Heterologous Transmembrane and Cytoplasmic Domains Direct Functional Chimeric Influenza Virus Hemagglutinins into the Endocytic Pathway

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Abstract. Chimeric genes were created by fusing DNA sequences encoding the ectodomain of the influenza virus hemagglutinin (HA) to DNA coding for the transmembrane and cytoplasmic domains of either the G glycoprotein of vesicular stomatitis virus or the gC glycoprotein of Herpes simplex virus 1. CV-1 cells infected with SV40 vectors carrying the recombinant genes expressed large amounts of the chimeric proteins, HAG or HAgC on their surfaces. Although the ectodomains of HAG and HAgC differed in their immunological properties from that of HA, the chimeras displayed the biological functions characteristic of the wild-type protein. Both HAG and HAgC bound erythrocytes as efficiently as HA did and, after brief exposure to an acidic environment, induced the fusion of erythrocyte and CV-1 cell membranes. However, the behavior of HAG and HAgC at the cell surface differed from that of HA in several important respects. HAG and HAgC were observed to collect in coated pits whereas wild-type HA was excluded from those structures. In the presence of chloroquine, which inhibits the exit of receptors from endosomes, HAG and HAgC accumulated in intracellular vesicles. By contrast, chloroquine had no effect on the location of wild-type HA. HAG and HAgC labeled at the cell surface exhibited a temperature-dependent acquisition of resistance to extracellular protease at a rate similar to the rates of internalization observed for many cell surface receptors. HA acquired resistance to protease at a rate at least 20-fold slower. We conclude that HAG and HAgC are efficiently routed into the endocytic pathway and HA is not. However, like HA, HAG was degraded slowly, raising the possibility that HAG recycles to the plasma membrane.

In most cell types endocytosis through coated pits is the major route by which cell surface proteins are internalized (2, 9, 22, 27). Coated pits have been estimated to occupy 2% of the cell surface and, depending upon the cell type, can internalize membrane equivalent to 50 to 200% of the plasmalemma each hour (2, 22, 27, 38, 59, 60). Uptake of proteins by this process is selective (2, 4, 22, 45, 60): certain surface proteins are apparently excluded from coated pits (8), and the presence of a ligand can stimulate the specific removal of a particular type of receptor from the cell surface whereas endocytosis of other plasma membrane proteins is unaffected (10, 36). The mechanisms responsible for this selectivity are at present unknown. It has been variously proposed that a component of the coated pit binds a specific site on proteins destined to be internalized (4, 33, 45) or that aggregation of surface proteins causes them to lodge in pits and be endocytosed (34, 38, 56).

By contrast to the scanty information available on the nature of the association between surface receptors and coated pits, the pathway followed by proteins after they are internalized is well known (9, 27, 60). In a process that requires only minutes, coated pits bud from the plasma membrane, lose their coats, and fuse with peripheral endosomes (17, 26, 29, 38, 43, 44). Here, proteins destined to return to the surface are sorted from those that are transported to intracellular locations (6, 17, 28, 57, 69). Recycling of proteins from endosomes to the cell surface is quite rapid, requiring only 5 to 15 min (6, 7, 9, 57). Endosomes are acidic (3, 16, 39, 63, 64), and low pH is required for the movement of many proteins from endosomes either to the cell surface (6, 23, 25, 62) or to lysosomes (58). Agents that raise intracellular pH, such as chloroquine, allow uptake of these proteins into endosomes but inhibit further transport (43).

Recently it has been shown that in the absence of the synthesis of other viral proteins, the G glycoprotein of vesicular stomatitis virus is efficiently endocytosed, enters endosomes, and is recycled (40, 46–48; Gottlieb, T., M. Rindler, and D. D. Sabatini, personal communication). Thus G protein resembles cell surface receptors and must contain some feature or features that cause it to be recognized by coated

pits. By contrast, when expressed from either bovine papilloma virus or SV40 vectors carrying a cloned cDNA gene (18, 54), the hemagglutinin (HA),[1] of the A/Japan influenza virus has a long lifespan on the cell surface and is internalized at a very low rate (Copeland, C., A. Helenius, I. Mellman, and J. Hearing, personal communication). Thus, HA is an example of a protein that appears to be excluded from the endocytic pathway. Exclusion of HA might occur through interactions between HA and an element of the cell surface that is anchored in some way, or might be due to the lack of a specific signal required for internalization through coated pits. If this latter possibility is the case, then transfer of an "internalization signal" to HA should target HA for endocytosis.

