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The E1 glycoprotein of an avian coronavirus is targeted to the cis Golgi complex

(intracellular protein trafficking)

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ABSTRACT **It was previously reported that the E1 protein of an avian coronavirus was targeted to the juxtanuclear region in COS cells expressing the protein from cloned cDNA, suggesting that the protein contains information for targeting to the Golgi complex. The first of three membrane-spanning domains was required for intracellular targeting, because a mutant E1 (Δm1,2) lacking this domain was delivered to the plasma membrane. We have used immunoelectron microscopy to localize the wild-type E1 protein within Golgi elements of COS cells and AtT-20 cells expressing these proteins from recombinant vaccinia vectors. By immunoperoxidase and immunogold labeling, the wild-type E1 protein was localized to one or two cisternae located on one side of the Golgi stack that could be identified as the cis side in AtT-20 cells. In contrast, the mutant E1 protein was detected in all cisternae across the stack as well as at the plasma membrane. When the E1 proteins were immunoprecipitated and subjected to digestion with endoglycosidase H, the majority of the wild-type E1 glycoprotein was endoglycosidase H sensitive, whereas the majority of the mutant E1 was processed to an endoglycosidase H-resistant, polylactosaminoglycan-containing form. The findings indicate that the wild-type E1 protein is specifically targeted to cis Golgi cisternae and are consistent with the assumption that the first membrane-spanning domain is required for targeting to the cis Golgi.**

The Golgi complex plays a key role in the processing and sorting of polypeptides that enter the exocytic pathway. This organelle consists of stacks of flattened cisternae that can be divided into at least three functionally distinct subcompartments: cis, middle, and trans (1–3). Each subcompartment is enriched in different glycosidases, glycosyltransferases, and other processing enzymes (4). This compartmentalization allows orderly and sequential processing of glycoproteins as they are transported vectorially through the Golgi (4). Little is known regarding the mechanism by which resident Golgi proteins are sorted from those that will reach the plasma membrane and how those resident proteins are retained in specific Golgi cisternae.

It was shown previously that the E1 glycoprotein of an avian coronavirus, infectious bronchitis virus (IBV), is targeted to the Golgi region of cells expressing the protein from cloned cDNA (5). Here we have used immunoperoxidase and immunogold labeling in conjunction with analysis of carbohydrate processing to determine the specific site [i.e., transitional elements of the endoplasmic reticulum (ER) or subcompartment of the Golgi] to which the E1 glycoprotein is targeted. We report that the E1 glycoprotein is concentrated in the cis Golgi of cells expressing the protein from cloned cDNA.

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MATERIALS AND METHODS

Cells and Viruses. AtT-20 and COS-1 cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum. The generation of recombinant vaccinia viruses encoding the wild-type E1 protein and the mutant Δm1,2 was previously described (5).

Anti-E1 Antibody. The polyclonal antibody recognizing the IBV E1 protein was generated in a rabbit immunized with a synthetic peptide corresponding to the 22 carboxyl-terminal amino acids of E1 and affinity purified as described (5).

Immunoperoxidase Labeling. AtT-20 cells were plated in 35-mm dishes 2 days prior to infection. Cells that were about 80% confluent were infected with recombinant vaccinia viruses at a multiplicity of infection of ≈20, with adsorption of 0.25 ml of serum-free medium for 30 min at 37°C. Medium containing serum was then added, and cells were fixed at 4 hr after infection in 3% paraformaldehyde/0.05% glutaraldehyde in phosphate buffer for 1 hr at room temperature. Labeling was carried out in the tissue culture dish as described (6). In brief, cells were permeabilized with 0.05% saponin in phosphate-buffered saline and then incubated sequentially with affinity-purified anti-E1 IgG (1:40 for 2 hr) and Fab fragments of sheep anti-rabbit IgG conjugated to horseradish peroxidase (1:50 for 2 hr), after which they were aldehyde fixed, allowed to react with diaminobenzidine, and processed for electron microscopy.

