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Chimeric influenza virus hemagglutinin containing either the NH_2 terminus or the COOH terminus of G protein of vesicular stomatitis virus is defective in transport to the cell surface

(integral membrane proteins/signal sequence/glycoproteins/intracellular processing/cDNA expression)

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ABSTRACT **Chimeric cDNA clones of influenza virus hemagglutinin (HA) were constructed in which the DNA encoding either the NH_2 terminus or the COOH terminus of HA was replaced with that of a vesicular stomatitis virus G protein. The chimeric cDNAs (GHA or HAG) were expressed in CV1 cells using the simian virus 40 late replacement promoter. Both chimeric proteins are synthesized, glycosylated, and transported to the rough endoplasmic reticulum. These results show that the NH_2-terminal sequences of vesicular stomatitis virus G protein can provide a signal function for translocation and the COOH-terminal sequences can provide the anchor function for the influenza virus HA, when substituted for similar sequences. However, the chimeric glycoproteins were not transported to the Golgi complex or the plasma membrane. The implication of these results in translocation, sorting, and transport processes is discussed.**

Viral glycoproteins such as vesicular stomatitis virus (VSV) G protein and influenza hemagglutinin (HA) have been extensively used to elucidate the biosynthetic and topological events involved in membrane biogenesis (1–4). Furthermore, temperature-sensitive mutations in these glycoproteins have indicated that specific structural features are required for recognition by cellular machinery involved in different steps of transport. In addition, because VSV and influenza viruses bud from different domains of the plasma membrane in polarized epithelial cells (1–4), the structural features of their envelope glycoproteins may provide clues to the process of sorting and directional transport of membrane glycoproteins. This was further supported by our recent observation that influenza HA expressed from a cloned cDNA exhibits the same polarized expression on the apical surface of monkey kidney (AGMK) cells (5) as is found in influenza virus-infected cells (2). To elucidate further some of the structural features involved in biogenesis, transport, and sorting of influenza HA, we have constructed and expressed chimeric cDNA clones in which the DNA encoding either the NH_2 terminus or the COOH terminus of HA has been replaced with that of VSV G. In this report, we describe the properties of these chimeric proteins that have been expressed in CV1 cells and discuss the implication of these observations for translocation, sorting, and transport of the HA glycoprotein.

MATERIALS AND METHODS

Virus Strains, Cells, and Plasmid Vectors. CV1P and CV1 cells were grown in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% fetal bovine serum. Virus stocks of A/WSN/33 (H1N1) strain of influenza virus were prepared in Madin–Darby bovine kidney (MDBK) cells as described (6). SVHA2 [simian virus 40 (SV40) late-replacement vector expressing WSN HA] and SVSal·32 (SV40 defective in T-antigen expression) were grown in CV1 cells (7). pG1 and pGR125 containing VSV G cDNA were obtained from John K. Rose (8, 9).

We expressed the chimeric HAG and GHA cDNA employing the late SV40 promoter as described for HA (10). To produce a lytic infection, SVSal·32, which provides late gene function, was used as a helper and virus stock was prepared as described (7).

Antibodies and Immunofluorescent Staining. Anti-HA monoclonal antibody (H15A13-18) against PR8 virus was obtained from W. Gerhard. Anti-WSN antibody was prepared from rabbits. Procedures for intracellular and surface staining using indirect immunofluorescence have been described (7).

Radiolabeling of Infected Cells and Analysis of Polypeptides. At 40–48 hr after infection, cells were labeled for 5–6 hr by using 3 ml of methionine-free DME medium supplemented with 2% dialyzed fetal bovine serum and 50 μCi of L-[^{35}S]methionine per ml (1 Ci = 37 gBq). For tunicamycin treatment, cells were first pretreated for 1 hr at 37°C with tunicamycin (2 μg/ml) in DME medium containing 2% fetal bovine serum and then labeled with L-[^{35}S]methionine (50 μCi/ml) in DME medium containing tunicamycin (2 μg/ml). Subsequently, cells were washed twice with cold Tris (25 mM)-buffered saline, scraped, pelleted, and lysed in RIPA buffer (0.05 M Tris, pH 7.4/0.15 M NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% $NaDodSO_4$) (11, 12) for 10 min at 0°C. Nuclei were removed by centrifuging 15 min in an Eppendorf centrifuge and the supernatant was incubated for 1 hr at 4°C with 2 μl of anti-WSN antibody. The antigen–antibody complexes were isolated by using protein A-Sepharose, washed four times, and analyzed on a 10% $NaDodSO_4$/polyacrylamide gel (12–14).

