Apical plasma membrane proteins are not obligatorily stored in secretory granules in exocrine cells

Veronica Colomer¹, Michael J. Rindler¹,* and Anson W. Lowe²

¹Department of Cell Biology and Kaplan Cancer Center, New York University Medical Center, New York, NY 10016, USA
²Department of Medicine and The Digestive Disease Center, Stanford University School of Medicine, Stanford, CA 94305, USA

*Author for correspondence

SUMMARY

Exocrine cells are epithelial cells in which secretory granules undergo fusion with the apical plasma membrane upon secretagogue stimulation. Several apical plasma membrane proteins have been found in secretory granules in cells from pancreas and salivary glands raising the possibility that incorporation into secretory granules followed by exocytosis of the granules accounts for their insertion into the apical plasma membrane. To test this hypothesis, we have expressed the influenza hemagglutinin (HA) in pancreatic AR42J cells, which make zymogen-like granules upon incubation with dexamethasone. The influenza virus HA is known to be specifically targeted to the apical plasma membrane of epithelial cells that lack a regulated pathway and is also known to be excluded from secretory granules in virally-infected pituitary AtT20 cells. Localization of the protein by immunofluorescence microscopy revealed that it accumulated at the plasma membrane of the transfected AR42J cells. HA was not observed in the amylase-rich secretory granules. By immunolabeling of ultrathin cryosections of the transfected cells, HA was also found exclusively on the cell surface, with label over secretory granules not exceeding that seen in control, untransfected cells. In addition, in cell fractionation experiments performed on radiolabeled AR42J cell transformants, HA was not detectable in the secretory granule fractions. These results indicate that HA is not efficiently stored in mature secretory granules and is likely to reach the cell surface via constitutive transport pathways.

Key words: secretory granule, apical sorting, epithelial cell, exocrine cell, pancreas

INTRODUCTION

Selective incorporation of proteins into storage granules involves their segregation from constitutively transported proteins in the trans-Golgi network or TGN (Rindler, 1992). Membrane proteins that accumulate in the plasma membrane have been shown in AtT20 pituitary or PC12 neuroendocrine cells to be absent from Golgi-derived secretory (dense core) granules (Gumbiner and Kelly, 1982; Orci et al., 1987; Rivas and Moore, 1989; Disdier et al., 1992), whereas granule-specific proteins, including P-selectin (Koedam et al., 1992; Disdier et al., 1992), peptidyl glycine α-amidating enzyme (Milgram et al., 1992), and the insulin responsive glucose transporter GLUT-4 (Hudson et al., 1993), are selectively included. Synaptic vesicle proteins may also be incorporated into the dense core secretory granules of PC12 cells, albeit at lower concentrations than in synaptic vesicles (Lowe et al., 1988).

