin and conditions for growth of the TA3-Ha and TA3-St ascites cells have been previously described[11]. Cells were harvested seven days following intraperitoneal injection of 8–12 week old male A/WySn mice (Jackson Laboratory, Bar Harbor, ME, U.S.A.) with 1×10^5 TA3-Ha or 1×10^6 TA3-St ascites cells. Animals were maintained in the animal housing facility at the Boston Biomedical Research Institute.

Materials. — Endo-*N*-acetyl-α-D-galactosaminidase[23] (O-Glycanase, 1.0 U/mL) was obtained from Genzyme, Inc. (Boston, MA, U.S.A.). Wheat germ agglutinin (WGA) was obtained from Sigma Chemical. Co. (St. Louis, MO, U.S.A.), and *Ricinus communis* agglutinin was purchased from EY Biochemicals (San Mateo, CA, U.S.A.). Specific immunocolloidal gold conjugates and streptavidin-gold conjugate of 5-nm diameter were purchased from Amersham Corp. (Arlington Heights, IL, U.S.A.). Biotinylated Jacalin was obtained from Vector Research (Burlingame, CA, U.S.A.).

Antibodies. — Monoclonal antibodies to epiglycanin were prepared as will be described[24]. In brief, C57BL/6J mice were immunized with asialoepiglycanin. Three days following hyperimmunization, spleen cells were fused with SP-2 mouse myeloma cells. After cloning and subcloning hybridoma cells, cloned cultured cells were injected (10^6 cells/mouse) into [CByB6] F1 hybrid mice (Jackson Laboratory), which had been preinjected with Pristane. Immunoglobulins were purified by precipitation in 50% saturated $(NH_4)_2SO_4$ solution and fractionation on a Sephadex G-200 gel-exclusion column[25].

Specimen preparation for cryo-electron microscopy. — After fixation for 1 h in 0.1% glutaraldehyde–2% formaldehyde at 4°, the cells were centrifuged off gently (800 *g*) resuspended in 3% gelatin (Sigma), and fixed for a further 60 min. The solid pellet was cut up into small cubes of ~1 mm^3 and prepared for immunoelectron microscopy[26].

Preparation of gold–lectin conjugates. — Gold colloids consisting of 5-nm and 15-nm particles were made by the "borohydride method" and "citrate method", respectively[27]. The conjugates were stabilized by lectins that were added in precise amounts, as judged by a colorimetric assay[28]. The colloid–lectin conjugate was stabilized with 0.5% poly(ethylene glycol) and centrifuged off at 15 000*g* for 45 min in the case of the 15-nm conjugate, and 30 000*g* for 1 h for the 5-nm conjugate. The supernatant was removed and the pellet gently resuspended in 0.1M phosphate-buffered saline (PBS), pH 7.4, containing 0.01% NaN_3, and stored at 4°. By use of these methods, it was found that the gold particles were sufficiently homogeneous to permit omission of differential centrifugation on a sucrose gradient to separate particles of different sizes.

Sectioning and labeling for immunoelectron microscopy. — Sections (80–110-nm thick) were cut on dry glass knives with a Sorvall MT5000 ultramicrotome fitted with an FS1000 cryochamber at −110 to −130°. The sections were lifted in a small droplet of 1.5M sucrose solution and placed on Formvar–carbon-coated grids (80 mesh/cm). The sugar solution was then removed by three washes with PBS and the sections were washed three times with PBS containing 0.5% bovine serum albumin (BSA) and 0.15% glycine, pH 7.4 (Buffer A). This was followed by a 30-min incubation with purified nonspecific goat IgG (50 μg/mL) at 25° and three additional washes with Buffer A. All the preceding steps were designed to ensure minimal, nonspecific reaction to the antibody. Sections were then incubated for 30 min with the primary marker (1–2 μg/mL), *i.e.*, an antiepiglycanin monoclonal antibody (AE-3) of the IgM class or a lectin [either WGA or *Ricinus communis II* lectin, conjugated to a 5- or 15-nm gold particles (10 μg/mL), or biotinylated Jacalin (10 μg/mL)].

The sections were washed six times (5 min/wash) with Buffer A. After incubation with the monoclonal antibody, sections were incubated with goat antimouse IgM coupled to a 5-nm gold particles for 30 min at 25°. In the case of the biotinylated Jacalin, labeled sections were incubated in streptavidin conjugated to 5-nm gold particles for 30 min at 25°. The sections incubated with lectin–gold markers were immediately washed and mounted, as described below, except that for double-labeling studies, sections were sequentially labeled. The sections were stained with 1% uranyl acetate for 2 min and mounted in a thin film of 1.25% methylcellulose (Fluka Chemical Co., Donkokomo, NY), allowed to dry, and examined at 60 kV with a Philips EM300 electron microscope, at a magnification of 10–40 000, using a 30-μm objective aperture.

Incubation of thin sections with enzymes. — Thin, frozen sections were mounted on grids and incubated with a solution of endo-*N*-acetyl-α-D-galactosaminidase (20 μL) (10 U/mL) in 0.1M Tris–maleate buffer, pH 6.8, at 25°. Incubations were continued for periods ranging from 30 s to 4 h. After removal of the enzyme by washing, the sections were immunolabeled as described above.

Labeling of intact TA3-Ha and TA3-St ascites cells for both external masking and internalization. — Viable cells were washed with PBS and incubated in PBS containing 0.5% BSA, pH 7.4, and monoclonal antibody AE-3 (1.0 μg/mL) at 4° for up to 2 h. The cells were washed thoroughly in PBS at 4° and incubated in a gold-conjugated second antibody specific to the AE-3 IgM class (5-nm probe, Amersham) for a further 60 min. Both labeled and control cells were then washed with PBS (4°) to remove any unbound, gold-conjugated antibody, and subsequently incubated for 1 h at 4° or 37° prior to being fixed in 2.5% glutaraldehyde and prepared for electron microscopy[29].