Endoglycosidase H (endo H) Treatment. At 40–48 hr after infection, cells were labeled for 2 hr with L-[^{35}S]methionine as described above. The cells were then washed with methionine-free DME medium and incubated further in DME medium containing 2% fetal bovine serum for 3 hr. Cells were subsequently lysed and immunoprecipitated as described above. The protein A-Sepharose samples containing antigen–antibody complexes were suspended in 200 μl of endo H buffer (14), heated for 2 min at 100°C, and centrifuged to remove protein A-Sepharose. Four hundred microliters of 0.3 M sodium citrate (pH 5.5) and 5 μl of endo H (33 ng/μl) were added to the supernatant and the mixture was incubated at 37°C for 17 hr. Finally, the mixture was desalted by passing

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Abbreviations: VSV, vesicular stomatitis virus; HA, hemagglutinin; SV40, simian virus 40; bp, base pair(s); endo H, endoglycosidase H; RER, rough endoplasmic reticulum.

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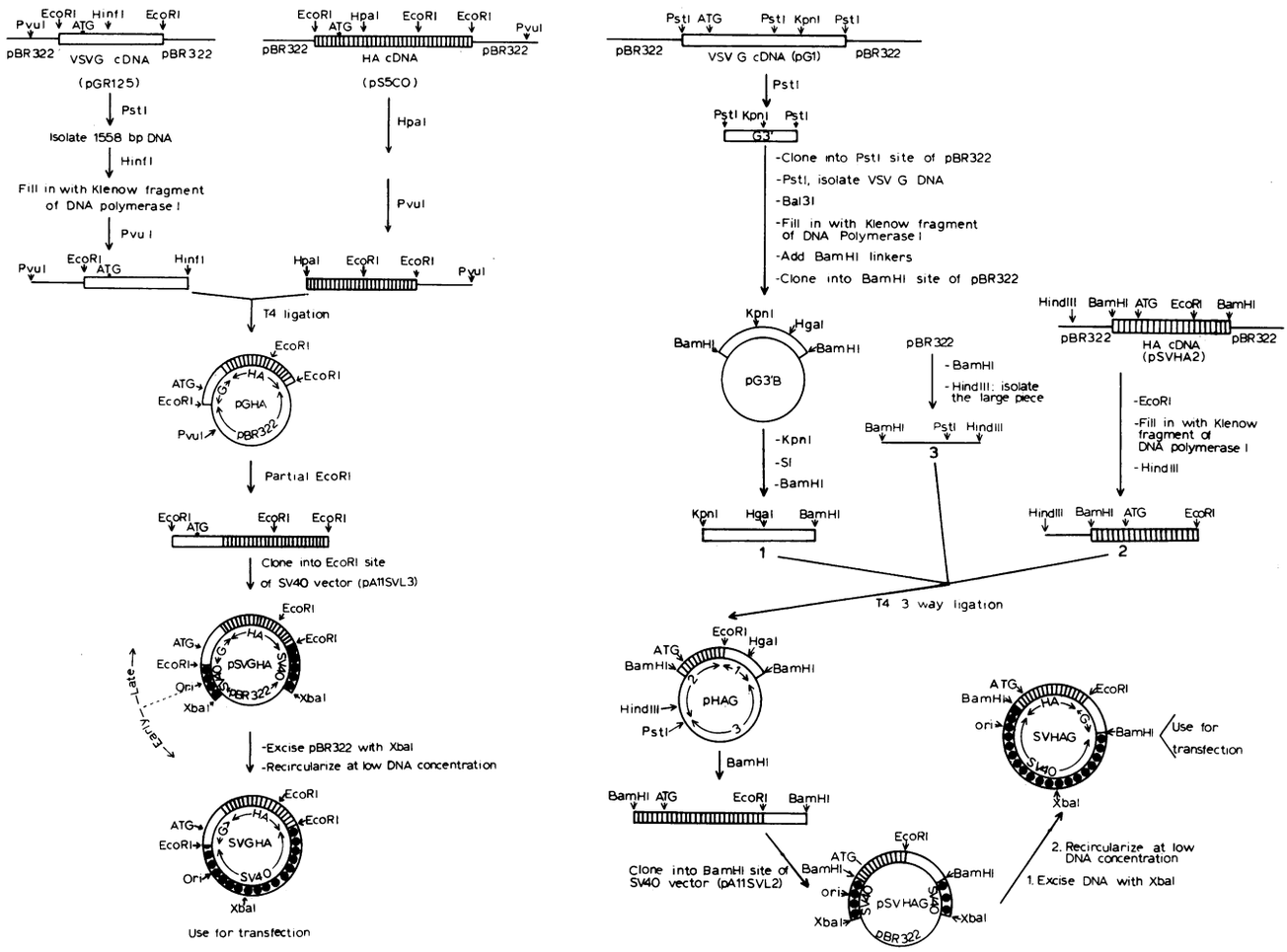

