Polarized Apical Targeting Directed by the Signal/Anchor Region of Simian Virus 5 Hemagglutinin-Neuraminidase*

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To examine the possibility of independent cytoplasmic/transmembrane domain-based apical sorting, we have investigated paramyxovirus SV5 hemagglutinin-neuraminidase (HN), a type II membrane protein with a small N-terminal signal/anchor region. In SV5-infected Madin-Darby canine kidney (MDCK) cells, >90% of HN is found on the apical surface. We have expressed chimeric proteins in which the N terminus of HN, including its signal/anchor region, is attached to a (normally cytosolic) reporter pyruvate kinase (PK). PK itself expressed immediately downstream from a cleavable signal peptide was converted to a 58-kDa N-linked glycosylated form, which was secreted predominantly (80%) to the basolateral surface of MDCK cells. By contrast, stably expressed PK chimeras, now anchored as type II membrane proteins with either the first 48 or 72 amino acids of HN, received similar N-linked glycosylation, yet exhibited polarized transport with a preferentially (75%) apical distribution. These results suggest that the N-terminal signal/anchor region of HN contains independent sorting information for apical specific targeting in MDCK cells.

Enveloped viruses have provided an extremely useful paradigm to understand polarized protein traffic in epithelial cells (1). Viral membrane glycoproteins, even when expressed without other viral constituents, contain information sufficient for selective transport to either the apical or basolateral surface (2–10). Indeed, analyses of both viral and endogenous glycoproteins expressed in epithelial cells, using recombinant DNA approaches, have shown that the information required for polarized transport depends on specific structural determinants contained in the newly synthesized glycoproteins (11). From these analyses, several common themes have emerged. Specifically, attachment of a glycosylphosphatidylinositol anchor to protein moieties that are otherwise luminal is sufficient for polarized surface delivery (12, 13) that is typically (but not in every case) (14) directed apically. In addition, studies using type I membrane glycoprotein chimeras as well as truncation mutants have suggested that the ectodomains of these membrane proteins possess signals for apical transport (9, 15–18). Most recently, this idea has been extended to suggest that both secretory glycoproteins and the ectodomains of membrane glycoproteins may use N-linked carbohydrates either indirectly (19) or directly (20) for polarized sorting. However, Roth et al. (21) and Green et al. (22) found that complete inhibition of glycosylation did not affect polarized viral glycoprotein expression. On the other hand, information for the basolateral transport of the vesicular stomatitis virus G-protein is contained within its cytoplasmic/transmembrane extension (23), and furthermore, specific motifs in the cytoplasmic tails of several mammalian membrane protein receptors and lysosomal membrane glycoprotein lgp120 are now recognized to be essential for the targeting of these proteins to the basolateral surface (24–28). Of course, it should be noted that most of these studies have employed single-spanning membrane proteins with a type I topology (29), and it has not been excluded that specific basolateral targeting information can also exist in protein luminal domains (9, 23, 30–32).

SV5, which is released by budding from the apical surface of infected epithelial cells (2), possesses two glycoproteins, the hemagglutinin-neuraminidase (HN) and fusion protein. HN is a type II membrane protein with a small N-terminal signal/anchor region and a large C-terminal ectodomain (33, 34). In recent years, chicken muscle pyruvate kinase (PK) has been a favored reporter for protein targeting, as chimeric genes containing specific sorting information can relocate the normally cytosolic PK moiety to the nucleus (35, 36), the membrane of the endoplasmic reticulum (37), or the mitochondria (38). With this in mind, a chimera including the signal/anchor portion of HN was found to redirect PK to the cell surface, suggesting that specific protein targeting information may reside in this region of HN (39). However, it has been unclear if this segment could also account for polarized apical delivery of HN. In this study, we have utilized HN-PK chimeras to examine this question in stably transfected MDCK cells.