We wished to determine whether the transmembrane and cytoplasmic domains of G protein contained features that could direct the external domain of HA into coated pits. To accomplish this, we constructed a chimeric gene in which the translational reading frame for the HA ectodomain was joined to that of the transmembrane and cytoplasmic domains of G protein. Previously we had constructed a chimera with a similar structure fusing HA sequences to those of the HSV-1 glycoprotein C and had demonstrated that the hybrid protein reached the cell surface (21). We have now expressed these genes in simian cells infected with SV40 vectors and have investigated the influence of the foreign domains on the interaction of HA with the endocytic pathway.

Materials and Methods

Enzymes and Reagents

Restriction enzymes and DNA ligase were purchased from New England Biolabs (Beverly, MA); T4 polymerase from P-L Biochemicals, Inc. (Milwaukee, WI); lactoperoxidase and calf intestinal phosphatase from Boehringer Mannheim Biochemicals (Indianapolis, IN); reagents for polyacrylamide electrophoresis from Bio-Rad Laboratories (Richmond, CA); fluorescent and ferritin-conjugated antibodies from CooperBiomedical, Inc. (Malvern, PA); and reagents for electron microscopy from Polysciences, Inc. (Warrington, PA). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of Chimeric Glycoprotein Genes

Recombinant DNA techniques were those described by Maniatis et al. (15). A pBR322 plasmid containing an *Eco*RI-BamHI fragment of HSV-1 gC DNA (0.633 to 0.643) was obtained from E. K. Wagner (37). This plasmid was digested with PvuI, at a site four nucleotides upstream from sequences encoding the hydrophobic amino acids of the gC transmembrane domain. To adjust the translational reading frame of the gC sequence with that of a sequence encoding the HA ectodomain, the two nucleotide 3′ extension at the PvuI site was removed with T4 polymerase. The gC plasmid was then digested with SalI at a site outside the gC gene and a fragment was isolated containing gC sequences from nucleotide 1692 at the PvuI site to a BamHI site at nucleotide 2208 in the 3′ noncoding region. Plasmid Xf3 (15) was digested with BamHI, treated with the Klenow fragment of DNA polymerase I to fill the recessed ends, and digested with SalI. The PvuI-SalI fragment containing the gC 3′ sequences was inserted between the SalI and filled BamHI sites of Xf3 and bacteria were transformed with the ligation mixture (24). Colonies were selected containing plasmids in which blunt-end ligation of gC nucleotide 1692 to the altered plasmid BamHI site had re-formed the BamHI site. Digestion of these plasmids with BamHI released a 514-bp fragment which was cloned into M13mp8 and sequenced by the dideoxy chain termination method (55), confirming the correct positioning of the reconstructed BamHI site. The SV40 vector SVEHA20-A$^-$ (19) containing a truncated gene encoding a secreted form of HA, (HA^{sec}) was cloned into pKSB$^-$ (12) through the unique SV40 KpnI site. The resulting plasmid, pKSVEHA^{sec}, was linearized with BamHI at a unique site at the 3′ end of the HA^{sec} gene, and the BamHI fragment containing the gC sequences was inserted. The orientation of the BamHI gC fragment relative to the HA sequences was determined by restriction enzyme analysis and plasmids with the correct orientation were isolated as the series pkSVEHAgC.

DNA coding for the VSV G protein was obtained from J. Rose (51), and nucleotides 1381 to 1665 (encoding the transmembrane and cytoplasmic domains of G protein) were isolated as a TaqI to BamHI fragment. In the course of previously reported experiments (12) we constructed M13-HA derivatives each having a ClaI linker inserted into HA sequences encoding the carboxy-terminal one-third of the HA protein. We identified a recombinant phage, mpHA351, in which the position of the inserted ClaI site at nucleotide 1590 of the HA sequence (20) allowed fusion of the authentic HA reading frame with that of the G sequences in the isolated TaqI-BamHI fragment. The HA sequences in mpHA351 between the ClaI and BamHI sites were replaced with the G sequences on the TaqI-BamHI fragment. Correct fusion of the HA and G sequences in mpHAG was confirmed by DNA sequencing (55). HA sequences in the vector pkSVEHA were replaced with the analogous sequences from mpHAG to create pkSVEHAG.