In exocrine pancreatic and salivary gland cells, which have secretory granules that undergo exocytosis at the apical plasma membrane, the existence of completely distinct constitutive and regulated pathways for membrane proteins has not yet been fully established. Indeed, several putative apical membrane proteins are known to be incorporated into secretory granule membranes. These proteins could then be transported to the apical surface indirectly as a consequence of fusion of the granule membrane with the apical membrane during exocytosis. γ-glutamyl transpeptidase is an apical membrane protein in kidney and intestinal epithelial cells whose activity is enriched in secretory granules in a variety of exocrine cells (Cameron and Castle, 1984). It has also been localized to pancreatic zymogen granules by immunolabeling at the electron microscopic level (Beaudoin et al., 1993). The precursor for epidermal growth factor (EGF) is a membrane protein of the apical membrane of renal distal tubular cells (Salido et al., 1986), while EGF itself is a granule content protein in the granular convoluted tubular cells of the mouse submandibular gland (Tanaka et al., 1981) and in other exocrine cells (Jacobs and Story, 1988; Fukuyama and Shimizu, 1991; Saga and Takahashi, 1992). In addition, GP-2, the major pancreatic granule membrane protein, is attached to the membrane via a glycosylphosphatidylinositol (GPI) linkage (LeBel and Beattie, 1988; Rindler and Hoops, 1990). GPI-linked proteins are known to be apical plasma membrane proteins in many other epithelial cell types (Lisanti et al., 1990). The GPI anchor itself serves as a signal for targeting to the apical cell surface domain (Brown et al., 1989; Lisanti et al., 1989). Thus, GP-2 must be considered a putative apical plasma membrane protein as well.
The requirements for membrane protein incorporation into secretory granules appear to differ in exocrine and endocrine cells. GP-2, for example, enters the zymogen-like granules of AR42J cells, derived from exocrine pancreas, but is excluded from the endogenous secretory granules of AtT20 and rat insulinoma cells (Hoops et al., 1993). This cell type specificity for granule entry is not unique to GP-2 but is a property of other GPI-linked proteins as well (V. Colomer, I. Caras, T. C. Hoops and M. J. Rindler, unpublished data). It is therefore reasonable to hypothesize that exocrine secretory granules may act as a transport intermediate for apical plasma membrane proteins in exocrine cells. These same proteins would not necessarily be incorporated into the secretory granules of endocrine cells, which generally do not maintain separate apical and basolateral membrane domains. To this end, we have expressed influenza HA in pancreatic AR42J cells and studied its subcellular distribution. HA was chosen because it has been shown to be an apical membrane protein in a variety of epithelial cells, including cell lines of renal and intestinal origin (Rodriguez Boulan and Sabatini, 1978; Rindler and Traber, 1988) and those such as cultured thyroid epithelial cells that have unorthodox membrane polarity (Zurzolo et al., 1992b). In addition, HA is excluded from the newly forming endogenous secretory granules in the TGN of virally infected AtT20 cells and hence is not found in the mature secretory granules in these endocrine cells (Orci et al., 1987). We observe that HA is also excluded from the zymogen granules of transfected AR42J cells.

MATERIALS AND METHODS

Cell lines and transfection
AR42J cells were obtained from the American Type Culture Collection and cultured in Dulbecco’s minimal essential medium in 10% fetal bovine serum as previously described (Hoops et al., 1993). The cDNA encoding HA from influenza strain X31 (Verhoeyen et al., 1980) was cloned into the retroviral vector pMV-7 (Kirschmeier et al., 1988). Recombinant retrovirus was prepared after transfection of psi2 cells (Mann et al., 1983). Following infection with the retrovirus, the AR42J cells were subjected to selection for resistance to G418 (0.2 mg/ml). Resistant colonies were subcloned and screened for expression by immunoprecipitation of metabolically labeled cells.

Immunofluorescence microscopy
AR42J cells were cultured on polylysine-coated glass cover slips for 2-3 days and then incubated with dexamethasone (10^{-6} M) for 4 days with 10 mM Na butyrate added for the final 40 hours (Gottlieb et al., 1986). The butyrate treatment does not affect the formation of secretory granules as assessed by several criteria including the continued ability of the cells to store amylase for release upon stimulation with CCK. After fixation in 4% paraformaldehyde in Dulbecco’s phosphate buffered saline (PBS) and permeabilization with 0.2% TX-100, antibody incubations were conducted as previously described (Hoops et al., 1993). Mouse monoclonal antibody against HA (supernatant of hybridoma originally from Dr J. Skehel, National Institute of Medical Research, London, UK) and rabbit anti-amylase (Sigma Chemical Co., St Louis, MO) were used sequentially at 1:5 and 1:200 dilution, respectively. FITC anti-mouse IgG and rhodamine anti-rabbit IgG were purchased from Jackson Immunoresearch (West Grove, PA) and used at a 1:50-1:100 dilution. Samples were mounted in Citiflour (London, UK) and photographed on a Zeiss Axio phot microscope.