EXPERIMENTAL PROCEDURES

Construction of Chimeric HN-PK and Secretory PK cDNAs—Construction of the HNαPK chimera via an in-frame EcoRI linker (5′-CGGAAT-TCC-3′), encoding the hydrophilic linker Arg-Ang-Ser) in pSV63 has been described (previously called APKO (39). Intact HNα-PK was excised from pSV63 with XhoI and subcloned first into pGEM7Z and then, using HindIII and XhoI sites, into pRF4CMV (Invitrogen). This produced the plasmid pRCHNα-PK, encoding a protein joining HN residues 1–48 to PK residues 17–529 by a 3-amino acid linker (encoding Arg-Ang-Ser), driven

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1 The abbreviations used are: HN, hemagglutinin-neuraminidase; PK, pyruvate kinase; MDCK, Madin-Darby canine kidney; PAGE, polyacrylamide gel electrophoresis; PNGase F, peptide N-glycosidase F.
by the cytomegalo virus promoter. To construct HN<sub>n</sub>PK, an oligonucleotide (5′-TTGGATCCAgAcGCTGTTGcAcAGAAGATGCCCCT-3′) containing a HindIII site followed by HN nucleotides 320–340 was used as an upstream primer, and an oligonucleotide (5′-GTCGACATTCTGTCAAGCAGAAGATGCCCCT-3′) containing HN nucleotides 521–538 followed by an EcoRI linker was used as a downstream primer to amplify the N-terminal fragment 1–72 of the HN gene by polymerase chain reaction as described previously (40). The amplified DNA fragment was isolated after digestion with HindIII and EcoRI, and then incubated with 10,000 units/ml PNGase F in 0.5% SDS plus 1% 2-mercaptoethanol at 100 °C for 10 min; cooled; and then incubated overnight at 4 °C with rabbit anti-PK sera. Subclones of transfected MDCK cells were induced with 10 μg/ml G418 (500 μg/ml; Life Technologies, Inc.) in Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum. Infected cells were maintained at 37 °C for 16 h (26) and then labeled for 2 h with 100 μCi of [35S]methionine/cysteine (see Fig. 4D) to establish a linear relationship of ECL densities in the range of 0.1–100 arbitrary units in 5% nonfat dry milk in phosphate-buffered saline and then for 2 h with primary antibodies diluted in the same buffer. Protein-bound radioactivity was quantified using a PhosphorImager. Immunoblot Analysis and Densitometric Quantitation—For immunoblotting, biotinylated samples were analyzed by reducing 8.5% SDS-PAGE. The proteins were transferred to nitrocellulose filters (0.2-μm pore size; Schleicher & Schuell) and incubated for 1 h at room temperature in 5% nonfat dry milk in phosphate-buffered saline and then for 2 h with primary antibodies diluted in the same buffer. Protein-bound antibodies were detected with horseradish peroxidase-conjugated specific secondary antibodies using enhanced chemiluminescence (ECL, Amersham Corp.). Immunoblots were quantified by scanning densitometry. The method was validated by using serial dilutions of protein samples to establish a linear relationship of ECL densities in the range of protein concentrations studied.

Antibodies—Monoclonal antibodies to SV5 HN were kindly provided by Dr. R. E. Randall (42). An antiserum to chicken PK was prepared in rabbits, which received a total of 250 μg of antigen (five × 50 μg). The first intramuscular injection of PK was emulsified in Freund’s complete adjuvant, followed by three boosts at weekly intervals emulsified in Freund’s incomplete adjuvant. Finally, a single intravenous dose of PK (without adjuvant) was administered 5 days before bleeding.