FIG. 1. Construction of chimeric DNAs. (*Left*) GHA. A DNA segment [1,558 (bp)] base pairs containing the 5′ portion of VSV G DNA was isolated from a *Pst* I digestion of pGR125. This DNA segment was further digested with *Hinf*I (nucleotide 219 of VSV G DNA), filled in with the Klenow fragment of DNA polymerase I to produce a blunt end, and then digested with *Pvu* I. The VSV G DNA segment from *Pvu* I to filled in *Hinf*I encoding the NH_2 terminus of G protein was ligated in phase with a HA DNA segment. The HA portion of the DNA segment (5,341 bp) was obtained from pS5CO by digesting with both *Pvu* I and *Hpa* I (nucleotide 171 of HA DNA). Finally, G DNA and HA DNA segments were ligated to create the chimeric GHA DNA and cloned into the *Eco*RI site of pBR322 to obtain pGHA. For expression, pSVGHA was constructed by ligating the GHA DNA into the *Eco*RI site of the SV40 vector pA11SVL3. Finally, pBR322 was excised with an *Xba* I digestion and the SV40 GHA recombinant DNA was recircularized and used for transfection in conjunction with the early mutant helper DNA of SVSal-32. HA DNA, striped bar; VSV G DNA, clear bar; SV40 DNA, bars with filled circles. (*Right*) HAG. The 3′ portion of the VSV gene was derived from pG1 by *Pst* I digestion and cloned into the *Pst* I site of pBR322. Subsequently, G·C tails were removed by BAL-31 treatment; *Bam*HI linkers were added to the 3′ VSV G DNA that was recloned into the *Bam*HI site of pBR322 to obtain pG3′B. This clone (pG3′B) was digested with *Kpn* I (nucleotide 1,039 of VSV G DNA), followed by nuclease S1 to create a blunt end at the *Kpn* I site, and finally treated with *Bam*HI. The *Kpn* I (blunt end) to *Bam*HI DNA segment of 627 bp was used for a three-way ligation in phase with a HA DNA segment and a pBR322 DNA segment (*Hin*dIII to *Bam*HI) to obtain pHAG. The HA portion of DNA (1,612 bp) was obtained from the pSVHA2 clone (15) by digesting with *Eco*RI (nucleotide 1,266 of HA), filling in with the Klenow fragment of DNA polymerase I to produce a blunt end, and then digesting with *Hin*dIII (nucleotide 29 in pBR322). For expression, pSVHAG was constructed by ligating the HAG DNA into the *Bam*HI site of the SV40 vector pA11SVL2. The pBR322 was excised as described above.

through a P6DG (Bio-Rad) column, lyophilized, and analyzed on a 10% $NaDodSO_4$/polyacrylamide resolving gel as above.