Immunolabeling of frozen ultrathin sections
AR42J cells cultured on 60 mm dishes and induced as described above were fixed in 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M cacodylate buffer for 1 hour, scraped from the dishes, pelleted and fixed for an additional 2 hours. Samples were processed for ultracytosectioning and double immunolabeling as described elsewhere (Rindler et al., 1984; Hoops et al., 1993). Briefly, double labeling was performed using as a first step rabbit anti-HA (a gift from Dr Ari Helenius, Yale University School of Medicine) at a 1:50 dilution for 3-13 hours followed by incubation for 2 hours with 5 nm Protein A gold (E-Y Laboratories, San Mateo, CA). Sections were then washed and fixed in 2% glutaraldehyde for 10 minutes and subsequently saturated for 20 minutes with 0.5 mg/ml Protein A. This step blocks effectively free binding sites as was determined in control experiments where 15 nm Protein A gold was added directly to the samples after fixation and saturation with Protein A. Rabbit anti-amylase antibody (1:75-1:200) was then added for 1 hour followed by 15 nm Protein A gold for 1 hour.

Quantitation of gold particles was performed on photomicrographs of immunolabeled sections from the permanently transformed AR42J cells having abundant cell surface labeling for HA. Sections of control or transfected cells were photographed at random. Only structures of the appropriate size, with a relatively dense secretory content, and labeled with antibodies to amylase were considered granules for the statistical analysis. Surface density measurements were performed using the COSAS computer program (Cornacchia and Black, 1988) on a Macintosh computer equipped with a digitizing pad. The particles over a total of 209.1 and 576.5 μm of plasma membrane surface area for untransfected and transfected cells, respectively, were counted.

Cell fractionation
AR42J cells expressing HA were plated on five 100 mm dishes, grown to confluency, and treated with dexamethasone as described above. One of the dishes was pulse labeled with 3 ml of 0.8 μCi/ml [35S]methionine for 1 hour and chased for 3 hours. Based on previously published observations concerning the half life of secretory granules in AR42J cells, this chase period should have been sufficient to allow HA either to enter a stable pool of secretory granules or to be transported to the cell surface (Sachs and Jamieson, 1992). Cells were removed from the plates by scraping, washed once in PBS, and the cell pellet was resuspended gently in 1 ml of homogenization buffer (0.25 M sucrose, 3 mM EDTA, 0.2 mM PMSF, 25 mM MOPS, pH 6.8) at 4°C. Lysates were obtained by homogenization using the Ball Bearing Cell Cracker and a 8.008 mm diameter ball (12 μm clearance) as has been described (Rosewicz et al., 1992). Post-nuclear supernatants (PNS) were then prepared after centrifugation at 100,000 g for 5 minutes. The PNS was then centrifuged for 5 minutes at 3000 g. The pellet was gently resuspended in 0.2 ml of homogenization buffer and loaded onto 20% Percoll gradients containing 0.25 M sucrose, 1 mM EDTA, 1 mM MOPS, pH 6.8, and protease inhibitors with a 0.2 ml cushion of 2 M sucrose, similar to the procedure used by Koedam et al. (1992) for separating granules in AtT20 cells. After centrifugation at 100,000 g for 25 minutes using a Ti50 rotor, sixteen fractions of ~0.6 ml were collected. The organelles were lysed by freeze-thawing and the membrane- and non-membrane-bound components were separated by centrifugation at 100,000 g for 25 minutes using a TL100 ultracentrifuge and a Ti100.3 rotor onto a 0.1 ml cushion of 1.7 M sucrose. After immunoprecipitation of HA, the samples were subjected to SDS/PAGE and fluorography. In the transformants HA was almost entirely cleaved to HA1 and HA2 subunits. The amount of HA1 in each membrane pellet (which accounts for all of the HA1 loaded on the gradient) was measured using NIH Image after scanning of the autoradiographs on a flatbed scanner connected to a Macintosh computer. α-amylase activity was used as a marker for secretory granules and was performed on a sample from each fraction (Bernfeld, 1955). In control experiments, the distribution of...
amylase activity correlated very closely to that of radiolabeled immunoprecipitated amylase (not shown). The density across the gradient was determined using isopycnic density marker beads (Sigma).

