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# Apical sorting of hepatitis B surface antigen (HBsAg) is independent of N-glycosylation and glycosylphosphatidylinositol-anchored protein segregation

MARÍA PAZ MARZOLO*, PAULINA BULL*, AND ALFONSO GONZÁLEZ†‡

†Departamento de Inmunología Clínica y Reumatología, Facultad de Medicina, and *Departamento de Biología Celular y Molecular, Facultad de Ciencias Biologicas, Pontificia Universidad Católica de Chile, Santiago, Chile

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**ABSTRACT** **We have used the hepatitis B surface antigen (HBsAg) as a tool to explore mechanisms by which polarized epithelial cells address specific proteins to their apical domain. It recently has been proposed that N-glycans can serve as apical signals recognized by lectin-like sorting receptors in the trans-Golgi network. We found, however, conclusive evidence that the HBsAg follows an apical pathway not mediated by N-glycan signaling. Neither tunicamycin treatment nor replacement of its single glycosylated residue, Asn-146, altered its predominant (>85%) apical secretion from transfected Madin-Darby canine kidney cells (MDCK). Although HBsAg is known to be secreted as a lipoprotein particle, our results suggest that the exocytic machinery involved in its N-glycan-independent pathway overlaps, at least partially, with that of other apically targeted proteins, including the endogenous gp80, as judged by the effects of brefeldin A. We also tested whether its sorting behavior could be ascribed to association with glycosylphosphatidylinositol (GPI)-anchored proteins, which, together with glycosphingolipids, primarily are targeted to the apical domain of MDCK cells. HBsAg was preferentially secreted from the apices of transfected Fisher rat thyroid cells, which, in contrast to MDCK cells, address GPI-proteins and glycosphingolipids to their basal domain. Moreover, complete inhibition of GPI biogenesis by mannosamine treatment did not impair the HBsAg apical secretion, discarding the possibility that HBsAg could be "hitchhiking" with a newly synthesized GPI-protein. Thus, the HBsAg provides a unique model system to search for yet-unknown apical sorting mechanisms that could depend on proteinaceous targeting signals interacting with cognate trans-Golgi network receptors that are at present unidentified.**

Polarized epithelial cells are characterized by the presence of apical and basolateral cell surface domains that are functionally specialized and are separated by tight junctions (for reviews see refs. 1–3). Such cells are capable of segregating distinct sets of plasma membrane proteins in each of these domains and of releasing specific secretory proteins into the medium surrounding one or the other surface.

In the polarized kidney-derived Madin–Darby canine kidney (MDCK) cell line, a widely used model to study the biogenesis of epithelial cell polarity (4, 5), newly synthesized plasma membrane proteins destined to the apical or basolateral cell surface domains have been shown to emerge from the Golgi apparatus in two different carrier vesicle populations that are targeted directly to the surfaces where the vesicular cargo proteins accumulate (6–10). The sorting of transmembrane proteins destined to the basolateral surfaces appears to be mediated by peptide signals present within their cytoplasmic tails, which must interact with as-yet-unidentified receptors, or adaptors, that mediate the incorporation of the proteins being sorted into basolaterally directed transport vesicles (reviewed in ref. 11). Abolition of this signal, by mutation or deletion, leads to either nonpolarized delivery of the protein or, in some cases, its rerouting to the apical domain, which is believed to result from the action of a cryptic apical targeting signal in the ectoplasmic portion of the modified protein (12). Certain apically targeted transmembrane proteins appear to contain sorting information within their ectoplasmic portions, because elimination of their membrane anchors leads to the apical discharge of the resulting truncated secretory versions (13, 14).

Two types of signals are thought to confer apical targeting specificity to plasma membrane or secretory proteins. First, evidence has been presented that N-linked oligosaccharide chains function as apical signals recognized by cellular lectins that serve as sorting receptors (12, 15). A role of N-glycans in mediating apical targeting initially was inferred from the effects of inhibiting the synthesis of N-linked oligosaccharides by tunicamycin treatment, which led to random secretion of some unglycosylated polypeptides that normally are secreted primarily from the apical surface (16–18). More direct evidence for a signaling role of N-glycans was provided by the finding that the addition of sites for N-glycosylation to growth hormone, a nonglycosylated protein that in transfected MDCK cells is secreted randomly from both surfaces (19), led to the preferential apical secretion of the resulting glycoprotein and that this also could be eliminated by tunicamycin treatment (15). The notion that N-linked oligosaccharides are sufficient to direct a secretory protein to the apical surface, however, is inconsistent with the observation that truncation of the vesicular stomatitis virus G and influenza hemagglutinin glycoproteins to remove their membrane anchors led to their random secretion from both surfaces of MDCK cells (20), and not primarily from the apical surface, as predicted from the N-glycan apical signaling model. Moreover, previous studies had failed to demonstrate an effect of tunicamycin on the sorting of either the vesicular stomatitis virus G or influenza hemagglutinin glycoproteins to the basolateral or apical surfaces, respectively (21, 22).