Immunogold Labeling. Cells were plated, infected, and fixed as described above, except that 100-mm dishes were used; the cells were fixed for 1 hr after which they were collected and processed for cryoultramicrotomy (7) and immunogold labeling as described (8, 9). Ultrathin cryosections were incubated in affinity-purified anti-E1 antibody (diluted 1:40 for 1 hr), followed by goat anti-rabbit IgG conjugated to 5-nm colloidal gold (diluted 1:50 for 1 hr). They were stained sequentially with 2% OsO_4 (15 min), 2% acidic uranyl acetate (15 min), and 0.002% lead citrate in 2.2% polyvinyl alcohol (5 min) (7).

Immunofluorescence. Semithin cryosections (0.5–1 μm) were incubated with affinity-purified anti-E1 antibody (1:50 for 2 hr), followed by rhodamine-conjugated sheep anti-rabbit $F(ab')_2$ (1:50 for 1 hr), mounted, and photographed as described (9).

Biosynthetic Labeling and Glycosidase Digestion. Infected cells were labeled from 2 until 4 hr after infection with 100 μCi (1 Ci = 37 GBq) of L-[^{35}S]cysteine per ml in cysteine-free medium. Detergent lysates were prepared, and E1 proteins were immunoprecipitated with an anti-E1 serum as described

Abbreviations: Endo H, endoglycosidase H; IBV, infectious bronchitis virus; MHV, mouse hepatitis virus; ER, endoplasmic reticulum.

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(5). For glycosidase digestion, immunoprecipitates were eluted from the *Staphylococcus aureus* pellets in 10 mM Tris, pH 8.6/0.5% SDS/0.1% 2-mercaptoethanol by incubation at 100°C for 2 min. The sample was split into three aliquots and either left untreated or digested with endoglycosidase (Endo H) (0.2 milliunit) or N-glycanase (0.05 milliunit) in a volume of 20 μl for 16 hr at 37°C as described (10). After addition of 6 μl of 4× sample buffer containing 2% 2-mercaptoethanol, samples were electrophoresed in 15% polyacrylamide gels and fluorographed.

RESULTS

By indirect immunofluorescence it was previously demonstrated that the E1 protein is expressed in the juxtanuclear or Golgi region of transfected COS-1 cells (5). To determine the precise localization of E1 among the multiple organelles found in the Golgi region, we explored several transient expression systems and found that use of recombinant vaccinia viruses was best for this purpose, since virtually all of the cells on a culture dish could be infected and expressed E1 at a high level. COS-1 cells were used in earlier experiments, but they have the disadvantage that their Golgi is small and its polarity is difficult to determine. Subsequently we turned to the murine anterior pituitary AtT-20 cell line, since these cells contain secretion granules (8), and the polarity of the Golgi can be determined morphologically by the presence of secretory granules marking the trans side of the stacks (11). When the wild-type and Δm1,2 mutant proteins were localized by indirect immunofluorescence in AtT-20 cells infected with recombinant vaccinia viruses, wild-type E1 was concentrated in the juxtanuclear region (Fig. 1*A*), whereas the mutant E1 protein was localized mainly to the plasma membrane (Fig. 1*B*). Thus, the distribution of E1 expressed in AtT-20 cells using a recombinant vaccinia virus was similar at the light microscope level to that observed earlier in COS cells using a simian virus 40 vector except that the number of cells expressing the E1 protein was much higher (>90%). No viral cytopathic effects were seen at early times (4 hr) after infection of COS cells or AtT-20 cells with the recombinant vaccinia viruses.