Absorption of radiolabeled epiglycanin by viable TA3-Ha cells. — Viable TA3-Ha ascites cells (>98% viable, Trypan Blue dye exclusion) at two-fold dilutions, ranging from 2.0×10^7 to 2.5×10^6 cells, in Dulbecco's Minimum Essential Medium (Gibco, Grand Island, NY, U.S.A.) with bovine calf serum (10%)(Flow Labs, McLean, VA, U.S.A.) (1.0 mL), were placed into 2-mL sterile plastic tubes. [^{125}I]Epiglycanin (15 μL), prepared as previously described[30], was added to each tube at 4°. The tubes contained 15 ng of labeled epiglycanin, with radioactivities ranging from 6500 to 6900 d.p.m., and were incubated with shaking at 37° for 2 h. After centrifugation, the supernatant solutions were removed, leaving a suspension containing the cell pellet (~ 50 μL). The cell pellets were washed once with PBS (1.0 mL), again leaving a residue of approximately 50 μL. The radioactivities in the two supernatant solutions and in the cell pellets were determined in a Beckman Gamma 5500 counter.

RESULTS

Immunoelectron microscopy using Epon and cryosections to demonstrate surface labeling of TA3-Ha and TA3-St cells. — At low magnification, the TA3-Ha cell appears as a large mononucleate cell with extensively long microvilli, as described previously[31,32]. At high magnification, other ultrastructural features become apparent (see Fig. 1). Large mambrane-enclosed sacs containing microvesicles are often seen within the cytosol, but may also be seen to be open to extracellular space (Fig. 1). A fuzzy coat consisting of abundant, long macromolecular filamentous structures can be resolved on the surface of the TA3-Ha cell (arrows).

Although about 20% greater in diameter than the TA3-Ha cell, the TA3-St cell shares many of its morphological characteristics[31]. However, the micro-extensions constituting the TA3-St cell surface consist mostly of folds and ridges, in contrast to the long, regularly spaced microvilli of the TA3-Ha cell. Also, following similar fixation, the fuzzy surface-coat seen on the TA3-Ha cell is not evident on the TA3-St cell.

Ultrathin cryosections of the TA3-St cell line showed no significant labeling with the antiepiglycanin antibody, AE-3, either at the cell surface (Fig. 2A), or within the cell itself. No membrane systems were labeled, nor was labeling of the virus particles or of the large, multivesiculate bags apparent. When TA3-St cells were labeled with the same immunogold probe prior to plastic embedment and sectioning, rare tufts of labeled material were observed at the surface of a very few cells (Fig. 2B).

Fig. 1. Epon sections of a TA3-Ha cell stained with uranyl acetate and lead citrate showing a micron-sized sac open to the extracellular medium, which appears to be bursting and liberating its contents, including vesicles, into the external environment. Epiglycanin is present on the cell surface (solid arrows). Immunocytochemistry results presented in subsequent figures show that epiglycanin antigen is present within the sacs as well as on the cell surface (solid arrows). Mouse mammary tumor virus or Rauscher leukemia virus is also present within the endoplasmic reticulum (open arrows) (bar = 0.2 μm).

In striking contrast, TA3-Ha ascites cells showed extensive labeling with AE-3 at the cell surface, either in plastic sections prepared by the same method as described above, or in ultrathin cryosections (Fig. 2C). Labeling was generally continuous around an entire cell profile or, in some cell populations, patchy, though locally intense, labeling occurred, which was not associated with any particular cell structure. For example, microvilli showed the same intensity of labeling as the general surface. However, when closely apposed cells showed regions of intercellular adhesion, epiglycanin labeling was absent (Fig. 2D).

When cryosections of the TA3-Ha ascites cell were examined with gold-conjugated WGA (Fig. 2E), *R. communis* lectin–gold (Fig. 2F), or gold-labeled Jacalin, the same homogeneous surface-labeling pattern was observed as was found with the anti-epiglycanin antibodies. Double labeling with the *R. communis* lectin and WGA showed these lectin receptors to be intermixed over the entire cell surface.

In order to learn whether the specificity of the monoclonal antibody AE-3 used in these experiments was similar to that previously found with purified epiglycanin[33], sections of fixed cells were treated with endo-*N*-acetyl-α-D-galactosaminidase[23] at a concentration of 1 U/mL in 0.1M Tris–maleate buffer, pH 6.8. After incubation for as