A second feature that can confer apical targeting to a protein is the presence of a glycosylphosphatidylinositol (GPI) membrane anchor at the C terminus of the polypeptide. Such

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Abbreviations: HBsAg, hepatitis B surface antigen; BFA, brefeldin A; PI-PLC, phosphatidylinositol-specific phospholipase C; GPI, glycosylphosphatidylinositol, MDCK, Madin–Darby canine kidney; TGN, trans-Golgi network; FRT, Fisher rat thyroid.

‡ To whom reprint requests should be addressed at: Departamento de Inmunología Clínica y Reumatología, Facultad de Medicina, Pontificia Universidad Católica de Chile, Casilla 114-D, Santiago, Chile. e-mail: agonzara@osler.med.puc.cl.

a feature determines the apical targeting of several proteins in most epithelial cells examined, including MDCK and Caco-2 cells (23–26). It has been proposed that the GPI moiety functions by associating with glycosphingolipid clusters in the trans-Golgi network (TGN), which are preferentially incorporated into apically targeted vesicles in these cells (2, 27–31). An apical sorting mechanism can, therefore, be envisaged for proteins that lack GPI anchors, which relies on their association with the ectoplasmic portion of a specific GPI-anchored protein or with glycosphingolipid clusters in the TGN (2).

Recently, we showed that transfected MDCK cells that express the hepatitis B surface antigen (HBsAg) provide a useful system to study mechanisms determining the preferential apical secretion of specific proteins from polarized epithelial cells (32). We found that the HBsAg is secreted predominantly from the apical surface of MDCK cells, and that this continues to occur even when the cells producing the antigens are treated with tunicamycin, which suggests that N-glycan moieties do not play a sorting role for this viral glycoprotein (32). However, because the HBsAg is secreted in the form of a lipoprotein particle that contains 100–200 monomeric HBsAg polypeptide subunits (33–35) it is possible that the presence of only a few glycosylated monomers within an individual lipoprotein particle could determine its association with a lectin-type sorting receptor responsible for its apical secretion. Therefore, to critically assess the possible role of carbohydrate moieties in the sorting of the hepatitis antigen-containing particles it was necessary to completely eliminate glycosylation of the monomeric subunits. In this paper we analyze the polarity of secretion of a mutant form of the HBsAg in which the single asparagine residue capable of accepting an N-linked oligosaccharide chain (35) was eliminated. The results unequivocally demonstrate that N-glycosylation is not required for the effective apical secretion of the viral antigen-containing particles.

We also examined the possibility that the apical sorting of the HBsAg could depend on its hitchhiking with a newly synthesized GPI-anchored protein traveling from the TGN to the apical surface. We also were able to eliminate this mechanism by showing a dissociation of the apical sorting of the HBsAg from that of GPI-linked proteins. Although the mechanism that effects the apical targeting of HBsAg remains to be elucidated, we provide evidence that it shares essential features with that operating on other apically directed proteins.

## MATERIALS AND METHODS

**Expression Plasmids and Site-Directed Mutagenesis.** A *Hin*dIII/*Bam*HI 1850 fragment containing the *S* gene was removed from $pSV_2TKneo$-HBsAg (32), inserted into the corresponding sites of a pBluescript SK(−), and subjected to oligonucleotide site-directed mutagenesis (36), by using the primer 5′-ACAGGTGCAATATCCATCCGTAGG-3′ to replace the Asn-146 codon by a Tyr codon. The mutated cDNA was assessed by dideoxynucleotide chain-termination sequencing (Sequenase, United States Biochemical) (37) and inserted between the *Hin*dIII/*Bam*HI sites under the cytomegalovirus promoter of the pcDNA3 expression vector (Invitrogen), generating the pcDNA-HB-Tyr146 plasmid.