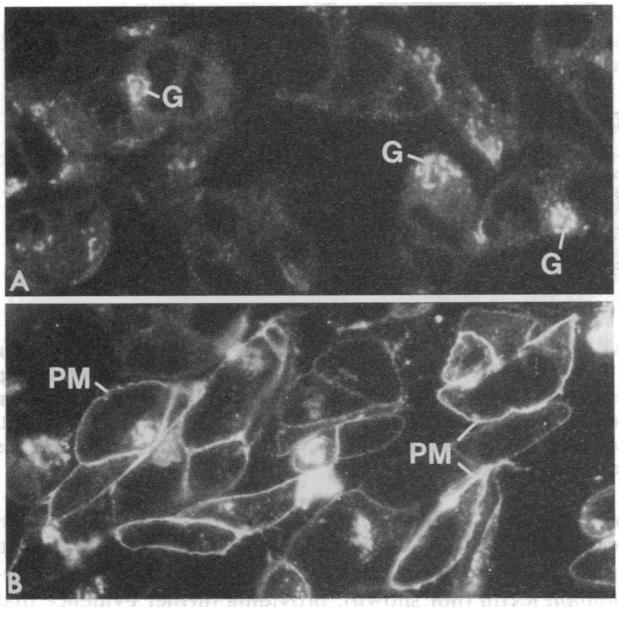

FIG. 1. Localization of the wild-type (*A*) and Δm1,2 mutant (*B*) E1 proteins in semithin cryosections of AtT-20 cells by indirect immunofluorescence. The wild-type E1 protein accumulates in the juxtanuclear Golgi region (G) of the cells, whereas the mutant E1 protein is transported to the plasma membrane (PM). (×200.)

Immunoelectron Microscopic Localization of the Wild-Type and Mutant IBV E1 Protein. After immunoperoxidase labeling of COS and AtT-20 cells with an affinity-purified anti-E1 antibody, peroxidase reaction product was found in the cytoplasm associated with the outer surface of one or two cisternae on one side of the Golgi stack (Fig. 2). The sidedness of the stack could not be reliably determined in COS cells; however, in AtT-20 cells the labeled cisternae could be identified as cis cisternae, based on the fact that they were situated on the opposite side of the stack from secretory granules. By definition (2, 3), secretory granules are found on the trans side of the stack (arrow, Fig. 2 *B* and *C*). The location of the reaction product in the cytoplasm, along side the cis cisternae, is in keeping with the fact that the antibody recognizes the cytoplasmic domain of E1, as it was raised against a synthetic peptide corresponding to its 22 carboxyl-terminal amino acids. Occasionally, peroxidase reaction product was also seen in association with ER cisternae.

To verify the immunoperoxidase results, we performed immunogold localizations on ultrathin cryosections prepared from AtT-20 cells infected with the recombinant vaccinia virus encoding E1 as described above. In cells fixed 4 hr after infection, gold particles were again found in close association with one or two of the cis-most cisternae of the Golgi stacks (Fig. 3), with vesicles on the cis side, and occasionally with ER cisternae. This labeling pattern was unchanged when cells were incubated an additional 2 hr in the presence of cycloheximide to prevent protein synthesis and to allow time for transport of E1 to its final destination. Thus by immunogold and immunoperoxidase methods E1 expression was confined to cis cisternae and was not detected in middle or trans cisternae.

We next localized the mutant E1 protein that lacks the first and second of three membrane-spanning domains. In contrast to the wild-type protein, this protein is transported to the plasma membrane as determined by indirect immunofluorescence (Fig. 1) and susceptibility to exogenously added protease (5). We reasoned that at early times after infection we should be able to detect the protein in transit through the Golgi cisternae as well as at the plasma membrane. In AtT-20 cells fixed 4 hr after infection with the mutant protein, gold particles were distributed across the entire Golgi stack as well as on the plasma membrane (not shown). Thus, whereas the wild-type E1 protein accumulated at a specific site (cis cisternae) in the Golgi complex, the mutant protein apparently had no such constraints on its movement.

Wild-Type E1 Protein Contains Incompletely Processed N-Linked Oligosaccharides. The E1 protein of the avian coronavirus IBV has two N-asparagine-linked oligosaccharides near its amino terminus (12, 13). We analyzed the processing of these oligosaccharides in cells expressing wild-type E1 from a recombinant vaccinia virus after biosynthetic labeling with [^{35}S]cysteine from 2 until 4 hr after infection. When E1 proteins were immunoprecipitated from detergent lysates of AtT-20 cells, the majority of the wild-type E1 protein remained Endo H sensitive during the labeling period, although about 20% was processed to a resistant form (Fig. 4, lanes 1–3). The fraction that was Endo H resistant was found to be somewhat variable in different experiments. Less than 5% of the wild-type E1 protein synthesized in COS cells was processed under identical labeling conditions (Fig. 4, lanes 7–9). These results indicate that most of the E1 glycoprotein has not been acted on by the middle Golgi enzymes α-mannosidase II and GlcNAc transferase I, which convey Endo H resistance.