RESULTS

Construction of HAG and GHA Chimeric DNAs. The detailed scheme of the construction of the DNA encoding chimeric glycoproteins is shown in Fig. 1. For GHA (Fig. 1 *Left*), the 173 nucleotides at the 5′ terminus containing the untranslated region and sequence coding for the signal sequence of HA plus 30 additional amino acids have been replaced, in phase, by the 222 nucleotides consisting of the 5′ untranslated region and the sequences coding for the signal sequence of G plus 48 additional amino acids. For HAG (Fig. 1 *Right*), DNA encoding the entire COOH terminus region of HA, which includes both the hydrophobic anchoring region and the hydrophilic cytoplasmic region plus 113 additional amino acids, has been replaced, in phase, by the DNA encoding the entire COOH-terminal region of G, which includes both the hydrophobic and hydrophilic regions plus 124 additional amino acids. HAG could be expected to code for a chimeric protein of 571 amino acids plus a leader sequence of 17 amino acids and GHA could be expected to code for a protein of 566 amino acids plus a leader sequence of 16 amino acids.

Fig. 2*A* shows the hydrophobic domains and the junction sites of the chimeric proteins as well as the hydrophobic and nonhydrophobic domains of WSN HA and VSV G proteins as predicted from the DNA sequences. In chimeric constructions, DNA sequences were confirmed by sequence analysis through the junctions and the predicted amino acids are shown in Fig. 2*B*. Note that the resulting constructions did not introduce new amino acids at the junction sites, and the sequences on both sides of the junction are precisely the

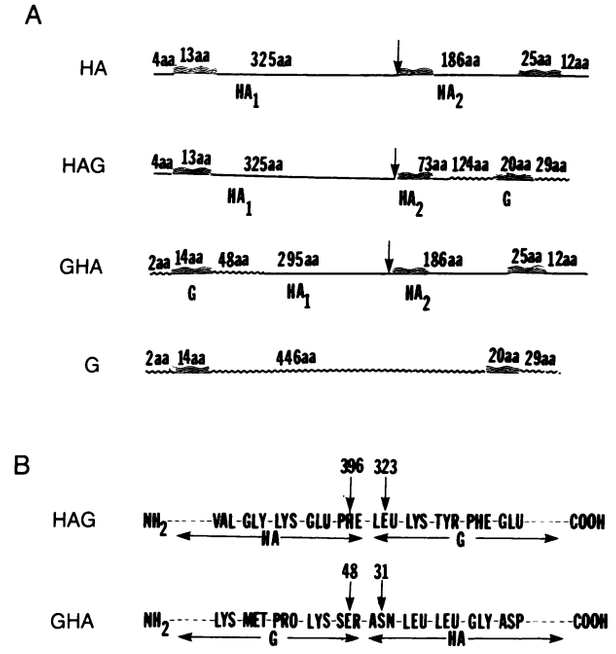

FIG. 2. Schematic representation of the primary structures of HA, G, and the chimeric proteins (HAG and GHA) as predicted from the DNA sequence (*A*) and the amino acid sequences at the junction sites of the chimeric proteins (*B*). (*A*) The hydrophobic domains (▬), HA (——), and G (~~~~). The arrows indicate the cleavage site of HA into HA_1 and HA_2. (*B*) The expected amino acid sequence as predicted from the DNA sequence. The numbers above the arrows give the amino acid numbers in the native HA (16) and G proteins (7).

same as expected in the native proteins. Both HAG and GHA are slightly larger than the native HA. The unprocessed HA, HAG, GHA, and G polypeptides would contain 565, 588, 582, and 511 amino aids, respectively, and the mature HA, HAG, GHA, and G should contain 548, 571, 566, and 495 amino acids, respectively. Polyacrylamide gel analyses of polypeptides from tunicamycin-treated cells agree with the expected molecular weight of signal minus polypeptide (see below). One glycosylation site was lost in each chimeric protein and one cysteine residue that is known to be involved in a disulfide bond (17) was removed in each construction. However, in the signal switch (GHA), a cysteine residue is gained from the VSV G NH_2 terminus.