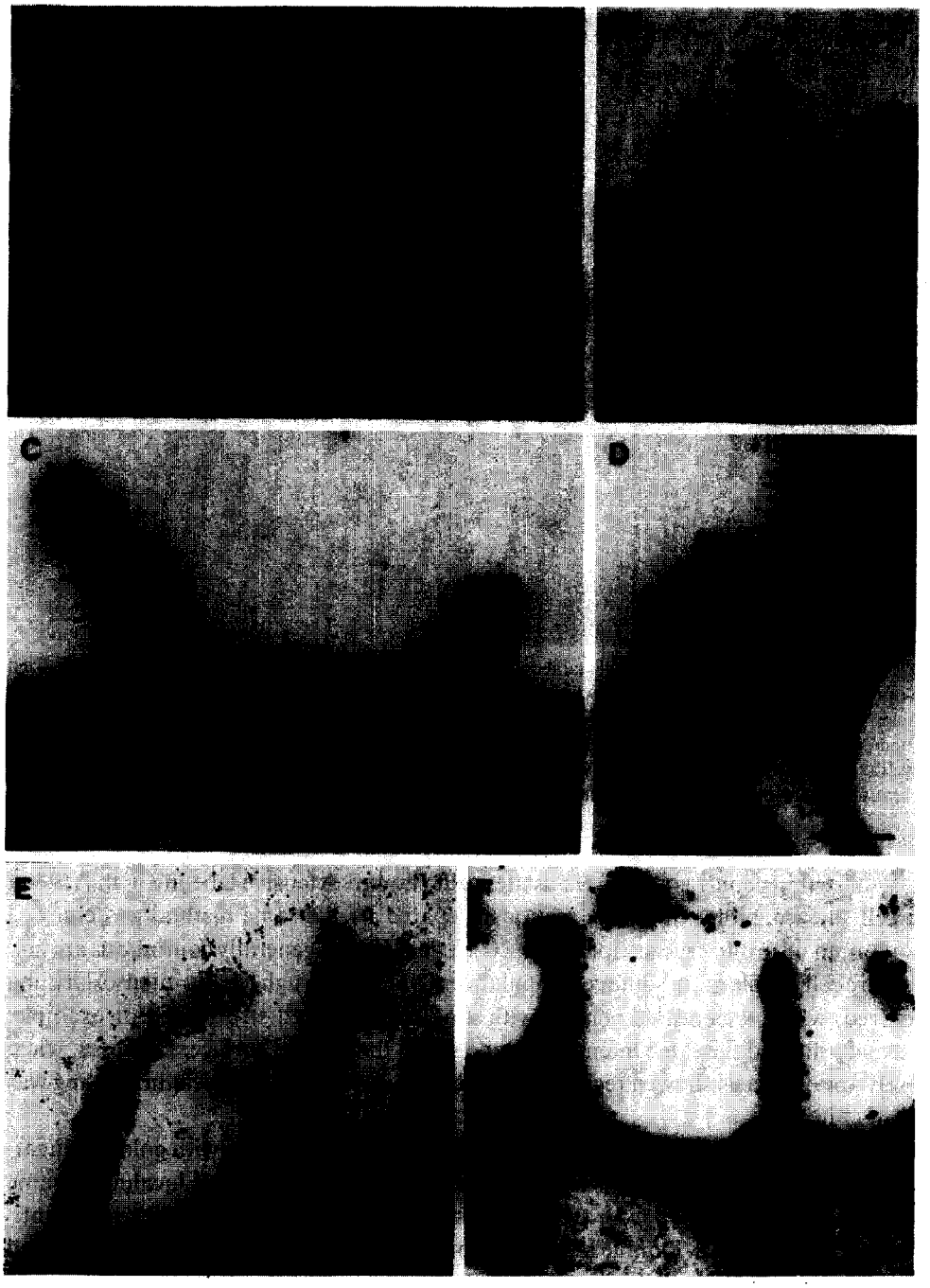

Fig. 2. (A) Thin-frozen section of a TA3-St cell which has been labeled with an antiepiglycanin monoclonal antibody showing no antibody binding (bar = 0.2 μm). (B) Epon section of a TA3-St cell showing a small tuft labeling positively for epiglycanin (arrow) (bar = 0.2 μm). (C) Thin-frozen section of a TA3-Ha cell showing significant surface labeling with antiepiglycanin antibody (bar = 0.2 μm). (D) Thin-frozen section of a cell junction (arrows) between two TA3-Ha cells showing no labeling for epiglycanin in the region of the junction (bar = 0.2 μm). (E) Thin-frozen section of a TA3-Ha cell labeled with WGA showing labeling of the external surface (bar = 0.2 μm). (F) Thin-frozen section of a TA3-Ha cell showing extensive surface labeling with *Ricinus communis* lectin (bar = 0.2 μm).

short a period as 60 s, no labeling either at the cell surface or in the cytoplasm was observed.

Viruses similar in appearance to Rauscher leukemia virus and mouse mammary tumor virus, both of which have been previously identified in this cell[11], are seen in the extensive membranous structures in both TA3-Ha and TA3-St cells (Fig. 1, open arrow).

Release of epiglycanin to the external environment. Uptake or absorption of [^{125}I]epiglycanin by viable TA3-Ha cells. — No significant uptake of [^{125}I]epiglycanin was observed in tubes containing viable TA3-Ha cells in concentrations from 2.5 to 20 $\times$ 10^6 cells/mL. Radioactivity in the cell pellets ranged from 4.0 to 8.5% of the total radioactivity detected in both pellets and supernatants.

Figure 3A shows an Epon section of coated pits at the cell surface. Epiglycanin is labeled in these structures at the externum of the cell. This implies that epiglycanin intermixed with transmembrane receptors whose transport from the cell surface occurs in coated vesicles. By surface labeling of the cells at 4° and subsequent incubation for 1 or 2 h at 37°, repeated attempts were made to show whether internalization of epiglycanin occurs. Under these conditions, the cells showed fewer coated pits than those incubated at 4°, and only a very limited amount of immunolabeled epiglycanin was seen within the cell, either in coated vesicles or within the large multivesiculate bodies. This