**Transfection and Polarity of the HBsAg Secretion.** The plasmids $pSV_2TKneo$-HBsAg (32) and pcDNA-HB-Tyr146 were transfected into type II MDCK cells and Fisher rat thyroid (FRT) cells (kindly provided by E. Rodriguez-Boulan, Cornell University, New York, NY) using the lipofectin (GIBCO/BRL) method according to the manufacturer's protocol. Briefly, 150,000–200,000 cells attached to plastic dishes were incubated with 2–5 $\mu$g of DNA and 10–20 $\mu$l of cationic liposomes for 8–16 h in media without serum or antibiotics. The cells then were trypsinized and plated in 10-$cm^2$ culture dishes (Nunc) in Coon's modified F12 medium containing 5% fetal calf serum for FRT cells and DMEM 7.5% fetal calf serum for MDCK cells, plus antibiotics. After 48 h of culture, G418 (GIBCO/BRL) was added to a concentration of 0.5 mg/ml for the FRT cells and 0.4 mg/ml for the MDCK cells. Permanent transformants were obtained after 2 weeks of selection.

To assess the polarity of the HBsAg secretion, 1–2 $\times$ $10^6$ trypsinized cells were plated on 24-mm Transwell filters, 0.4-$\mu$m pore size (Costar), and grown until the transepithelial resistance reached 5000–8000 ohm/$cm^2$ for FRT cells (5–7 days) and 200–300 ohm/$cm^2$ for the MDCK cells (3–6 days), as measured with an EVOM electrometer (World Precision Instruments, Sarasota, FL). The cells then were treated 12–24 h with 10 mM sodium butyrate. Pulse–chase experiments were made by incubating the cells for 30 min in methionine- and cysteine-free DMEM followed by 30 min with 150–200 $\mu$Ci (1 Ci = 37 GBq) of $^{35}$S-labeled methionine and cysteine (ICN) in 120 $\mu$l of medium applied to the basolateral side of the inverted filter. After 6–8 h of chase in medium containing 10-fold increased concentrations of methionine (3 mg/ml) and cysteine (6.5 mg/ml), immunoprecipitations with polyclonal antiserum against HBsAg (32) or gp80 (antiserum kindly provided by T. Gottlieb, New York University Medical Center, NY) or with monoclonals anti-laminin (GIBCO/BRL) or anti-fibronectin (Sigma), were made for the apical and basolateral media, as described (20, 32). The immunecomplexes were resolved by SDS/PAGE (5% polyacrylamide for laminin, 7.5% for fibronectin, 10% for gp80, and 12% for HBsAg). Fluorograms were developed on preflashed Kodak AR X-Omat films (Eastman) and digitalized using a VISTA-T630 UMAx scanner with Adobe Photoshop 3.1. The relative intensity of the bands was estimated with NIH Image 1.55.

**Analysis of GPI-Anchored Membrane Proteins.** Cell surface domain-specific biotinylation, extraction and partitioning of hydrophobic proteins with Triton X-114, and isolation of total GPI proteins by cleavage with phosphatidylinositol-specific phospholipase C (PI-PLC) to induce their transition from hydrophobic to hydrophilic state were carried out exactly as described (24, 38). Streptavidin-horseradish peroxidase conjugate and the enhanced chemiluminiscence (ECL) detection kit (Amersham) were used to detect the biotinylated proteins.

## RESULTS

**The Presence of N-Linked Oligosaccharide Chains on HBsAg Molecules Is Not Required for Their Apical Secretion.** Normally, HBsAg is secreted as a lipoprotein particle containing 100–120 monomeric subunits, about 50% of which have been reported to bear an N-linked oligosaccharide chain (35). When synthesized in transfected MDCK cells, approximately 20% of the HBsAg monomeric subunits are glycosylated and, therefore, have a significantly lower electrophoretic mobility than the unmodified subunits (Fig. 1, lane 1). To establish whether the N-linked oligosaccharides on these subunits have a signaling function in determining the apical targeting of the HBsAg lipoprotein particles in which they are contained, the effect of tunicamycin, an inhibitor of N-glycosylation, on the polarized secretion of the viral antigen has been examined using monolayers of transfected cells expressing the antigen grown on polycarbonate filters that separated apical and basolateral chambers (32). As illustrated in Fig. 1, tunicamycin treatment of the cultures eliminated the band corresponding to the glycosylated subunits (lanes 1 and 3), but did not measurably impair the nearly exclusively apical release ($\approx$82%) of pulse-labeled HBsAg molecules. On the other hand, as previously reported (16), the glycosylation inhibitor abolished the polarized apical release of the well known endogenous secretory glycoprotein gp80 (clusterin) and provoked a significant decrease (90%) in its total secretion (Fig. 1, lanes 3 and 4).