Expression of the Chimeric Proteins in CV1 Cells. Monolayers of CV1 cells were infected with each of the SV40-chimeric recombinant viruses and subsequently analyzed for intracytoplasmic and surface antigen expression by using indirect immunofluorescence. Fig. 3 shows that both the HAG and GHA chimeric proteins are expressed in the infected cells and become concentrated in perinuclear regions. In each instance, ≈20% of the cells are positive. However, neither of the chimeric proteins is expressed on the cell surface (not shown), whereas the native HA is clearly present on the cell surface (Fig. 3*D*). Furthermore, cells infected with the GHA or HAG recombinants were negative for cell surface hemadsorption, whereas cells expressing complete HA were positive.

To further localize the intracytoplasmic antigen, infected cells were doubly stained by using lectins that bind to saccharides in specific cellular compartments (18). The distribution of GHA antigen (Fig. 4*B*) appears to coincide generally with the rough endoplasmic reticulum (RER) as seen by concanavalin A staining (Fig. 4*A*), suggesting that the chimeric glycoprotein (GHA) is predominately localized in this compartment. However, when wheat germ agglutinin was used

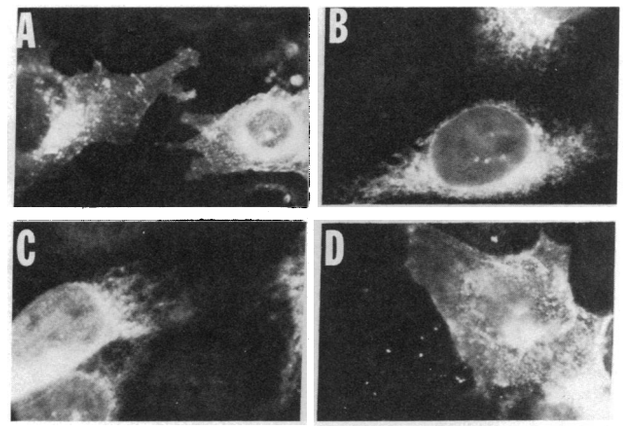

FIG. 3. Expression of native and chimeric HA in cells infected with recombinant viruses. Monolayers of CV1 cells were infected with recombinant viruses for 48 hr and used for either intracytoplasmic (acetone fixed) or surface staining (paraformaldehyde fixed) by using anti-WSN rabbit immunoglobulins and fluorescein-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). (*A*) HA intracytoplasmic; (*B*) HAG intracytoplasmic; (*C*) GHA intracytoplasmic; (*D*) HA surface. Surface immunofluorescence was absent for both HAG and GHA infected cells. (×210.)

to stain *N*-acetyl-D-glucosamine or sialic acid residues, which are added in the Golgi apparatus, the staining patterns showed little similarity with that observed for GHA. The diffuse fluorescein staining of GHA (Fig. 4*D*) was markedly different from the punctate foci of rhodamine staining (Fig. 4*C*). Similar results were obtained with cells expressing the HAG chimeric protein (data not shown). These results suggest that both HAG and GHA are predominately present in the RER, and are not concentrated in the Golgi apparatus.