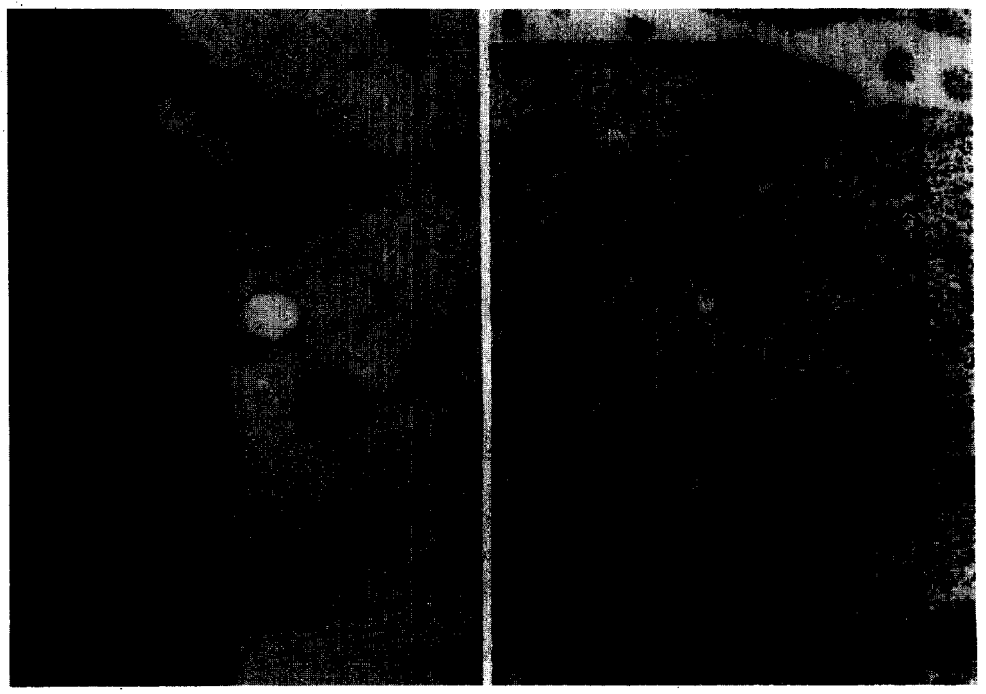

Fig. 3. Thin-Epon sections of TA3-Ha cells: (A) Coated pits are seen to fuse with the cell surface (arrows); gold markers labeling epiglycanin are also seen here, implying an intermixing of surface receptors for the antibody (bar = 0.2 μm). (B) Vesicles (0.1 μm; arrows) are seen in the vicinity of the *trans*-Golgi network (bar = 0.2 μm).