Generation of Recombinant SV40 Stocks

Several isolates of pkSVEHAG and pkSVEHAgC were selected and digested with KpnI. Viral sequences were isolated from plasmid sequences, recircularized, and introduced into CV-1 cells with an equal amount of D11055 helper virus DNA (49). Virus stocks were prepared and assayed for HA production as previously described (12, 18, 55). No significant differences were observed between independently derived stocks of the same recombinant virus.

Hemagglutination and Cell Fusion Assays

At 36 to 40 h after infection with recombinant viruses, CV-1 cells were overlaid at 23°C with a 1% solution of washed guinea pig erythrocytes in PBS. After 10 min unbound cells were removed by washing and the cells were photographed with a Nikon diaphot inverted microscope. Cell fusion induced by low pH was assayed by the procedures of Doxey et al. (11). In brief, human erythrocyte ghosts loaded with horseradish peroxidase were allowed to bind to vector-infected cells which had been previously treated with 10 μg/ml trypsin in Dulbecco's modified Eagle's medium (DME) at 37°C to cleave HA. The medium containing trypsin was replaced with medium buffered to pH 4.8–5.0. After several min this medium was replaced with DME at pH 7.2 and the cells were left at 37°C for 1 h to allow the peroxidase to diffuse from the erythrocytes into the CV-1 cells. The cells were fixed with 1% glutaraldehyde, stained with diaminobenzidine, and photographed.

Immunofluorescence and Electronmicroscopy

After 30–36 h, cells infected with SV40-HA recombinant viruses were fixed with 2% formaldehyde and 0.1% glutaraldehyde for surface labeling, or with formaldehyde alone if they were to be permeabilized. The fixative was quenched with 20 mM ammonium chloride in PBS (5). For surface labeling, nonspecific adsorption of the fluorescent antibody was blocked by treating fixed cells for 30 min in PBS containing 1.0% rehydrated skim milk, and all subsequent washes and incubations with antibodies were performed with this solution. For intracellular labeling, fixed cells were treated with 1.0% Triton X-100 in NET/GEL 150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 0.25% gelatin, 0.05% Nonidet P-40, 0.01% NaN_3 for 15 min. Subsequent washes and antibody incubations were performed with NET/GEL from which the Triton X-100 had been omitted. Cells were observed and photographed with a Leitz Ortholux II microscope fitted with N2 and L2 filters and an Orthomat W camera.

For electron microscopy, cells were fixed and reacted with anti-HA IgG as described for immunofluorescence and then labeled with a ferritin-conjugated goat anti-rabbit IgG. After labeling, monolayers of cells were fixed with 1% glutaraldehyde for 30 min and postfixed with 1% osmium tetroxide for 1 h at 4°C. After dehydration through 70, 90, and 100% ethanol, cells were removed from the plastic culture dish by dissolving the plastic in propylene oxide (53). The sheets of monolayer released from the plastic were collected in glass tubes, washed twice with propylene oxide and impregnated with Polybed (Polysciences Inc.). Cells in liquid polybed were placed in Beem capsules, inserted into microfuge tubes, and heated to 40°C for 10 min to make the polybed less viscous. The cell sheets were spun to the bottom of the Beem capsule in a microfuge and the plastic hardened at 60°C overnight. Thin sections were cut using a Sorvall M2-B microtome (Dupont Instrument Co., Wilmington, DE), stained with uranyl acetate and lead citrate, and mounted on 300 mesh copper grids. Samples were photographed with a Philips 201 electronmicroscope.

[1] *Abbreviations used in this paper:* DME, Dulbecco's modified Eagle's medium; HA, hemagglutinin; NET/GEL, 150 mM NaCl, 50 mM Tris, pH 8.0, 1 mM EDTA, 0.25% gelatin, 0.05% Nonidet P-40, 0.01% NaN_3.