To examine the sizes of the GHA and HAG polypeptides,

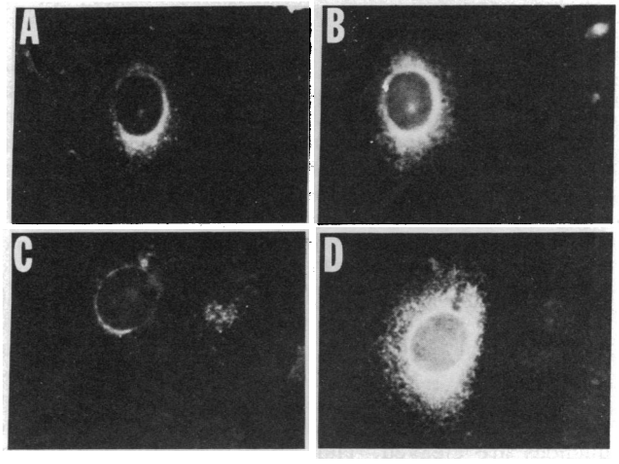

FIG. 4. Intracellular localization of native and chimeric HA by fluorescent staining. At 72 hr after infection, infected CV1 cells were fixed with methanol for 5 min and stained with rhodamine-conjugated concanavalin A or rhodamine-conjugated wheat germ agglutinin for 15 min at room temperature. After extensive washing with phosphate-buffered saline, the cells were reacted with rabbit antiserum to A/WSN virus (1:20) for 20 min at 37°C, washed, and then stained with fluorescein-conjugated anti-rabbit IgG (1:10) for 20 min at 37°C. (*A*) Cells stained with rhodamine-conjugated concanavalin A; (*C*) Cells stained with rhodamine-conjugated wheat germ agglutinin. Cells were photographed with a Nikon Optiphot microscope equipped with a modified G cube for effective visualization of rhodamine staining. (*B* and *D*) The same cells as in *A* and *C*, respectively, were photographed with a modified B2 cube for effective visualization of fluorescein staining, showing the distribution of viral antigen. (×210.)

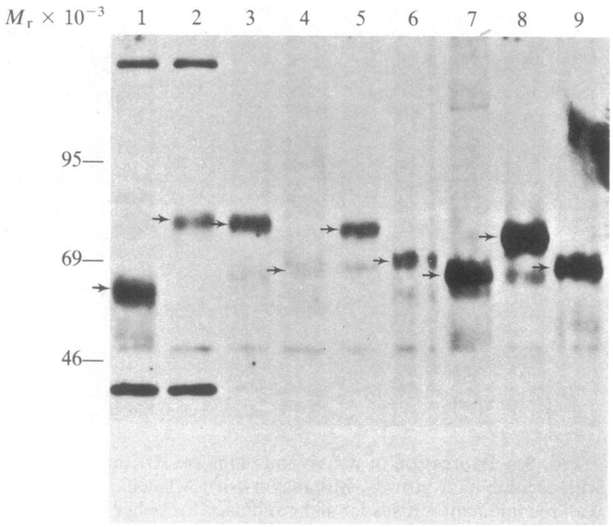

FIG. 5. $NaDodSO_4$/polyacrylamide gel electrophoresis of native HA and chimeric HA polypeptides. CV1 cell monolayers were infected with either SVHA (lanes 1, 2, and 3), SVHAG (lanes 4, 5, and 6), or SVGHA (lanes 7, 8, and 9). At 48 hr after infection, cells were labeled with L-[^{35}S]methionine with or without tunicamycin. Subsequently, cells were washed twice with cold Tris-buffered saline and lysed in RIPA buffer, and the lysates were used for immunoprecipitation using rabbit anti-WSN IgG and protein A-Sepharose. Lanes 1, 4, and 7, tunicamycin treated; lanes 3, 6, and 9, endo H treated; lanes 2, 5, and 8, without either endo H or tunicamycin treatment. Arrows show the positions of the immunoprecipitated HA, HAG, and GHA proteins.

CV1 cells were infected with each of the SV40 recombinant viruses and radiolabeled polypeptides were analyzed by immune precipitation and electrophoresis on a $NaDodSO_4$/polyacrylamide gel. HAG and GHA both have a M_r of ≈70,000, which is the same as that of uncleaved HA (Fig. 5, lanes 2, 5, and 8).

Examination of the DNA sequences of HAG and GHA shows that, in each case, one glycosylation site is lost as compared to complete HA. Because both chimeric proteins migrate to almost the same region as native HA, it appears that both GHA and HAG are glycosylated. This was confirmed by comparison of the glycosylated molecules with unglycosylated proteins obtained in the presence of tunicamycin, an antibiotic known to block primary glycosylation of the nascent polypeptide (15). In the presence of tunicamycin, the molecular weight of each of the GHA and HAG polypeptides was reduced from M_r ≈70,000 to M_r ≈64,000, whereas the molecular weight of the HA was reduced from M_r ≈70,000 to M_r ≈62,000 (Fig. 5, lanes 1, 4, and 7). These results agree with the molecular weights of GHA and HAG predicted from the hybrid DNA sequences if the leader sequences are cleaved. Thus, both chimeric proteins expressed in the infected cells are glycosylated and their leader sequences are probably cleaved.