RESULTS

Expression and Polared Exocytotic Discharge of Secretory PK—Previous studies have established that for chimeras that position the normally cytosolic PK moiety in the lumen of the transmembrane polypeptide, a major fraction of the expressed chimera undergoes N-linked glycosylation and intracellular transport to the cell surface (39). Since our goal was to attach putative signals for polarized protein targeting to the PK receptor, we first set out to examine the fate of a chimeric protein expressed after ligating a cDNA fragment encoding the N-terminal 31-amino acid signal peptide of preprolactin directly to PK itself.
Fig. 2. Synthesis and glycosylation of secretory PK in stable MDCK clones. Transfected MDCK cells were labeled with [35S]Met/Cys for 8 h. A, the cells were lysed in TNT lysis buffer with 0.1% SDS, immunoprecipitated with anti-PK serum, and then analyzed by 8.5% SDS-PAGE. Mock, cells transfected with empty vector; PK, cells transfected with recombinant secretory PK; P-P, secretory PK after digestion with PNGase F. B, the labeling medium was collected, clarified by centrifugation, and then immunoprecipitated with anti-PK serum. Mock, medium derived from cells transfected with empty vector; PK, medium bathing cells expressing recombinant secretory PK; P-P, secreted PK digested with PNGase F. The second and third lanes were collected from clonal MDCK cells after early passage (<10), whereas the fourth and fifth lanes represent clonal MDCK cells after later passage (>25), indicating stability of expression and secretion of 58-kDa PK.

("secretory PK") (Fig. 1). This signal peptide contains the information necessary for normal cleavage by signal peptidease (43, 44), resulting in a threonine residue as the new N terminus of the mature protein. After G418 selection of stably transfected MDCK cells, clones expressing this form of PK were identified by immunoprecipitation and SDS-PAGE analysis.

Unglycosylated secretory PK with an uncleaved signal is predicted to be a polypeptide of 547 amino acids with a molecular mass of ~60 kDa, whereas unglycosylated secretory PK after cleavage of the signal peptide is 517 amino acids with a predicted molecular mass of ~56 kDa. Indeed, when we expressed a leaderless cytosolic form of PK in COS cells, this unglycosylated polypeptide lacking a signal peptide migrated on SDS-PAGE at the predicted size of ~56 kDa (data not shown). For expression in the lumen of the secretory pathway, the PK cDNA sequence contains one potential N-linked glycosylation site (45). With this in mind, we observed that in MDCK cells expressing secretory PK, intracellular immunoreactive forms of PK included different closely migrating species: the majority ran as an ~58-kDa species, whereas a smaller portion ran at ~56 kDa (Fig. 2A, second lane).2 To see if the larger form occurred as a consequence of N-linked glycosylation, the secretory PK forms expressed in MDCK cells were treated with PNGase F. In this case, all forms collapsed into a single species of ~56 kDa (Fig. 2A, third lane). Because PNGase F removes N-linked glycans that are added in the lumen of the secretory pathway, these molecular mass data strongly suggest that secretory PK had indeed been translocated into the endoplasmic reticulum lumen and underwent removal of the prolactin signal peptide and that most of the secretory PK received N-glycosylation as has been reported for the membrane-bound form (39). Such N-linked glycosylation has been proposed to have important benefits in favoring protein stability and export through the secretory pathway (46–49).

We therefore examined the stability and export of newly synthesized secretory PK in MDCK cells over a 12-h chase. As expected at the zero chase time, secretory PK was quantitatively intracellular (Fig. 3A). There was intracellular loss of a portion (~50%) of labeled PK during the first 2 h of chase (Fig. 3B) that is not understood; loss was seen for both the 58- and 56-kDa forms (Fig. 3A). Following this, however, between 2 and 12 h of chase, the remaining immunoprecipitable 58-kDa PK was progressively released from the cells to the medium, with τ1/2 ~ 4 h and with an efficiency of ~90% over this time course (Fig. 3C). As expected, PNGase F treatment converted PK in the medium back to an ~56-kDa species (Fig. 2B). The release of ~58-kDa PK was not due to nonspecific cell leakage or lysis, as the 56-kDa form of PK was retained quantitatively within the MDCK cells (Fig. 3A). Moreover, secretory PK recovered free in the medium was not sedimentable in a 1-h spin at high speed (data not shown). Thus, the data in Figs. 1–3 indicate that the prolactin signal-PK chimera enters the lumen of the secretory pathway and undergoes N-linked glycosylation. While a portion of the PK protein is apparently degraded intracellularly, the remaining glycosylated 58-kDa form is competent for intracellular transport and behaves as a valid secretory protein marker.