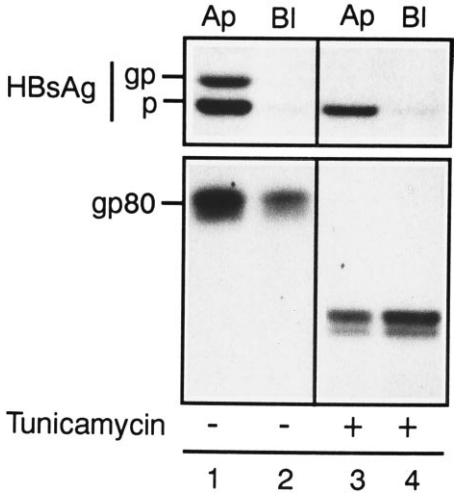


FIG. 1. Apical secretion of HBsAg is insensitive to tunicamycin inhibition of N-glycosylation, in contrast to endogenous gp80, in transfected MDCK cells. Permanent transfectants of MDCK cells forming impermeable monolayers in Transwell chambers were treated in the absence (lanes 1 and 2) or presence of tunicamycin (lanes 3 and 4), incubated 30 min with 100 mCi/ml [$^{35}$]S-labeled methionine and cysteine, and chased for 6 h. Tunicamycin was added at low concentration (2 $\mu$g/ml) for 18–20 h and was increased to 12 $\mu$g/ml 2 h before the pulse-labeling period and maintained thereafter during the chase. Immunoprecipitates from the apical (Ap) and basolateral (Bl) media were resolved by SDS/PAGE, in either reducing or nonreducing conditions for HBsAg or gp80, respectively. The fluorograms of the gp80 secreted from cells treated with tunicamycin were exposed for longer periods to compensate for the inhibition of its secretion (16). Glycosylated (gp) and unglycosylated (p) forms of HBsAg are indicated.

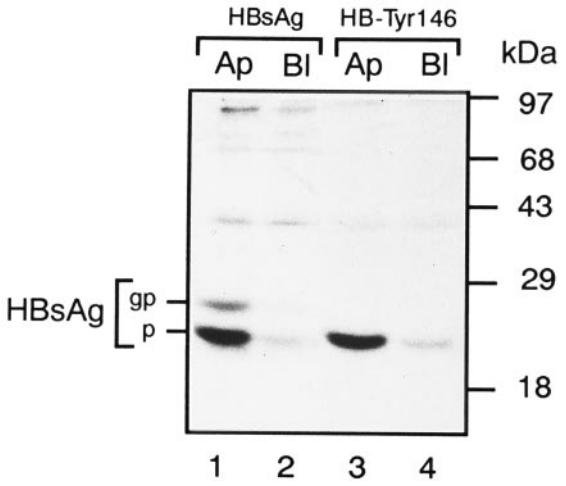


FIG. 2. Apical secretion of the mutant (HB-Tyr146) lacking N-glycosylation from permanently transfected MDCK cells. MDCK cells expressing either the wild-type HBsAg (lanes 1 and 2) or the HB-Tyr146 mutant (lanes 3 and 4), in which the the unique Asn-146 N-glycosylation site was replaced by Tyr, were grown on filters in Transwell chambers and pulse–chase labeled. Densitometric analysis of the fluorograms of the immunoprecipitated proteins resolved in SDS/PAGE indicated better than 80% apical distribution in both cases.

To eliminate the possibility that a small number of unglycosylated monomers within the viral antigen particles, undetectable in the biochemical assay, were sufficient to drive their apical secretion we analyzed, in a pulse–chase experiment, the polarity of secretion of a mutant HBsAg protein in which the single asparagine residue (Asn-146) that normally provides the acceptor site for the oligosaccharide chain was replaced by tyrosine. Fig. 2 (compare lanes 1 and 3) shows that, as expected, only the more rapidly migrating nonglycosylated form of the antigen was produced by permanently transformed MDCK cells expressing the mutant polypeptide and, yet, this was secreted with the same apical preference (>80%) as the wild-type one from control cells. Measured by ELISA, its apical polarity was 86 ± 3.6% ($n$ = 7). This finding definitively rules out a role of N-glycosylation as a determinant of the apical secretion of HBsAg-bearing particles.

O-glycosylation of HBsAg was found to take place when the polypeptide, expressed in a particular expression system, contains 25 extra N-terminal residues derived from part of the pre-S2 region preceding the *S* gene (39). O-glycosylation, however, has not been described when the antigen is expressed exclusively from the *S* gene (35, 39–42), as was the case in our experiments. We also were able to discard that O-glycosylation of the HBsAg occurs in our expression system, because in pulse–chase experiments with FRT and MDCK cells in conditions that abolished N-glycosylation of HBsAg, either by tunicamycin treatment or Asn-146 replacement, the HBsAg did not change in apparent molecular mass (24 kDa) during its intracellular traffic and secretion (not shown).