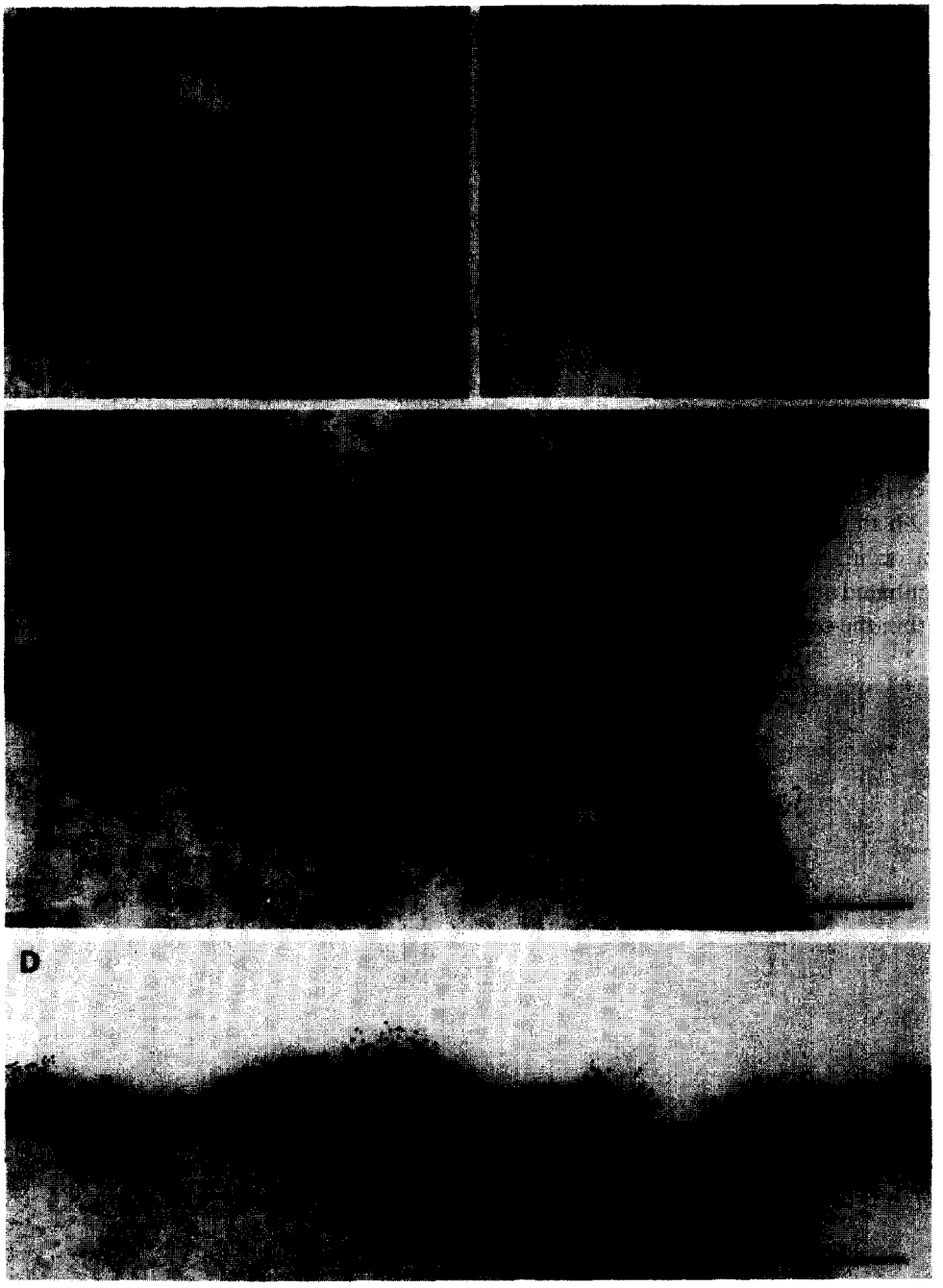

Fig. 4. Thin-frozen sections of TA3-Ha cells showing antiepiglycanin antibody-labeled structures at various stages of transport to the sell surface: (A) Typical labeling at the Golgi apparatus is shown, while the Golgi cisternae remain unlabeled (closed arrows). The immediately proximal compartment, the *trans*-Golgi network, is strongly labeled (open arows) (bar = 0.2 μm). (B) Another example of *trans*-Golgi network showing the intense label found in this locale (bar = 0.2 μm). (C) A cross-section suggesting transport from the *trans*-Golgi network to the cell surface along a radial pathway (bar = 0.1 μm). (D) Fusion of vesicles which label positively with antiepiglycanin antibody in the process of fusing with the plasma membrane (arrows) (bar = 0.2 μm).

suggests that very little if any internalization of surface-located epiglycanin does occur.

Figure 3B shows vesicular structures about 0.1 μm in diameter, probably interconnected to and forming part of the *trans*-Golgi network (TGN) region (see arrows)[34]. When ultrathin frozen sections of TA3-Ha cells were incubated with anti-epiglycanin antibodies (Fig. 4A), these vesicles were immunolabeled, whereas the Golgi cisternae remained negative. No labeling was apparent over the rough endoplasmic reticulum.

Apart from labeling in the TGN, labeling in cryosections also was found within other cytoplasmic vesicular structures (Fig. 4B), some of which appeared to be in the process of fusing with the cell-surface membrane (Fig. 4 C,D). These vesicles showed intense labeling with the antiepiglycanin antibody. Extensive labeling occurred also in the large sacs (3 μm) (Figs. 5A, 6A, and B) which appeared to contain some small (0.1 μm) vesicles. Close examination of the labeled material within the sacs revealed that the label is predominantly on the outside of the vesicles, or is free in intervesicular spaces, rather than within the vesicles. Not all of the sacs were labeled.

In contrast, WGA and Jacalin labeling was universally heavy within all sacs (Figs. 5C and 6C), although the WGA labeling was more intense than the Jacalin one. Labeling with WGA-gold paralleled that observed with the monoclonal antibody (Fig. 5C), inasmuch as TGN and the *trans*-Golgi cisternae were strongly labeled. In addition, WGA labeled the medial and *trans*-cisternae of the Golgi apparatus, which were not immunolabeled by antiepiglycanin. Labeling of the Golgi apparatus by WGA–gold took the form of occasional foci rather than a dense concentration of label, as seen in the vesicles or at the cell surface (Fig. 5D). Labeling with Jacalin mimicked the AE-3 monospecific IgG labeling more closely than did labeling with WGA, inasmuch as the Golgi apparatus remained unlabeled (Fig. 6D), although the TGN was labeled (Fig. 6E). Futhermore, the Jacalin labeling was of an intensity similar to that of the AE-3 labeling.

The large sacs were occasionally observed at the cell surface, open to the extracellular milieu (Figs. 1 and 6A). At this point, the sacs appeared to be discharging their contents from the cell. Figs. 6A and 6B illustrate a sac positively labeled with gold–AE-3. At low magnification (Fig. 6A), the sac was seen to have been extruded almost entirely outside the cell, as compared with a sac which is almost entirely inside the cell (Fig. 1). The labeling for epiglycanin is clearly associated with the sac contents (Fig. 6B). Efforts to show whether internalization of labeled antibody occurs were attempted repeatedly by surface labeling of cells at 4° *in vitro*, followed by incubation for up to 2 h. These experiments were unsuccessful as only minimal labeling (1–2 gold grains/sac) was found within the cells, inplying that only a very limited uptake of epiglycanin from the cell surface occurred in culture.

DISCUSSION

Viewed by electron microscopy, both as isolated molecules[14,35] and as cell membrane-attached molecules[29], epiglycanin towers over other membrane glycoproteins in