Next, we investigated the polarity of PK secretion from filter-grown MDCK cells. Stable MDCK transfectants expressing secretory PK were continuously labeled for 8 h, and apical and basolateral media were collected and analyzed by immunoprecipitation, SDS-PAGE, and fluorography (Fig. 4A, upper panel). Interestingly, only 20% of secreted PK (~58 kDa) was found on the apical side of transfected cells, with 80% recovered in the basolateral medium (Fig. 4A, lower panel). By contrast, the endogenous MDCK secretory protein gp80/clusterin (50) was released with apical predominance (data not shown). To exclude possible rapid apical secretion of PK followed by redistribution and transcytosis to the basolateral side, polar-
HN48PK chimera (Fig. 1), in which the HN N terminus including the signal for apical specific transport in MDCK cells. We next examined the expression of the HN48PK expression in CV1 cells, where the glycosylation in MDCK cells, as is the case for secretory PK was followed as a function of chase time (Fig. 4). Evidently, from the moment that PK secretion is first detected, the protein is directly delivered with a fixed polarized ratio leading to apical specific transport in MDCK cells. Filter-grown MDCK cells transfected with the secretory PK gene were metabolically labeled for 5 min with [35S]Met/Cys and chased for the times indicated. At each chase time, the apical and basolateral media were immunoprecipitated with anti-PK serum and analyzed by SDS-PAGE and fluorography (left panels). Quantitation by scanning densitometry is also shown (right panel). arb. units, arbitrary units.

Expression and Polarized Distribution of the HN48PK Protein in MDCK Cells—We next examined the expression of the HN48PK chimera (Fig. 1), in which the HN N terminus including its signal/anchor is linked to the luminally oriented PK protein (39). Immunoprecipitates with anti-PK serum from cells transfected with pRC/HN48PK revealed two closely migrating species, a major band at ~67 kDa and a slightly less intense band at ~65 kDa (Fig. 5A). When treated with PNGase F to remove N-linked oligosaccharides, the two different species migrated with the same mobility as a nonglycosylated form at ~63 kDa (Fig. 5B), in agreement with previous reports (39). Thus, the luminal domain of HN48PK underwent N-linked glycosylation in MDCK cells, as is the case for secretory PK (Fig. 2) and for HN48PK expression in CV1 cells, where the chimera underwent glycosylation en route to residence at the cell surface (39).

To examine the polarized surface distribution of the HN48PK chimera in filter-grown MDCK cells, we utilized cell-surface biotinylation. As a control, in SV5-infected MDCK cells, ~95% of blottable HN was found on the apical surface (Fig. 6, left). Interestingly, linking the N-terminal region containing the signal/anchor of HN to PK (Fig. 1) caused a change in the targeting of the glycosylated PK reporter (Fig. 5) from a basolateral (Fig. 4) to an apical surface distribution. Specifically, ~75% of HN48PK was now present on the apical surface of transfected cells (Fig. 6, right). The data indicate that the N-terminal region of HN, even when transplanted onto a basolaterally targeted luminal domain, by itself is sufficient for preferential apical distribution, although this steady-state distribution is less completely apical than that found for the full-length HN protein in the setting of expression with other viral components.