It also is worth noting that the newly synthesized nonglycosylated mutant HBsAg molecules were secreted with the same high efficiency and kinetics (>80% in a 7-h chase period; half-time of 4–5 h) as the wild-type molecules (not shown). This indicates that the amino acid change did not render the mutant polypeptides sufficiently abnormal as to lead to their intracellular retention and/or degradation in the endoplasmic reticulum by a cellular quality-control mechanism (12).

**Apical Secretion of HBsAg Does Not Result from Cosegregation with GPI-Anchored Proteins.** Because in many polarized epithelial cells GPI-linked proteins are apically segregated (24, 25, 30, 38), we considered the possibility that one or more such proteins served as carriers or receptors for the delivery of HBsAg particles to the apical surface. We, therefore, assessed the polarity of HBsAg secretion in FRT cells, a line of epithelial cells in which GPI-anchored proteins and glycosphingolipids are known to be preferentially delivered to the basolateral plasma membrane domain (31, 43). Measurements of the distribution of GPI-linked proteins using the domain-specific biotinylation procedure—followed by Triton X-114 extraction and PI-PLC treatment (24, 38)—indicated that sorting of GPI-linked proteins to the basolateral membrane was maintained in permanently transformed FRT cells that expressed either the wild-type or the HBsAg-Tyr146 mutant proteins (shown in Fig. 3*A* for the wild-type transformants). Nevertheless, the viral antigens were still secreted almost exclusively from the apical surfaces of these cells (Fig. 3*B*). This preferential apical secretion also was demonstrated by measuring, using ELISA, the amount of HBsAg accumulated in apical (78 ± 3.9%, $n$ = 6) and basolateral culture chambers after 12 to 24 h periods of incubation.

That the sorting of HBsAg proceeds independent of the delivery of newly synthesized GPI-linked proteins also was made clearly evident by the unaltered behavior of the pulse-labeled antigen in MDCK or FRT cell transformants that were treated with mannosamine, an inhibitor of the synthesis of the GPI anchor (44). This treatment completely suppressed the formation of GPI-anchored proteins (Fig. 4*A*, compare lanes 2 and 4 for MDCK cells, and lanes 6 and 8 for FRT cells) without affecting the preferential (>80%) apical secretion of the HBsAg particles in either cell type, as assessed in a pulse–chase experiment (Fig. 4*B*), or by measurement of the total antigen secreted in each compartment using ELISA (not shown). It is worth noting that inhibition of GPI-anchor synthesis also had no effect on the polarity of secretion of the endogenous protein gp80, indicating that this was also independent of GPI-anchor-mediated sorting.