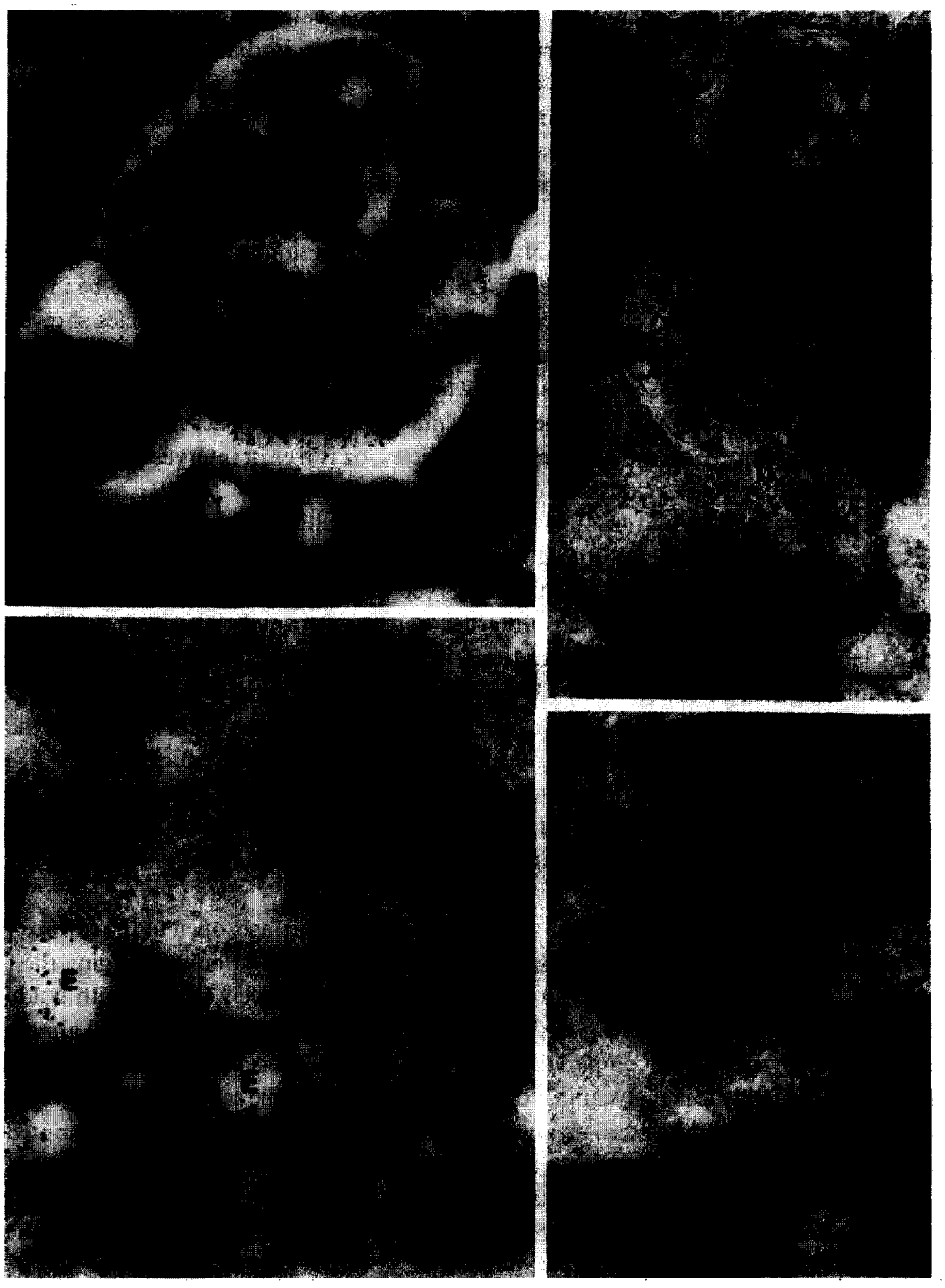

Fig. 5. Thin-frozen sections of TA3-Ha cells: (A) A large sac containing small vesicles, many of which, but not all, are labeled with antiepiglycanin antibody (bar = 0.2 μm). (B) A large sac in which the internal components are largely labeled with WGA (bar = 0.2 μm). (C) Other compartments labeled with WGA are the *trans*-Golgi network (arrows) and possibly endosomes (E) (bar = 0.2 μm). (D) The Golgi apparatus shows significant uptake of WGA label (M, medial cisternae; T, *trans*-cisternae) (bar = 0.2 μm).