Radiolabeling of Cells and Immunoprecipitation

Vector-infected cells were metabolically labeled with [^{35}S]methionine at 30–36 h after infection as previously described (12). Cell surfaces were labeled with ^{125}I at 0°C using lactoperoxidase and glucose oxidase (30). To assay for the acquisition of temperature-dependent resistance to trypsin, cells that had been labeled with ^{125}I at 0°C were washed extensively and placed at 37°C in DME for various intervals. After the 37°C chase periods, the cells were placed into ice-cold DME containing 10 mM Hepes, pH 7.2, and 100 μg/ml trypsin for 1 hr. At the end of this period the trypsin was removed and the cells were washed five times during 30 min with DME containing 10% calf serum. Samples were scraped into 50 mM Tris, pH 8.0, 1.0% Nonidet P-40, 1 mM p-hydroxymercuribenzoate, containing 0.1 U aprotinin and 10 μg a-macroglobulin/ml. After removal of nuclei and large debris by centrifugation at 10,000 *g* for 10 min, an equal volume of NET/GEL was added to the cell lysates, and samples were immunoprecipitated.

Results

Structure of HAG and HAgC

The HA, G, and gC polypeptides each have a large external domain, a single hydrophobic transmembrane domain, and a short carboxy-terminal cytoplasmic region (Fig. 1). Genes coding for chimeric glycoproteins were constructed such that DNA encoding the large external domain of HA was joined in translational reading frame to that coding for the transmembrane and cytoplasmic domains of G or gC (see Fig. 1 and Materials and Methods for details). The predicted amino acid sequences for the transmembrane and cytoplasmic domains of the resulting chimeras are shown in Fig. 1. Analysis of the A/Japan HA sequence with the hydropathy program of Kyte and Doolittle (32) indicates that the transmembrane domain of HA begins within one or two amino acids of glutamine 525. The HAG protein consists of the first 516 residues of HA, two new residues contributed by a synthetic linker used in cloning, and carboxy-terminal sequences from G. The HAgC protein consists of 524 amino acids of the HA ectodomain, a pentapeptide of novel sequence, and the gC transmembrane and cytoplasmic domains. Also shown for comparison in Fig. 1 are the sequences of the parent molecules from which the chimeras were derived.

As a consequence of their construction, both chimeric proteins contain changes in the ectodomain close to the boundary with the transmembrane domain. To establish that changes in this portion of the HA ectodomain have little effect on HA function, HA351, the direct precursor for HAG, was included as a control in all of the experiments with the two chimeric proteins. In HA351, five amino acids have been deleted and two new residues inserted into the HA ectodomain at a position four residues from the ectodomain-transmembrane domain boundary (Fig. 1). In all of the experiments described below HA351 was both functionally and antigenically equivalent to wild-type HA. Thus, changes can be introduced into this region of the HA ectodomain without observable effect.

Expression of Functional HAG and HAgC Glycoproteins from Recombinant SV40 Vectors

Recombinant virus stocks SVEHAG, SVEHA351, or SVEHAgC were developed by the procedure described previously (12). Fresh virus stocks of the vector carrying the wild-