To analyze the processing of oligosaccharides of the chimeric glycoproteins, HA, GHA, and HAG polypeptides were treated with endo-H, which is known to cleave carbohydrate side chains of the high mannose type but not complex carbohydrate side chains (14, 19, 20). Complete HA was found to be resistant, whereas both GHA and HAG were endo H sensitive (Fig. 5, lanes 3, 6, and 9). A slight increase in molecular weight of the endo H-treated chimeric proteins as compared to the totally unglycosylated proteins from tunicamycin-treated cells is probably due to the two or three sugar residues remaining on the protein after endo H cleavage. Because the processing steps involved in formation of complex oligosaccharides occur in the *trans*-region of the Golgi complex (21), these results indicate that the chimeric proteins are not traversing the Golgi complex.

DISCUSSION

Influenza HA and VSV G proteins possess similar structural features for interacting with membranes. Both HA and G contain a hydrophobic domain at the NH_2 terminus, which is proteolytically cleaved during the translocation process, and a hydrophobic domain near the COOH terminus, which functions to anchor the protein in the membrane. Both HA and G are synthesized on membrane-bound polyribosomes and translocated via the RER to the Golgi apparatus. Both proteins undergo extensive modification in the form of glycosylation in the RER and in the Golgi. Eventually they are directed to the different domains of the plasma membrane (1, 2, 22) of polarized epithelial cells. The present report shows that the signal sequence of G can replace the signal sequence of HA in translocating HA across the RER. Similarly, the COOH-terminal hydrophobic sequence of G appears to provide the anchor function for HA because none of the HAG protein was secreted into the medium (data not shown). This indicates that hydrophobicity rather than the sequence specificity is important for both the signal as well as the anchor function and that these functions can be provided by a similar sequence of heterologous proteins. Lack of a sequence conservation in the signal and anchor regions of various membrane proteins has been reported previously (23). Additionally, the signal sequence of GHA appears to be cleaved, suggesting that the information for cleavage is present in the signal and the adjacent sequence. Alternatively, structural features for cleavage of the G signal may be provided by the HA portion of GHA. However, it should be noted that in *Escherichia coli* prolipoprotein a mutation in the signal peptide can prevent its cleavage (24, 25). However, the transport and topological orientation of the protein on the outer membrane was only partially dependent on cleavage.

Structural features necessary for transport, sorting, and localization of HA appear to be more complex. Both HAG and GHA were found to be blocked in the RER, suggesting that neither the COOH terminus of HA nor the NH_2 terminus of HA sequences along with the rest of the HA molecule present in the chimeric proteins was capable of directing the chimeric protein to the final destination of HA. Similarly, neither the COOH terminus of G nor the NH_2 terminus of G along with the adjacent G sequences present in the chimeric proteins was capable of directing the protein to the G-specific destination. It should be noted that parts of G or HA that have been fused to produce the chimeric proteins do not possess any altered sequences. Additionally, the observed block in transport could not be due to a gross alteration of the size of the chimeric proteins because they gained only an additional 23 or 18 amino acid residues compared to wild-type WSN HA, and variations in size have been observed among the HA polypeptides of different influenza viruses (23). Similarly, the loss of a glycosylation site *per se* could not be the major factor in the block in transport, because chimeric proteins were glycosylated (Fig. 5) and the number of glycosylation sites varies in HAs of different strains. Furthermore, in native HA, sorting and transport to the cell surface can proceed when glycosylation is prevented with tunicamycin (26). Similarly, the loss of the major part of HA2 in HAG cannot be solely responsible for blocking transport, because others have shown that anchorless HA containing a deletion similar to that of HAG can be transported and secreted (27, 28). This suggests that the heterologous sequences may have caused a structural alteration in the HA glycoprotein and thus affected the transport. The NH_2 terminus in GHA is clearly different from that of HA. GHA introduces, in this region, three pro-

line and two glycine residues that are known to have a profound effect on the secondary structure. Furthermore, this region becomes more positively charged (four lysine and three histidine in GHA compared to one lysine and two histidine in native HA). Finally, one or more cysteine residues that are either lost (HAG) or replaced (GHA) may also have affected the structure of chimeric HA by altering the disulfide bonds. These results imply that the tertiary structure of the protein may play a vital role in the process of intracellular transport.

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