RESULTS

Immunofluorescence labeling of AR42J-HA cells

AR42J cells are derived from a pancreatic tumor and maintain differentiated properties of acinar cells (Logsdon et al., 1985; Hoops et al., 1993). They produce zymogen-like granules and are able to secrete amylase, the major content protein of zymogen granules, in response to incubation with cholecystokinin (CCK). A clone of G418-resistant AR42J cells expressing the influenza HA was isolated after transfection and subjected to labeling for immunofluorescence microscopy with antibodies to HA and to amylase. As shown in Fig. 1, the label corresponding to HA was almost exclusively localized to the cell surface, especially to regions of cell-cell contact. This is expected as the viral membrane protein is known to bind tightly to terminal sialic acid residues on glycoproteins of target cells to mediate viral adhesion and penetration (Higa et al., 1985). The stable binding of HA molecules to glycoproteins on adjacent cells would tend to make them behave like cell adhesion molecules and accumulate at the sites of cell-cell contact. HA was not, however, detectable in amylase-containing vesicular structures taken to be the secretory granules (compare Fig. 1a and b), even in overexposed photographs.

Immunolabeling of frozen ultrathin sections of AR42J-HA cells

The relatively strong cell surface staining could have potentially masked a weak localization of HA in secretory granules. To definitively determine its distribution, immunolabeling of cryosections of control and transfected cells was performed for viewing at the electron microscopic level. In double labeling experiments with antibodies to HA and to amylase, gold particles corresponding to HA were abundant over the plasma membrane (Fig. 2). As was observed by immunofluorescence microscopy, the level of labeling was variable in different regions of the plasma membrane. It was most abundant in regions of cell-cell contact (Fig. 2c) and at times was very scarce in adjacent segments. The small secretory granules characteristic of these cells, identified both by their dense appearance and by the presence of gold particles corresponding to amylase, were labeled for HA at very low levels. These levels were similar to those found over the secretory granules of

![Fig. 1. Localization of influenza HA to the plasma membrane at sites of cell-cell contact in transfected AR42J cells. Immunofluorescence microscopy was performed as described in Materials and Methods using mouse monoclonal anti-HA and rabbit anti-amylase followed by rhodamine-conjugated anti-mouse and FITC-conjugated anti-rabbit IgG. Shown is the same field photographed using rhodamine (a), FITC (b), or Nomarski filters (c). HA was confined to the cell surface especially in regions of cell-cell contact (arrows). It was not observed in the amylase-rich secretory granules (arrowheads). ×800.](image-url)
control, untransfected cells (Fig. 3). Plasma membrane labeling for HA was also very low in the sections from control AR42J cells. To quantitate these results the distribution of gold particles representing HA was determined using sections from both transfected and control cells. As shown in Fig. 4, the statistics confirmed that labeling over the secretory granules did not differ between the transfected and untransfected cells (0.27 grains/granule in both cases, corresponding to a density of ~0.7 grains/µm of granule membrane surface based on the average granule section diameter of ~130 nm). By contrast, the overall density of gold particles, averaged over the entire cell surfaces from the same photomicrographs, was 0.8 and 18.4 grains/µm for control and HA-expressing AR42J cells, respectively. We conclude that HA did not accumulate in secretory granules at significant levels.

**DISCUSSION**

The results of the localization of HA in AR42J cells as well as the lack of HA in the secretory granules fractions during cell fractionation demonstrate that this protein is not efficiently