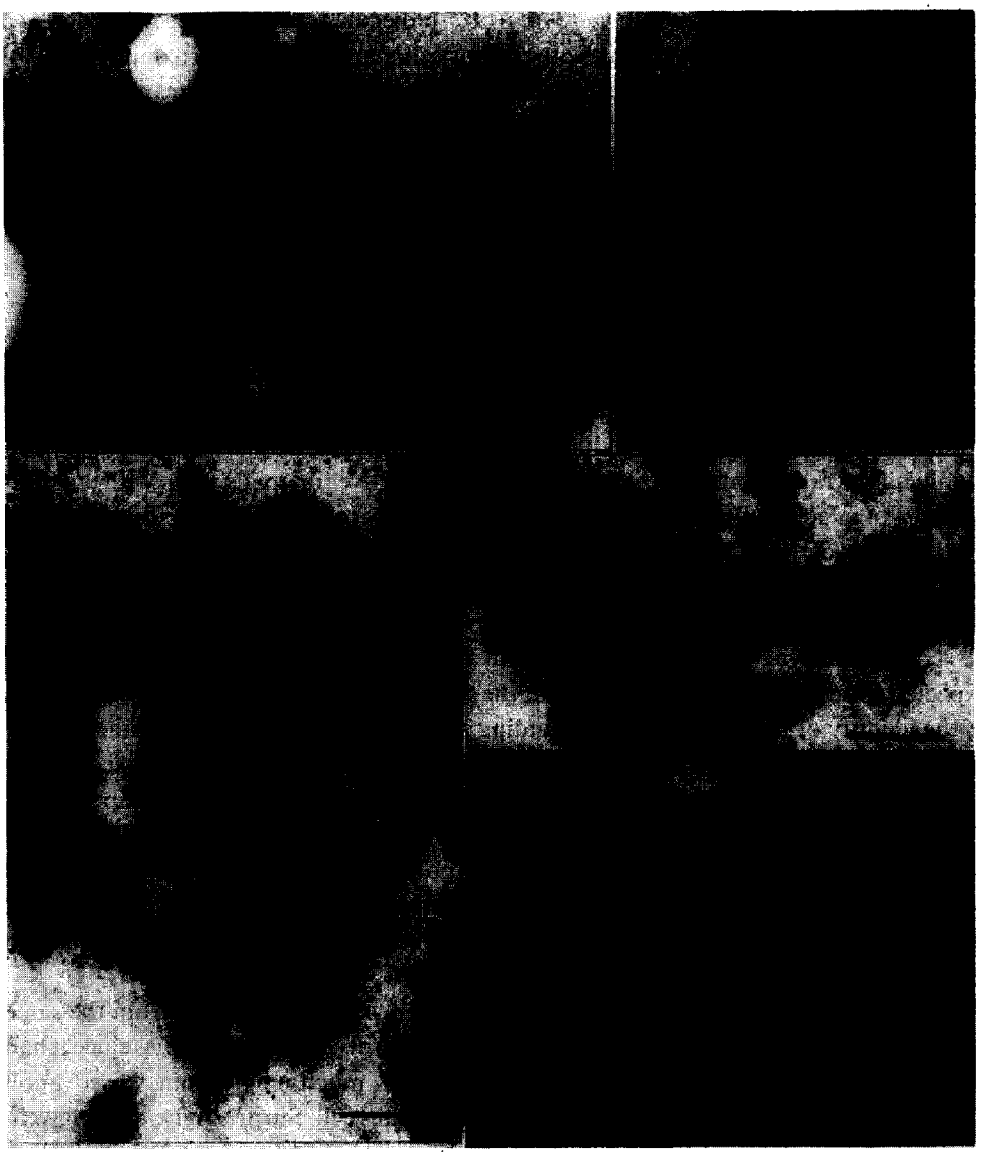

Fig. 6. Thin-frozen sections of TA3-Ha cells labeled with Jacalin lectin: (A) A large multivesiculate sac, almost entirely without the cell, showing positive labeling for AE-3. Boxed area is displayed in Fig. 6B (bar = 1.0 μm). (B) Boxed area of multivesiculate sac in Fig. 6A. Labeling for AE-3 is clearly apparent within this area (bar = 0.2 μm). (C) A large multivesiculate sac containing small vesicles showing labeling with Jacalin lectin (bar = 0.2 μm). (D) Golgi-TGN region showing no labeling within the Golgi cisternae (closed arrows), although some labeling of lateral sacs is apparent (bar = 0.2 μm). (E) Another compartment labeled with Jacalin is the *trans*-Golgi network (bar = 0.2 μm).

length (400–500 nm) and numbers (3–4 × 10^6 molecules/cell)[14]. In sheer bulk, it is a dominant feature on the TA3-Ha ascites tumor cell, representing approximately 1% of the cell's dry weight[10]. Because of these unique features, this glycoprotein appeared to be a good choice for studies of the intracellular pathway taken by a mucin-type glycoprotein, since its high concentration would facilitate its detection, and its reported biological role of masking cell surface antigens[13,15,36] would add significance to the findings. It has been demonstrated[37] that epiglycanin is present in the ascites fluid and serum of mice bearing the TA3-Ha ascites tumor, and the presence of glycoprotein material bearing a major epiglycanin epitope has been identified in the serum of cancer patients[30].

The monoclonal antibody selected for these studies was AE-3, which appears to recognize a glycopeptide structure containing a β-D-Gal*p*-(1→3)-D-GalNAc chain. This epitope appears to be closely related to a major epitope recognized by a previously reported polyclonal antibody induced in the rabbit[30]. In a manner similar to that involving the rabbit antibody, incubation of cells positive for epiglycanin with endo-*N*-acetyl-α-D-galactosaminidase[23] removed their capacity to bind to AE-3. Furthermore, the inability of the antibody to bind to any significant degree with other proteins containing the same disaccharide chain suggests the involvement of peptide in the combining site[30,38].

By means of a gold-labeled second antibody, epiglycanin was identified, as expected from previous reports[29,31,36,39], at the surface of the cell (Fig. 2C). Confirmation of the identity of the surface antigen binding AE-3 as epiglycanin was obtained by the removal of all binding activity as a result of a brief incubation with endo-*N*-acetyl-α-D-galactosaminidase. It had previously been demonstrated that this enzyme specifically cleaved β-D-Gal*p*-(1→3)-D-GalNAc chains from epiglycanin and destroyed 99% of the detectable capacity of epiglycanin to bind the polyclonal rabbit antibody[33], as well as AE-3. Previous studies[40] have suggested that there are only one or two such epitopes, recognized by the rabbit antibody, in epiglycanin. Similar studies have not been reported for antiepiglycanin monoclonal antibodies.

The nonallotransplantable TA3-St ascites cell, a cell line derived from the same spontaneous mammary carcinoma, was used as a control cell in these studies since it had been reported[10] that this cell contains only 0.5% as much epiglycanin, based upon absorption studies, on its surface as the TA3-Ha ascites cell. Surprisingly, the epiglycanin on the surface of the TA3-St cell was found to exist in occasional small clusters (Fig. 2B), in contrast to the epiglycanin at the TA3-Ha cell surface, which was in general found to exist in high concentration over the entire cell surface.

The presence of epiglycanin was observed in the *trans*-Golgi network (Fig. 4A), but it was not observed in the Golgi apparatus. Similar results were obtained with the lectin Jacalin (Figs. 6C, D, and E), which has been reported to bind to similar disaccharide structures[41]. This result was surprising since the completion of the disaccharide chain required for antibody binding would be expected to occur within the *trans*-Golgi cisternae. That the biosynthesis of epiglycanin differs from that of certain other glycoproteins within the cell was demonstrated by labeling with a less specific lectin, WGA, which has been shown to bind to structures earlier in the biosynthetic

pathway, namely the medial and *trans*-cisternae of the Golgi apparatus (Fig. 5). The WGA results were not unexpected, since previous studies of the biosynthesis of glycoproteins have reported the presence of D-galactosyltransferases in the *trans*-most Golgi cisternae[42,43], D-glucosaminyltransferases in the medial Golgi cisternae[44,45], and a sialyltransferase in the *trans*-Golgi cisternae[46]. WGA has been reported to bind to both 2-acetamido-2-deoxy-β-D-glucopyranosyl and *N*-acetylneuraminyl (sialyl) groups[47], both present in epiglycanin, but since sialic acid (14–18% by weight) is more abundant than 2-acetamido-2-deoxy-D-glucose and is more favorably located in terminal positions, it is probable that the intense labeling in TGN more distal-secretory compartments (Fig. 5C) can be attributed mainly to the binding to sialic acid units in epiglycanin, by far the most abundant glycoprotein in the cell[14]. It is not clear why the biosynthesis of these structures occurs prior to that of the glycopeptide structure involved in antibody binding. The possibility of the synthesis, in the TGN, of a unique structrual feature required for antibody binding must be considered.