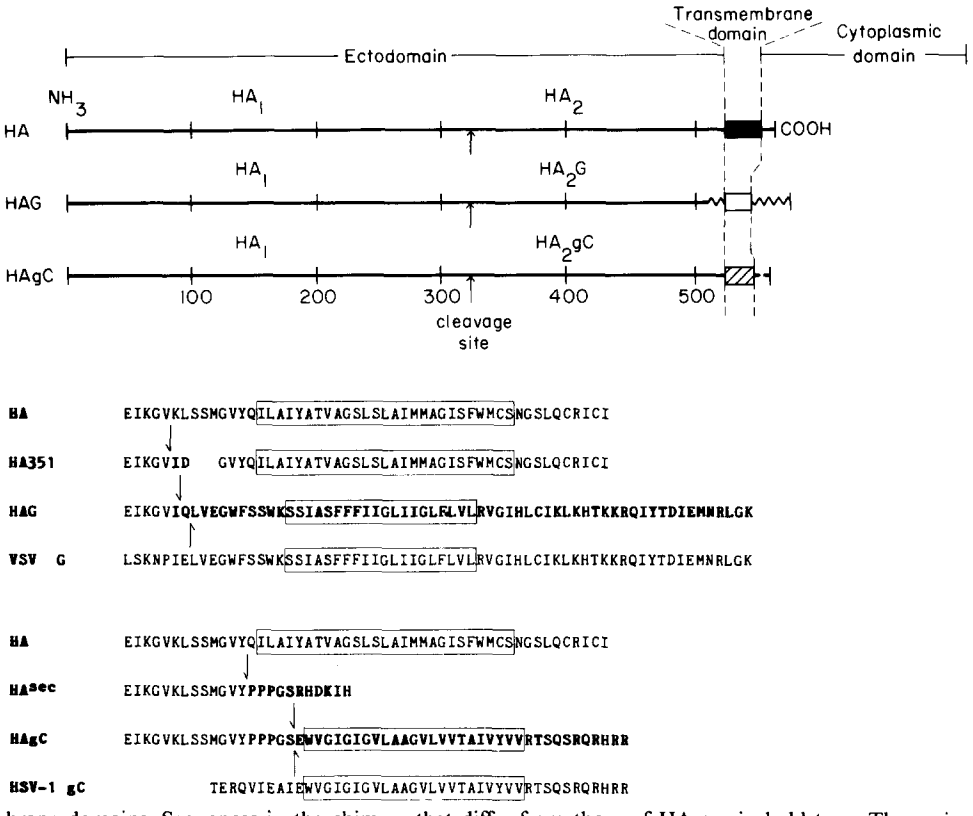
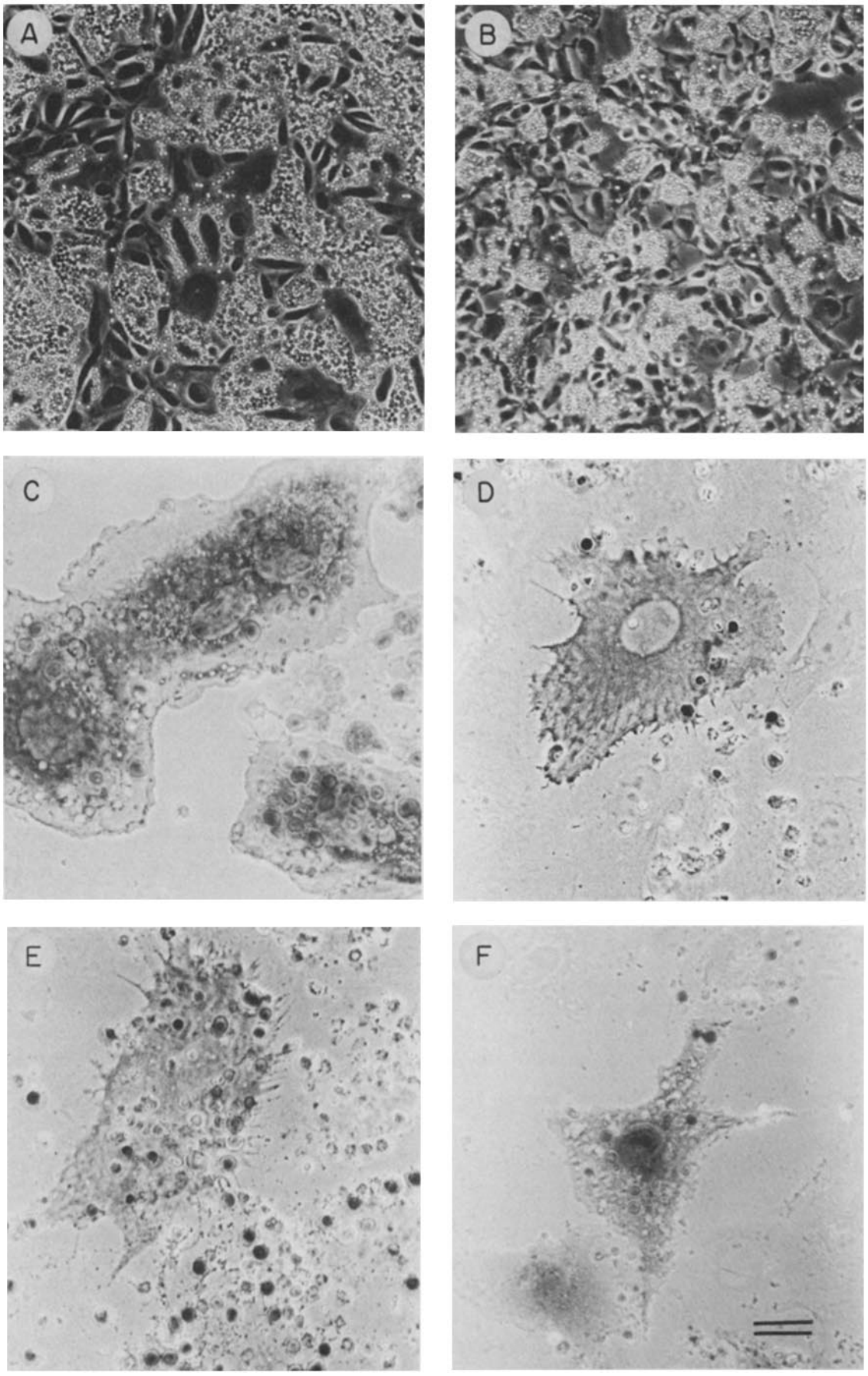