**Cell fractionation of AR42J-HA cells**

In order to confirm these microscopic observations, the AR42J cells expressing HA were subjected to cell fractionation (see Materials and Methods). The cells were metabolically labeled for 1 hour with [35S]methionine and chased for 3 hours before homogenization. The final step in the protocol is a Percoll gradient that separates the lighter membranes (which should contain large microsomal vesicles and plasma membrane fragments) from the denser secretory granules, which were identified by the amylase activity (Fig. 5). Immunoprecipitated HA was observed only in the lighter fractions and was virtually undetectable in fractions 14-16, which is where the amylase peak was located. These results support the localization data and show that HA does not accumulate in secretory granules in the AR42J cells.
stored in secretory granules. These cells represent the best available in vitro model of exocrine cells and maintain the ability to store amylase in small zymogen-like granules that undergo regulated exocytosis. The behavior of HA contrasts sharply with that of the pancreatic granule membrane protein GP-2. When AR42J cells are transfected with GP-2, which is not made in significant amounts by the cells, it is routed to secretory granules (Hoops et al., 1993). The accumulation of GP-2 in secretory granules, in contrast to HA, was readily detectable both by immunofluorescence microscopy and by immunolabeling of ultrathin cryosections. The findings with HA are also dissimilar to those reported by Beaudoin et al. (1993) for the immunolocalization of the apical membrane protein, γ-glutamyl transpeptidase, which was observed to be present in zymogen granules in pancreatic acinar cells, as expected from the cell fractionation results of Cameron and Castle (1984). In the current study, we cannot formally exclude the possibility that a portion of HA reaches the secretory granules and is then rapidly degraded. We consider this unlikely, however, since lysosomes that were noted in occasional sections were often labeled with antibody to HA. Even if partial degradation had occurred, we would have expected some immunoreactivity.

Because HA is a prototypical apical membrane protein of epithelial cells, these results imply that some apical membrane proteins bypass the secretory granules in exocrine cells and reach the cell surface via constitutive transport pathways. Two types of constitutive transport pathways have been reported in cells that make secretory granules. Some proteins, like coronavirus particles that originate from the cis-Golgi/intermediate compartment of AtT20 cells, are excluded from secretory granules in the TGN (Tooze et al., 1987). Other proteins may be initially segregated with the granule content proteins to nascent immature granules and subsequently removed during the course of granule maturation for transport directly to the cell surface (von Zastrow and Castle, 1987; Grimes and Kelly, 1992; Kulihawat and Arvan, 1992). GP-2, which is partially diverted to the constitutive pathway in resting acinar cells, may also follow this route (Havinga et al., 1984; Beaudoin and Grondin, 1987). Orci and collaborators (Orci et al., 1987) showed in endocrine AtT20 cells infected with influenza virus that HA was segregated away from the condensing granule content proteins in the TGN, implying that it does not enter immature granules at all in this cell type. The paucity of sig-

![Fig. 3. Labeling of untransfected AR42J cells by immunoelectron microscopy. Ultrathin cryosections of AR42J cells were prepared as described in Fig. 2. In the untransfected cells, the secretory granules (arrows) label with anti-amylase but HA labeling is low both in the granules and on the cell surfaces. Insets (b and d) show higher magnification views of selected secretory granules (*). Bars: 0.5 μm (c); 0.3 μm (a); 0.1 μm (b and d).](image)

![Fig. 4. Lack of significant labeling for HA in secretory granules in transfected as compared to untransfected AR42J cells. Analysis of labeling density for HA was conducted on photomicrographs of samples incubated with anti-HA and anti-amylase antibodies as described in Fig. 2. The number of 5 nm gold particles over granules was counted on a total of 116 granules in sections from control cells and 154 granules of transfected cells from two separate experiments. The data is plotted as the percentage of granules having the specified number of particles/granule. The overall density of label in each case is 0.27 grains/granule (0.65 grains/μm of granule membrane surface).](image)
experiments. Depicted is one of two similar secretory granule fractions (14-16). HA was found exclusively in the lighter density gradients are, from left to right: 1.048, 1.055, 1.067, 1.098 and 1.128 g/ml. HA was found exclusively in the lighter density marker beads (α₂) across the gradients are, from left to right: 1.048, 1.055, 1.067, 1.098 and 1.128 g/ml. HA was found exclusively in the lighter density membrane fractions and was absent from the denser amylase-rich secretory granule fractions (14-16). Depicted is one of two similar experiments.

significant labeling for HA in the secretory granules of transfected AR42J cells, while not conclusively settling this issue, would be consistent with its complete exclusion from the immature granules and, by inference, its direct transfer to the plasma membrane from the TGN.