The Δm1,2 Mutant E1 Protein Becomes Endo H Resistant. In contrast to the results with wild-type E1 protein, the mutant protein was efficiently processed in AtT-20 and COS cells; ≈85% became Endo H resistant in AtT-20 cells and

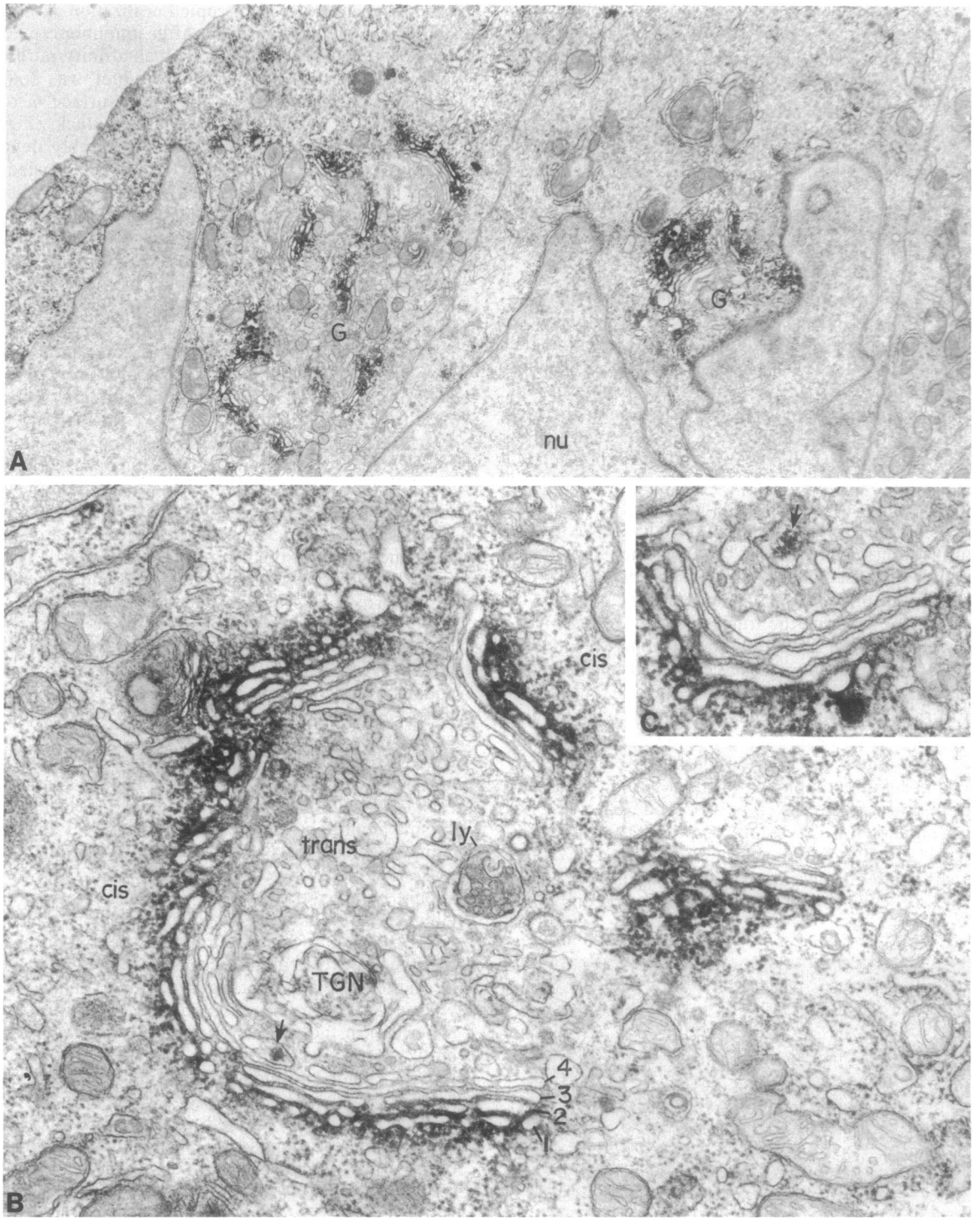

FIG. 2. Immunoperoxidase localization of the wild-type E1 protein in AtT-20 cells fixed 4 hr after infection with the recombinant vaccinia virus encoding E1. In all cases, peroxidase reaction product is found in the Golgi region (G) where it is concentrated along the first two Golgi cisternae (1 and 2) on the cis side (cis) of the Golgi stack. The trans side (trans) can be readily identified by the presence of condensing secretion granules (arrows in *B* and *C*). (*A*) A more irregular cisterna probably corresponding to the trans Golgi reticulum or network (TGN) is also present. The reaction product is located in the cytoplasm adjacent to the cis cisternae, which is in keeping with the fact that the antibody recognizes the carboxyl-terminal cytoplasmic domain of E1. ly, Lysosome; nu, nucleus. (*A*, ×15,000; *B*, ×32,000; *C*, ×38,000.)

60% became resistant in COS cells during the 2-hr labeling period. In immunoprecipitates from cells expressing Δm1,2 the Endo H-resistant material appeared as a broad band (Fig. 4, lanes 4 and 5 and lanes 10 and 11). However a discrete band was obtained after N-glycanase digestion (Fig. 4, lanes 6 and 12), indicating that the heterogeneity was due to the presence of N-linked oligosaccharides. The size heterogeneity of the Endo H-resistant material resembled that described for several other membrane proteins with polylactosaminoglycan type N-linked oligosaccharides (10, 14, 15). The heterogeneous, Endo H-resistant form of Δm1,2 bound immobilized tomato lectin (*Lycopersicon esculentum*) and *Datura stramonium* lectin (not shown), providing further evidence that this modification was polylactosaminoglycan (16, 17). Although the enzymes involved in this modification have not been purified or localized, they are assumed to be located in the middle or trans subcompartments of the Golgi complex (14).