To determine whether the apical distribution of HN48PK was accomplished by direct polarized delivery from the trans-Golgi network, we examined the kinetics of first appearance of the newly synthesized protein on the apical and basolateral surfaces. Filter-grown monolayers were pulse-labeled with [35S]methionine/cysteine for 20 min and chased for various times. At each chase interval, replicate cell monolayers were biotinylated on either the apical or basolateral side. As a control, virally infected MDCK cells were examined, and it was found that initial delivery of intact HN was ~90% to the apical surface (Fig. 7A), whereas the HN48PK protein arrived at 90 and 180 min with an apical preference in the 80–85% range (Fig. 7B) (and this apical delivery was already detectable at 45 min (data not shown)). The ratio of polarized initial delivery of HN48PK (apical/basolateral ~4:1) to the cell surface was slightly higher than the ratio of protein distribution at steady state (apical/basolateral ~3:1), just opposite of what would be observed in the integrally targeted membrane.
expected if this protein distribution were derived from basolateral to apical transcytosis. Thus, these results indicate that the polarized sorting of HN48PK occurs largely at the \textit{trans}-Golgi network. Although the apical polarity of newly delivered HN48PK was impressive, by 6 h after synthesis, a substantial decline in the level of newly synthesized HN48PK on the cell surface was observed (Fig. 8, dashed line). Since most of the delivery of HN48PK to the cell surface was apical, the subsequent loss of most of the HN48PK from the cell surface also occurred on the apical side (Fig. 7B). No HN48PK was recovered in the bathing medium, indicating that the protein was not shed from trans-fected cells.\textsuperscript{3} However, clathrin-coated internalization of HN from the cell surface and its subsequent degradation in the endocytic pathway have been described (51); the kinetics of turnover of newly synthesized HN48PK protein in MDCK cells (Fig. 8, solid line) suggested the possibility of a similar fate for the HN signal/anchor-containing chimera. Although the ordinates for the two graphs plotted on Fig. 8 are not the same, we note that $\geq 75\%$ of newly synthesized HN48PK has been reported to reside on the plasma membrane at the 3-h chase time (39). Taken together, the data suggest that despite cellular events that transpire after surface externalization (which may diminish the apparent apical protein distribution), attachment of the HN signal/anchor causes newly synthesized, glycosylated PK protein to be redirected from the basolateral to the apical surface.

Expression and Polarized Surface Distribution of HN72PK—Few if any apical sorting signals for membrane proteins have been previously described to exist in cytoplasmic/transmembrane domains, as there has been greater emphasis on the possibility that such signals are contained in the ectoplasmic domain (29). Thus, we constructed HN72PK (Fig. 1) to investigate whether including an increased portion of the HN N terminus, containing more of the juxtamembrane ectoplasmic region, could enhance the efficiency of apical transport. To determine the expression of HN72PK in stable MDCK transfectants, radiolabeled cells were immunoprecipitated with anti-PK serum and analyzed by SDS-PAGE and fluorography. As shown in Fig. 9A, HN72PK was expressed as a predominant band of $\approx 71$ kDa, with a minor band at $\approx 69$ kDa. When the same sample was treated with PNGase F, the two species migrated with the same mobility as a nonglycosylated form at $\approx 67$ kDa, indicating that the HN72PK chimera undergoes N-linked glycosylation. We therefore proceeded to examine the polarized surface distribution of HN72PK in transfected MDCK cells by selective cell-surface biotinylation (Fig. 9B). Quantitatively, $\approx 75\%$ of the HN72PK chimera was apically distributed, with $\approx 25\%$ of the surface protein found basolaterally (Fig. 9C). Evidently, the HN72PK protein behaved similarly to the HN48PK chimera (Fig. 6). Thus, as for the nerve growth factor receptor (16), inclusion or exclusion of luminal juxtamembrane residues has no specific effect on apical protein distribution.

Of note, using the same pRC/CMV expression vector to produce G418-resistant MDCK cells, several attempts to obtain clones stably expressing the full-length HN protein were unsuccessful, possibly because the hemagglutinin-neuraminidase activities produce a selective growth disadvantage in comparison with MDCK cells that do not express this protein. Thus,

\textsuperscript{3} R. A. Lamb, unpublished results.
we cannot exclude that additional information directing apical targeting may reside in more distal portions of the ectoplasmic domain of the HN protein.