In contrast to HA, apical membrane proteins that enter secretory granules, such as γ-glutamyl transpeptidase and GP-2, as well as other GPI-linked proteins (see Introduction), may have specific sorting information for zymogen granule packaging that is not utilized in cells lacking a regulated pathway. The granule packaging information must override the signals specifying direct transport to the apical plasma membrane. This conclusion is consistent with evidence in the literature indicating that proteins destined for insertion into the apical surface of polarized epithelial cells represent a heterogeneous group, with subgroups having their own trafficking information. This information is recognized somewhat differently depending on the cell type. For example, in thyroid FRT cells, it has been shown that proteins, such as the Semiliki Forest virus p62/E2 envelope protein, normally found on the basolateral membrane of other epithelial cells, are directed to the apical surface in this cell type (Zurzolo et al., 1992b), whereas GPI-linked proteins, efficiently transported to the apical surface in other cell types, are diverted to the basolateral domains (Zurzolo et al., 1993). Still other apical membrane proteins, such as influenza HA (Zurzolo et al., 1992b) and dipeptidylpeptidase (Zurzolo et al., 1992a), maintain their polarity in FRT cells. Furthermore, in nonexocrine epithelial cells, two different pathways of transport of proteins to the apical plasma membrane from the Golgi exist, one involving direct delivery and the other based on transcytosis after insertion into the basolateral membrane (Hubbard, 1991). The extent to which individual proteins are transported via either route is determined in part by the cell type, with hepatocytes having only the transcytotic system (Bartles et al., 1987). But in cells such as intestinal epithelial cells, in which both pathways are prominent, some proteins follow the direct TGN-apical membrane route while others preferentially use the indirect circuit (Le Bivic et al., 1990; Matter et al., 1990).

By analogy, another view of the lack of concerted sorting of exocrine apical membrane proteins is that the destination of each subgroup of proteins is determined by the cell’s sorting machinery. Some apical membrane proteins transit primarily through the storage granules while others preferentially use the constitutive pathway.

The authors thank Dr V. Black for guidance on the use of the morpophony program; Dr I. Ivanov, H. Pleken, and I. Gumper for assistance with ultrathin cryosectioning; M. Parameswaran and M.-J. Hannocks for technical assistance; and J. Culkin and F. Forcino for photography. V. Colomer is the recipient of an Ella Fitzgerald Fellowship from the American Heart Association, NYC affiliate. M. Rindler is an Established Investigator of the American Heart Association, NYC affiliate, and is also supported by NIH grant DK44238. A. Lowe is supported by grant DK43294 from the NIH.

Fig. 5. HA is not recovered in secretory granule fractions prepared from transfected AR42J cells. AR42J cells expressing HA were pulsed with [35S]methionine for 1 hour and chased for 3 hours. A post-nuclear supernatant (PNS) was prepared and centrifuged for 5 minutes at 3000 g. The pellet was resuspended in homogenization buffer and fractionated by centrifugation on an isosmotic 20% Percoll gradient as described in Materials and Methods. Fractions of approximately 0.6 ml were collected, the organelles were lysed by freeze-thawing, and the membrane and soluble components were separated by ultracentrifugation. (A) HA was recovered by immunoprecipitation from solubilized membrane pellets of each fraction as well as from 1/10 of the PNS. After SDS-PAGE, the radioactivity in the HA 1 subunit (arrowhead), which ran as a doublet of 41-43 kDa, was quantitated. (B) The HA data (●) is plotted as the percentage in each fraction of the total recovered in the PNS along with the percentage of α₂-amyrase activity (▲), a marker for secretory granules. The positions of isopycnic density marker beads (▼) across the gradients are, from left to right: 1.048, 1.055, 1.067, 1.098 and 1.128 g/ml. HA was found exclusively in the lighter density membrane fractions and was absent from the denser amylase-rich secretory granule fractions (14-16). Depicted is one of two similar experiments.

REFERENCES