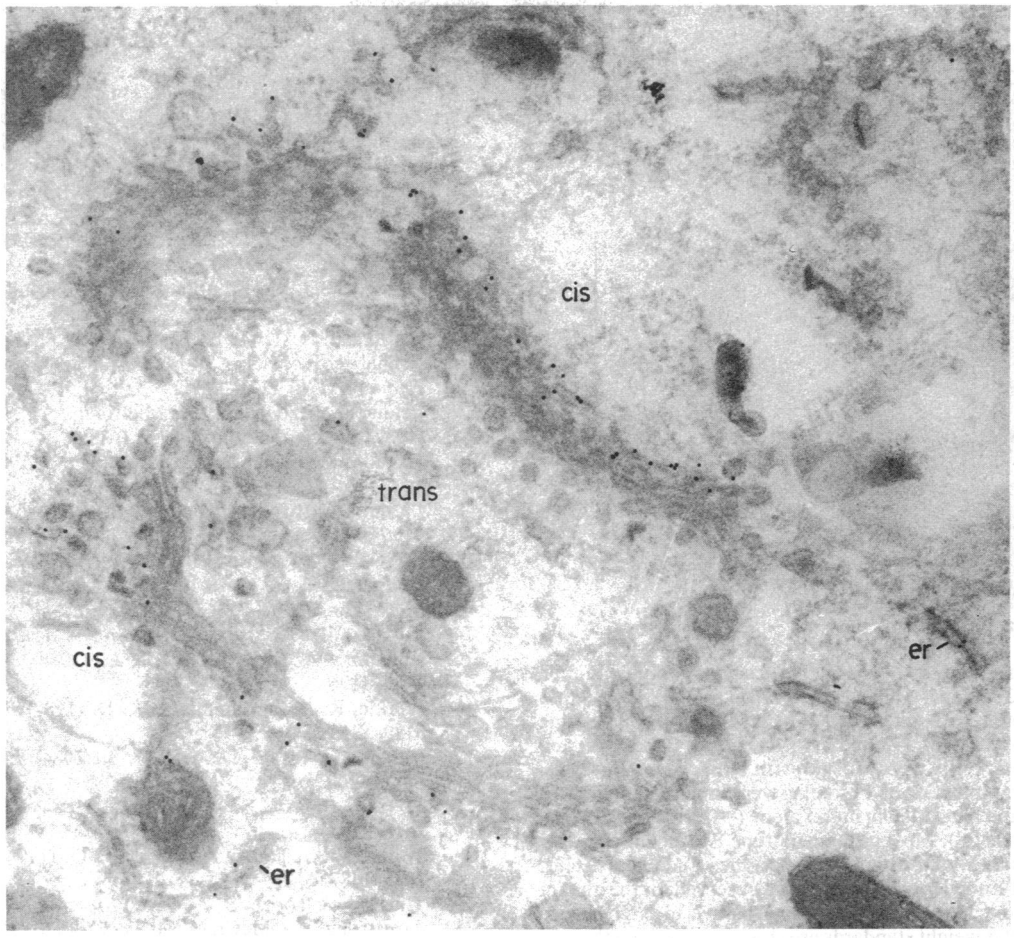

FIG. 3. Immunogold labeling of the wild-type E1 protein in ultrathin cryosections of AtT-20 cells fixed 4 hr after infection with the recombinant vaccinia virus encoding wild-type E1. In this field (which is representative) the vast majority of the 5-nm gold particles are found over 1 or 2 of the cis-most cisternae and associated vesicles. Of the 74 gold particles present, 81% (60) are associated with cis elements, 5% (3 or 4) are associated with trans, and 5% (4) are associated with ER cisternae. (×36,000).

DISCUSSION

The data presented in this paper demonstrate that the E1 protein from the avian coronavirus is retained in cis Golgi cisternae when expressed from cloned cDNA in animal cells. Earlier work using indirect immunofluorescence microscopy suggested targeting to elements in the Golgi region, but the specific site of targeting could not be determined. To precisely localize the E1 glycoprotein among the numerous organelles found in the Golgi region, we utilized immunoperoxidase and immunogold labeling at the electron microscope level. Use of recombinant vaccinia virus vectors has a distinct advantage for this type of analysis since a high level of expression is obtained in >90% of the cells early after infection. We found that the E1 protein accumulated in the first one or two cisternae of the Golgi stack. The extent of processing of the N-linked oligosaccharides on E1 was consistent with this localization, as the majority of E1 expressed in AtT-20 cells was Endo H sensitive, although a fraction (about 20%) was processed to an Endo H-resistant form. In COS cells, the fraction of processed oligosaccharides was <5%. Processing to an Endo H-resistant form occurs with the addition of the first GlcNAc residue to the trimmed core oligosaccharide and removal of one of the five remaining mannose residues (4). This is believed to occur in the middle (medial) Golgi cisternae, as *N*-acetylglucosamine transferase I has been localized to this region of the Golgi (1). The fact that some processing of E1 occurs even though it is concentrated on the cis side of the Golgi suggests that there is some overlap in its distribution with GlcNAc transferase I.

The results obtained with a mutant E1 protein called Δm1,2, which lacks the first and second of the three E1 membrane-spanning domains, were quite different. Early after initiating expression of Δm1,2, it could be observed in all cisternae throughout the Golgi stack as well as at the plasma membrane. In addition, the oligosaccharides were efficiently processed to an Endo H-resistant form. Thus, in contrast to the wild-type protein, there was no block in transport of the mutant protein along the exocytic pathway. The heterogeneity and lectin-binding properties of its processed oligosaccharides suggested they were of the polylactosaminoglycan type (10, 14). This particular processing occurred on the mutant E1 protein as it was transported through the Golgi en route to the plasma membrane.