Two possible pathways for the passage of epiglycanin to the cell surface have been suggested by this study. One involves the transport in 0.1-μm vesicles, a route which appears to result in the incorporation of epiglycanin into the plasma membrane. The other consists of large multivesicular-storage sacs, which were sometimes observed to open into the external medium and appeared to be in the process of discharging their contents.

Transport of epiglycanin from the TGN to be incorporated into the plasma membrane by 0.1-μm vesicles. — Epiglycanin-labeled vesicles, typical of those seen in constitutive secretion[48] were commonly observed in this study. In plastic sections of immune-labeled cells, epiglycanin-containing coated pits were also apparent (Fig. 3A). This implies that internalization of epiglycanin may also be occurring, as surface-associated coated pits are generally considered endocytotic in nature[48]. Given the very high concentration of the glycoprotein at the cell surface, it would seem that some surface-associated epiglycanin would be expected to be trapped during receptor-mediated endocytosis. To determine whether the coated pits were internalizing epiglycanin, viable TA3-Ha cells were incubated at 37°, following surface labeling with the AE-3 antibody at 4°. The locations of binding were revealed by means of a 5-nm gold probe. Little labeling was seen within any intracellular compartment. This contrasts with experiments performed similarly by us for the study of transferrin receptors on lymphocytes[50] and Class I receptors on macrophages[51], which revealed extensive intracellular binding. A possible reason for this is that the extensive epiglycanin coat, known to extend approximately 400 nm from the cell surface, may mask receptors, thereby limiting specific receptor-mediated endocytosis and, hence, the postulated nonspecific internalization of epiglycanin by this route.

Micron-sized sacs transfer epiglycanin to the extracellular environment. — Large multivesicular sacs were labeled with the AE-3 antibody (Fig. 5A), WGA (Fig. 5B), and Jacalin (Fig. 6A). As large as 3 μm in diameter, these sacs contained vesicles of about 0.1 μm in diameter. Labeling appeared to be consistent with the two lectins, but occasionally labeling was not possible with the antibody. Morphologically, the large sacs appear to

be similar to the multivesicular sacs observed in a human hepatoma cell line[43]. While fusion of cytoplasmic membrane vesicles into the large vesicles was apparent (Fig. 5B, for example), the ultimate origin of these sacs cannot be defined within the framework of the results presented here. It is possible that they represent a type of endolysosome, similar to the secondary lysosomes described previously[52], or the intermediate endosomal–lysosomal compartment described by Griffiths *et al.*[53], and may contain phagocytosed material, autophagocytosed material, as well as material originating from the TGN following fusion and release of hydrolytic enzymes into the large sacs. From the experimental approaches taken here, it is likely that in the TA3-Ha cell the large sacs function primarily exocytotically. Unfortunately, cell surface markers, such as α_2-macroglobulin or transferrin[53], used to study subsequent internalization, showed little or no label on the TA3-Ha surface, presumably owing to the extensive epiglycanin coat masking surface receptors for these proteins. However, no labeling was observed within the cell following surface labeling of intact cells by gold-labeled AE-3, followed by incubation at 37° under metabolic conditions. Internal labeling would have been observed if the process was phagocytotic or endocytotic. Furthermore, in another experiment, incubation of viable cells under metabolic conditions with [^{125}I]epiglycanin resulted in no significant uptake or absorption of the glycoprotein. Thus, these large sacs appear to be responsible for the release of major amounts of epiglycanin from the cell (Fig. 1), a process which had previously been attributed to shedding directly from the cell surface[54]. The experimental procedures utilized in this study do not allow comment as to whether shedding of cell surface epiglycanin occurs.

In a study of the biosynthesis of a mucin-type glycoprotein at the surface of the 13762 rat mammary carcinoma ascites cell, Spielman *et al.*[8] confirmed previous observations by that laboratory that the initiation of the synthesis of *O*-linked carbohydrate chains commences at the endoplasmic reticulum and continues until the material reaches the cell surface. Although the present results do not contradict this conclusion, no evidence was obtained to suggest an increase in labeled epiglycanin en route from the *trans*-Golgi network to the plasma membrane.

To our knowledge, the secretion of glycoproteins from mammalian cells by means of multivesicular sacs has not been previously reported. It is of interest, however, that in their studies of the biosynthesis of epiglycanin in the TA3-Ha cell, Miller and Cooper[54] found evidence for the presence of two independent pathways for the loss of epiglycanin from the cell, one with a half-life of more than 70 h, and a second with a half-life of only about 0.75 h. Further work will be required to determine whether or not this rapid secretory process involves the large sacs. Because of the important role played by glycoprotein tumor antigens in the body fluids of cancer patients[20,21] and of mucin-type glycoproteins in cancer[1], more information regarding the mechanisms of secretion of these molecules is urgently needed. Whether or not secretion by means of large sacs, as noted for epiglycanin, represents a significant secretory pathway for all tumor antigens, for only mucin-type glycoprotein antigens, or is unique to the TA3-Ha ascites cell, must await further experimentation.

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