Figure 1. The HA, HAG, and HAgC polypeptides consist of three topological domains. The topographic orientation of the linear amino acid sequences of HA, HAG, and HAgC are diagrammed to scale. The boxed areas indicate the hydrophobic residues predicted to span the lipid bilayer by hydropathy analysis (32). To the left of the boxed region extends the large amino-terminal external domain. A unique trypsin sensitive site is indicated at which the HA ectodomain can be cleaved into HA_1 and HA_2 subunits. To the right of the hydrophobic transmembrane domain are residues exposed to the cytoplasm. Carboxy-terminal amino acid sequences of parent, mutant, and chimeric glycoproteins are displayed below the diagram. The construction of the genes encoding these proteins is described in Materials and Methods. The arrows indicate where the sequences of the parent molecules are joined in the chimeras. The boxed sequences indicate the hydrophobic transmembrane domains. Sequences in the chimera that differ from those of HA are in bold type. The amino acid sequence of HA351, the parent molecule for the HAG chimera, differs from that of HA by the deletion and substitution shown; a synthetic DNA linker was inserted into the HA gene at that position. HA^{sec}, the parent molecule for HAgC, contains amino acids coded by sequences added to the HA^{sec} gene during the cloning process. Five of these amino acids are retained by HAgC.


A
B
C
D
E
F

type HA gene, SVEHA3 (18), were also prepared. The amount of HA-related glycoprotein produced by CV-1 cells infected with the various virus vectors was measured by radioimmunoassay of culture supernatants and cell lysates; after 68 h the amount of each protein was equivalent to 10^8 molecules per cell (data not shown). The chimeric proteins were detected at the cell surface by immunofluorescence (see below) and by hemadsorption. At 68 h after infection, 100% of CV-1 cells in cultures expressing either wild-type HA or the chimeras bound erythrocytes. Thus, HAG and HAgC were equivalent to wild-type HA both in the amount of protein present at the cell surface and in the capacity to bind to sialic acid on red cells. In experiments to be reported elsewhere, we observed that HAG was transported through the exocytic pathway as rapidly as HA. HAgC moved to the cell surface more slowly.

In addition to the ability to bind sialic acid, HA has the capacity to induce fusion between the membrane in which it is anchored and another, closely apposed membrane (35, 41, 67). This fusion potential is generated by a post-translational cleavage at a single site in the ectodomain (see Fig. 1). To determine whether HAG and HAgC retained the membrane fusion activity of wild-type HA, CV-1 cells infected with recombinant viruses were activated for fusion by mild trypsin treatment. Human erythrocyte ghosts loaded with horseradish peroxidase were allowed to bind to the cells (11). Upon brief treatment with medium buffered to pH 5.0, the erythrocytes fused with the infected CV-1 cells and delivered peroxidase into the cell cytoplasm. The results of such an experiment are shown in Fig. 2. After fixation and reaction with diaminobenzidine, cells expressing the chimeric or mutant HAs were stained (Fig. 2, *C–F*) but uninfected controls were not (not shown). The pH at which fusion occurred was the same for both wild-type and chimeric HAs; fusion was not observed above pH 5.2. Thus, HAG and HAgC bound sialic acid and induced membrane fusion, retaining both major biological activities of the HA glycoprotein.