**DISCUSSION**

In SV5-infected MDCK cells, the viral HN protein resides almost exclusively on the apical surface (Fig. 6) (2). Since viral membrane proteins contain autonomous information regulating transport to either the apical or basolateral surface (52), there is reason to think that the HN protein may also contain such signals. In recent years, the cytoplasmic tails of certain membrane proteins have been the focus of attention as sites for basolateral sorting signals (24–28), whereas the ectodomains of membrane proteins have been the focus of attention as sites for apical sorting signals (9, 15–18). Nevertheless, in MDCK cells, truncation of the cytoplasmic tail of the vesicular stomatitis virus G-protein to only 1 amino acid still leads to a predominantly basolaterally targeted protein (23), whereas addition of the cytoplasmic tail with or without the transmembrane domain of influenza hemagglutinin to the truncated vesicular stomatitis virus G-protein may (53) or may not (9, 30) disrupt the basolateral targeting. Moreover, the “rules” that suggest that cytoplasmic/transmembrane domains are relevant only for basolateral targeting may not apply to the polarized sorting for type II membrane glycoproteins since a chimera involving the cytoplasmic/transmembrane domain of influenza virus neuraminidase (normally an apically expressed protein (5)) redirects the ectodomain of the human transferrin receptor (normally a basolaterally expressed protein) to the apical surface (54).

To identify possible polarity signals contained in the N-terminal region of the HN protein including the signal/anchor, we used chimeric constructions with PK, which has been commonly employed by others as a successful reporter for protein targeting studies (35–39). Remarkably, when secretory PK was expressed after attaching a cleavable prolactin signal peptide to its N terminus, in which the “consensus” information for signal peptide cleavage is conserved, the PK protein was secreted from the basolateral surface of transfected MDCK cells with high fidelity, approaching or equal to that of secreted proteins with defined basolateral sorting signals (31, 32). This finding is all the more remarkable in light of the fact that the basolateral secretion of this “neutral reporter” was exclusively restricted to those forms that had undergone N-linked glycosylation, which has recently been suggested to play an indirect (19) or direct (20) role in apical specific trafficking. Thus, considering the earlier studies indicating that complete inhibition of glycosylation did not affect polarized glycoprotein expression (21, 22), it appears that a role for N-glycans in apical specific targeting in MDCK cells exists only in the context of some proteins and not others (55). The 58-kDa form of secretory PK, although glycosylated, exhibits an apparently dominant basolateral sorting signal. With this in mind, the luminal PK domain serves as a useful recipient for testing potential apical targeting information in the N-terminal region of HN.

We expressed two HN-PK chimeras that contained the N-terminal signal/anchor of HN plus increasing segments of the HN ectodomain (Fig. 1). The results with both chimeras were similar: the apical distribution of the proteins was ≈75% (Figs. 6 and 9). Although one can never exclude the possibility that the conformation of basolaterally secreted PK (i.e., after cleavage from the signal peptide) is subtly different from that of PK tethered to the signal/anchor of HN, there is no evidence to indicate this, suggesting that the N-terminal domain of HN contains signals for apical sorting. We note that, in general, expression of the membrane protein chimeras was not as polarized as that of their endogenous counterparts, which could reflect competing apical and basolateral signals in different protein regions, and this might also be influenced by internalization of the chimeras after delivery to the apical cell surface (Figs. 7B and 8). Thus, we hypothesize that in this type II membrane protein, a dominant apical signal exists in the N-terminal domain containing the signal/anchor, which can account for most if not all of the polarized targeting of the HN protein to the apical surface. Further studies are needed to define more precisely the nature of apical specific targeting signals in this region of HN. The finding of apical specific targeting signals in the cytoplasmic domain would be of special interest in light of recent developments in the identification of new adaptor proteins that may play roles in molecular sorting of apical membrane proteins at the level of the trans-Golgi network (56).

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