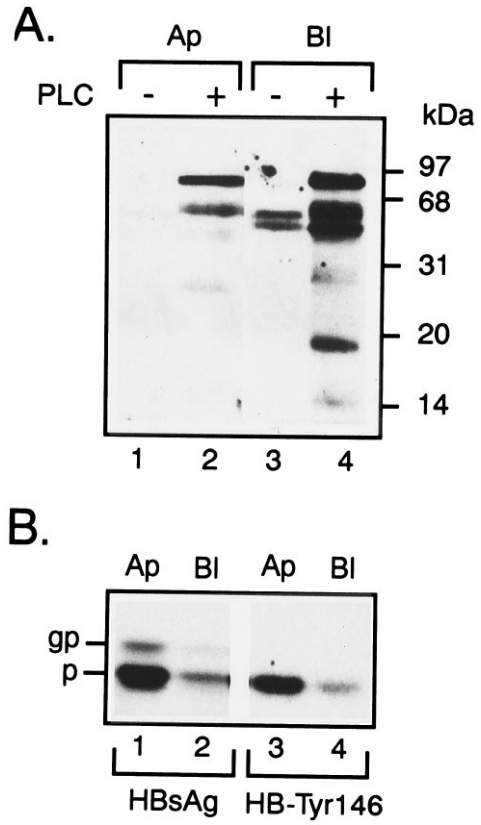


FIG. 3. Permanently transfected FRT cells still distribute most of their GPI-anchored proteins to the basolateral domain but secrete HBsAg apically. (*A*) FRT cells transfected with the $pSV_2$-TKneo-HBsAg plasmid and selected by neomycin resistance were analyzed by domain-selective biotinylation of apical (lanes 1 and 2) and basolateral (lanes 3 and 4) surface proteins. Triton X-114 extraction and temperature-induced phase separation, followed by PL-PLC (6 units/ml) treatment of the detergent phase, distinguishes the GPI-proteins by their PI-PLC-induced partitioning shift into the aqueous phase (lanes 2 and 4). Biotinylated proteins were resolved by SDS/PAGE, electroblotted onto nitrocellulose filters, and revealed by streptavidin-horseradish peroxidase and enhanced chemiluminiscence detection. After PI-PLC treatment, the aqueous phase showed most GPI-proteins predominantly basolateral (compare lanes 2 and 4); (*B*) Fluorograms show the preferential apical secretion of both the HBsAg (lanes 1 and 2) and HB-Tyr146 mutant (lanes 3 and 4) after pulse–chase labeling and immunoprecipitation.

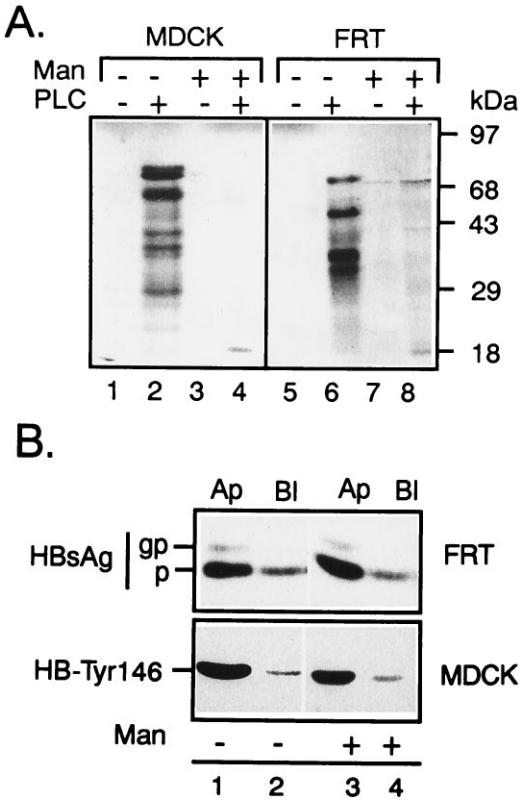


FIG. 4. Mannosamine treatment inhibits the biogenesis of GPI-anchored proteins in FRT and MDCK cells without affecting the apical secretion of HBsAg. (*A*) MDCK and FRT cells were preincubated for 6 h in glucose-free RPMI medium 1640 in the presence (+) or absence (−) of 10 mM mannosamine and then metabolically labeled for 18 h with $^{35}S$-labeled methionine and cysteine at 100 $\mu$Ci/ml in glucose, methionine, and cysteine-free RPMI medium 1640, 1% FBS, and 20 mM Hepes, in the corresponding preincubation conditions. The cells were extracted with 1% Triton X-114 and subjected to temperature-induced phase separation, and the detergent phases were incubated with PI-PLC (6 units/ml) for 1 h at 37°C to provoke the partition shift of GPI-proteins into the aqueous phases (lanes 2, 4, 6, and 8). Metabolically labeled GPI-proteins become undetectable after mannosamine treatment (lanes 3, 4, 7, and 8). (*B*) The secretion of HBsAg from FRT cells and HB-Tyr146 from MDCK cells during mannosamine treatment (lanes 3 and 4) was indistinguishable from control conditions (lanes 1 and 2).

**MDCK and FRT Cells Expressing the HBsAg Retain a Basolateral Secretory Pathway, and Treatment with Brefeldin A (BFA) Redirects the Antigen to That Pathway.** The behavior of HBsAg observed in the transfected cells can be interpreted as reflecting a sorting mechanism only if it can be demonstrated that normal basolateral secretory pathways are still operative in the cloned cell lines expressing the viral antigens. We, therefore, examined in the transfected cell lines the polarity of secretion of several endogenous extracellular matrix proteins that in the parental cell lines are secreted from the basolateral surface. Fig. 5 (lanes 1 and 2) shows, in a pulse–chase experiment, that laminin and fibronectin were secreted almost exclusively from the basolateral surfaces of transfected MDCK and FRT cells, respectively, despite the simultaneous apical secretion of the HBsAg. This established the preservation of intact basolateral routes in the transfected cells.