The simplest interpretation of our results is that the IBV E1 protein possesses a signal for retention in the cis region of the Golgi complex. The protein was efficiently transported out of the ER but stopped when it reached the first few Golgi cisternae. In contrast, the mutant protein apparently lacks this retention signal and was transported through the Golgi complex to the plasma membrane. The retention signal may reside in the part of the polypeptide that has been deleted or may be in another region but structurally disrupted by the deletion. Further work is necessary to define the molecular nature of this retention signal.

The intracellular site of budding of IBV has not been investigated in detail. However, considerable work has been done with another coronavirus, mouse hepatitis virus (MHV). In most MHV-infected cell lines examined, the

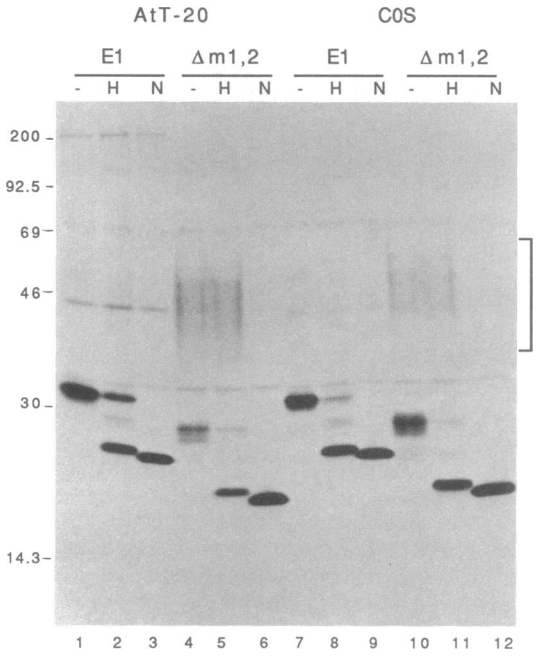

FIG. 4. Processing of the N-linked oligosaccharides on wild-type E1 and the Δm1,2 mutant. AtT-20 (lanes 1–6) and COS cells (lanes 7–12) were infected with recombinant vaccinia viruses encoding either the wild-type (lanes 1–3 and 7–9) or mutant (lanes 4–6 and 10–12) E1 proteins. Cells were labeled with [^{35}S]cysteine for 2 hr, starting at 2 hr after infection. E1 proteins were immunoprecipitated from cell lysates and incubated in buffer alone (−), with Endo H (H), or with N-glycanase (N). Most of the wild-type E1 remains Endo H sensitive whereas most of the mutant E1 becomes Endo H resistant. The broad zone of Endo H-resistant material observed for the mutant E1 protein (bracket, lanes 5 and 11) was sensitive to N-glycanase digestion. Molecular weight standards are shown ($\times 10^{-3}$) on the left.

major site of budding is a pre-Golgi compartment termed the transitional or intermediate compartment (18, 19). However, some budding occurs in the Golgi complex in MHV-infected AtT-20 cells (20). By indirect immunofluorescence MHV E1 was also found to be localized in the Golgi region of several different cell lines expressing the protein from cloned cDNA (21–23). Its precise localization among Golgi elements has not yet been investigated.

Recently cDNA clones encoding several endogenous trans Golgi glycosyltransferases have been isolated. These include β-1,4-galactosyltransferase (24–27), α-1,3-galactosyltransferase (28, 29), α-2,6-sialyltransferase (30), and α-1,2-fucosyltransferase (31). A partial clone encoding a middle Golgi enzyme, α-mannosidase II, has also been reported (32). Although several proteins resident in the cis Golgi have been identified (33, 34), no cDNAs encoding endogenous cis Golgi proteins are yet available. Thus the avian IBV coronavirus E1 protein should prove to be a useful marker for the cis region of the Golgi complex. In addition, the availability of mutant E1 proteins that are not retained in the Golgi may provide insight into the mechanisms of intracellular protein traffic through this organelle.

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