The possibility also was considered that after traversing the Golgi apparatus the viral antigen particles are sorted in the TGN by a mechanism that is totally distinct from that which operates on other apically destined proteins, including the glycoproteins, such as the gp80, whose sorting is dependent on their N-linked oligosaccharides. We took advantage of the finding that treatment of MDCK cells with BFA preferentially inhibits protein secretion from the apical surface and redirects apical proteins to the basolateral route (45–47). We found (Fig. 5, lanes 3 and 4) that BFA treatment totally reversed the apical polarity of secretion displayed by both the nonglycosylated hepatitis surface antigen (HB-Tyr146) and the gp80 glycoprotein, whereas the basolateral sorting of laminin and fibronectin was not affected, as described for several other basolaterally targeted proteins (45, 46). These observations also confirmed that a functional basolateral route exists in the transfected cells and, more importantly, because BFA is known to act by preventing the activation of ADP-ribosylation factor, a factor required for transport vesicle formation (48, 49), that similar molecular components are involved in the processes of formation of the apical transport vesicles that carry HBsAg or gp80.

## DISCUSSION

The preceding results demonstrate that the preferentially apical secretion of HBsAg in polarized epithelial cells does not require N-glycosylation of the polypeptide and, therefore, is

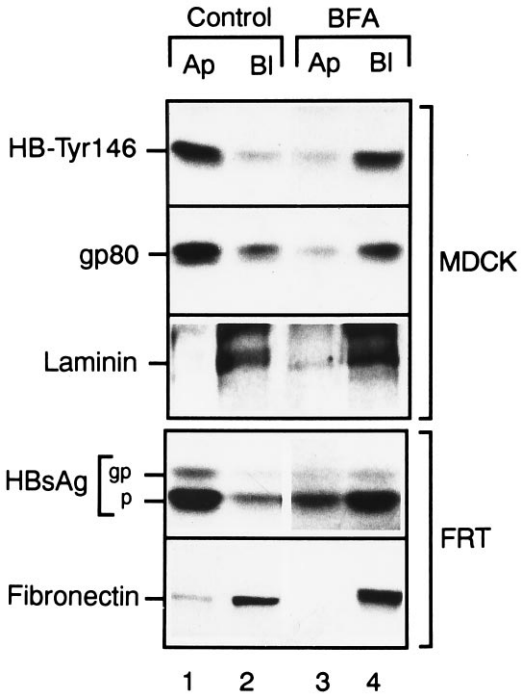


FIG. 5. HBsAg and HB-Tyr146 were secreted predominantly basolaterally when the apical pathway was blocked by BFA. MDCK and FRT cells grown in Transwell chambers were pulse-labeled with $^{35}$S-labeled methionine and cysteine for 30 min and chased for 6–8 h. Immunoprecipitations from apical and basolateral media of laminin, HB-Tyr146, and gp80 secreted by MDCK cells, and fibronectin and HBsAg secreted by FRT cells were made. Control conditions (lanes 1 and 2) showed a preferential apical secretion of HBsAg and the N-glycosylation negative mutant. Transfected MDCK and FRT cells still possess an intact basolateral pathway, disclosed by their secretion of laminin and fibronectin, respectively. BFA (10 $\mu$g/ml), added 30 min before pulse labeling and maintained during the chase (lanes 3 and 4), did not affect basolateral secretion of these proteins, whereas apical secretion of HBsAg and HB-Tyr146 was inhibited, redirecting them to basolateral.

not mediated by an interaction of the antigen with a cellular lectin that serves as a sorting receptor, as has been proposed to be the case for other secretory glycoproteins (12). Thus, removal, by mutagenesis, of the single site for N-glycosylation in HBsAg did not affect the extent of its apical secretion (>80%). This finding is consistent with the failure of tunicamycin treatment to alter the polarity of secretion of HBsAg and differs radically from the behavior of other glycoproteins, including the endogenous major secretory product of MDCK cells, gp80 (16), and several exogenous proteins that have been expressed in MDCK cells, such as erythropoietin (18), and the human corticosteroid-binding globulin (17), which are misdirected by tunicamycin treatment or by elimination of specific glycosylation sites (18).

It should be noted that in the latter cases the N-glycans may not necessarily serve as a sorting signal, but rather may function to stabilize a transport-competent conformation of the protein or to make accessible a discrete peptidic signal within it, which otherwise may be masked. The importance of glycosylation for proper protein folding is apparent from the fact that many glycoproteins synthesized in the presence of tunicamycin are retained intracellularly or degraded (see ref. 12). Stabilization of protein conformation as a result of dynamic interactions between the polypeptide and its attached glycan has been described by x-ray diffraction for the plasma membrane protein CD2 (50). The fact that one of the three N-glycan chains in erythropoietin plays a much more important role in determining the apical sorting of the protein than the other two (18) also suggests that the N-glycan does not simply act by interacting with a lectin receptor. These considerations do not exclude, of course, the possibility that N-glycans could at least in some cases directly convey sorting information, possibly contained in the core region of the oligosaccharide chain (51). This is suggested by the finding that whereas growth hormone—which in endocrine cells enters the regulated secretory pathway (52) and, when expressed in MDCK cells, is constitutively secreted in an unpolarized fashion (19)—when mutated to contain N-glycan acceptor asparagine residues manifests an increasing apical polarity concomitantly with the acquisition of more than one N-glycan chain (15). However, the apical-signaling properties of an oligosaccharide chain would appear to be relatively weak, because addition of more than one chain to growth hormone was required to achieve its efficient (80%) apical secretion (15). The relative weakness of glycan-related signals with respect to other basolateral signals in the same protein would explain why many transmembrane and secretory proteins are targeted basolaterally, despite the presence of N-linked oligosaccharides.

We also have ruled out a mechanism for apical delivery of HBsAg that depends on its cotransport to the apical surface together with GPI-anchored membrane proteins. These proteins are exclusively targeted to the apical domains of various kinds of epithelial cells, including kidney- and intestine-derived cell lines, such as MDCK and Caco-2 (24, 38). Furthermore, chimeric proteins containing GPI anchors linked to ectodomains of basolateral membrane proteins, or to secretory proteins that are not secreted in a polarized fashion, are preferentially targeted to the apical surface of MDCK cells (23, 26, 53). Although the polarized apical distribution of GPI-anchored proteins is highly conserved across species and tissue types (24), a striking exception is provided by FRT cells, in which most GPI-anchored proteins are targeted to the basolateral membrane (43). We used these unusual cells to analyze the relationship between the apical sorting of HBsAg and the sorting mechanism that relies on GPI anchoring. We found that in these cells HBsAg was targeted apically, despite the preferential basolateral targeting of the lipid-anchored proteins. Additionally, we found that in both MDCK and FRT cells treatment with mannosamine for 24 h, which completely eliminated the acquisition of GPI anchors, had no effect on the apical secretion of the HBsAg. This clearly demonstrated that delivery of the HBsAg to the apical surface is not dependent on the concomitant transport of newly synthesized GPI-anchored proteins, making it unlikely that a GPI-linked protein serves as the apical receptor for HBsAg. Furthermore, because glycosphingolipids are sorted preferentially to the apical surface of MDCK cells (27), whereas the reverse occurs in FRT cells (31), it seems that the apical-targeting mechanism of HBsAg is also independent of any other protein that could cosegregate with glycosphingolipid clusters in the TGN (2, 30).

The possibility that HBsAg follows a secretory pathway from the TGN to the cell surface that is radically different from that of other well studied secretory proteins seems unlikely, although the early biosynthetic stages of HBsAg have some unique features. The antigen is synthesized in the endoplasmic reticulum (ER) as a transmembrane precursor protein (41, 54), and its maturation includes rapid formation of disulfide dimers in the ER, transport to the ER-Golgi intermediate compartment where higher oligomers are formed, and assembly into particles that bud into the lumen of this compartment (42). Subsequently, the particles are transported to the Golgi apparatus, where their oligosaccharides are modified to endo H-resistant forms (40, 42). It is not apparent what aspect, if any, of this complex assembly process may contribute to the specific non-glycosylation-dependent sorting of this protein that takes place in the TGN. The selective targeting of HBsAg to the apical surface, nevertheless, seems to involve a pathway

that, at least partially, overlaps with that followed by gp80, and presumably other apically targeted proteins (45–47, 55). Thus, treatment of MDCK cells with BFA redirected both HBsAg and gp80 to the basolateral surface, implying that the apical transport of these proteins relies on a common or related BFA-sensitive component (48, 49), most likely a member of the ADP-ribosylation factor family of GTP-binding proteins (56).

Although expression of HBsAg did not affect the polarity of secretion of the endogenous gp80, it is still possible that the two proteins share the same TGN sorting receptor, but whereas recognition of a peptide or conformational signal in gp80 requires the presence of the N-glycan in the protein, the corresponding signal in HBsAg is fully expressed even when this antigen lacks an oligosaccharide chain. In any case, it is clear that the HBsAg provides a good model for undertaking the identification of both the glycan-independent signal responsible for apical targeting and, ultimately, its cognate cellular receptor.

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