The Ectodomains of HAG and HAgC Can Be Distinguished Immunologically from That of Wild-Type HA

Although HAG and HAgC displayed the biological activities of HA, we wished to determine whether the presence of heterologous carboxy-terminal domains in the chimeras had influenced the HA ectodomain in some other way. Previously we had observed that HA^{sec}, the parent molecule for the HAgC protein, could be distinguished immunologically from the wild-type HA. HA^{sec} secreted into culture medium was recognized by an antiserum, designated IH-2, that had been raised against denatured HA isolated by electrophoresis on cellulose acetate in the presence of SDS (unpublished results). Data summarized in Table I indicate that IH-2 serum recognizes sites on the HA ectodomain that are cryptic in the mature, fully glycosylated wild-type HA. When used for immunofluorescent staining of permeabilized cells expressing wild-type HA, IH-2 produced a reticular pattern with none of the perinuclear staining characteristic of the Golgi apparatus, and no surface fluorescence (not shown). HA (Fig. 3, *a* and *b*) or HA351 (not shown) at the surface of nonpermeabilized cells was completely unreactive with IH-2 but was detected by a rabbit polyclonal serum that recognizes all forms of HA. By contrast, IH-2 labeled the HAG and HAgC present at the cell surface (Fig. 3, *D* and *F*). Thus, the ectodomains of HAG and HAgC differed in some way from that of wild-type HA, either in shape or in stability. Although the transmembrane and cytoplasmic domains in the chimera anchored the proteins to the cell membrane and allowed the transport of a functional HA ectodomain to the cell surface, they did not fully substitute for the wild-type HA domains in maintaining HA structure.

*Table I. Specificities of IH-2 Antiserum**

Form recognized	Type of HA			
	HA	$HA^{sec‡}$	HAG	HAgC
Tunicamycin	+	+	ND	ND
Endo-H^s 1	+	+	+	+
Endo-H^s 2	–	–	–	–
Endo-H^r	–	+	+	+
$Trypsin^s$	–	+	+	+

Specificities of IH-2 antiserum were measured by immunoprecipitation. Tunicamycin designates the nonglycosylated polypeptide synthesized by cells treated with tunicamycin. Endo-H^s 1 refers to the form of the molecule that appears first during metabolic labeling with [^{35}S]methionine and that contains high mannose oligosaccharides sensitive to digestion with endoglycosidase H. Endo-H^s 2 indicates a polypeptide of slightly lower apparent molecular weight than endo-H^s 1 which appears after endo-H^s 1 during labeling and which has high mannose oligosaccharides sensitive to digestion by endo-H. Endo-H^r designates polypeptides containing oligosaccharides resistant to endo-H. $Trypsin^s$ refers to polypeptides that have been cleaved into HA_1 and HA_2 subunits by trypsin added to cell culture medium indicating those molecules that have reached the cell surface.

* A detailed analysis of the specificities of IH-2 antiserum will be published elsewhere.

‡ HA^{sec} is a truncated form of HA lacking the transmembrane and cytoplasmic domains and is secreted.

HAG and HAgC Are Concentrated in Coated Pits, HA Is Not

To determine whether the G and gC domains in the chimeric proteins would direct the HA ectodomain into the endocytic pathway, we compared the extent to which the HAG and HAgC localized in coated pits. In a first set of experiments, CV-1 cells expressing wild-type HA, HAG, HAgC, or HA351 were fixed with glutaraldehyde before immunospecific labeling in order to exclude any redistribution of the proteins that might be induced by cross-linking antibodies. In a second series of experiments, unfixed cells expressing the wild-type or chimeric proteins were labeled at 0°C and samples were subsequently placed into medium at 37°C to allow endocytosis

Figure 2. The chimeric glycoproteins retain HA functions of hemadsorption and cell fusion. (*A*) 40 h after infection CV-1 cells expressing HA bind guinea pig erythrocytes. (*B*) CV-1 cells expressing HAgC 40 h after infection bind erythrocytes at levels similar to those in wild-type HA. To demonstrate cell fusion, at 40 h after infection cells expressing HA, HA351, HAG, or HAgC were briefly treated with trypsin to cleave the glycoproteins into their subunits, then allowed to bind to human erythrocyte ghosts which had been loaded with horseradish peroxidase. The pH of the medium was lowered to 5.0, and cell fusion delivered the peroxidase into the cytoplasm of infected cells. Cells containing peroxidase were stained with diaminobenzidine. (*C*) HA. (*D*) HA351. (*E*) HAG. (*F*) HAgC. Bar: *A* and *B*, 22.5 μm; *C–F*, 6 μm.

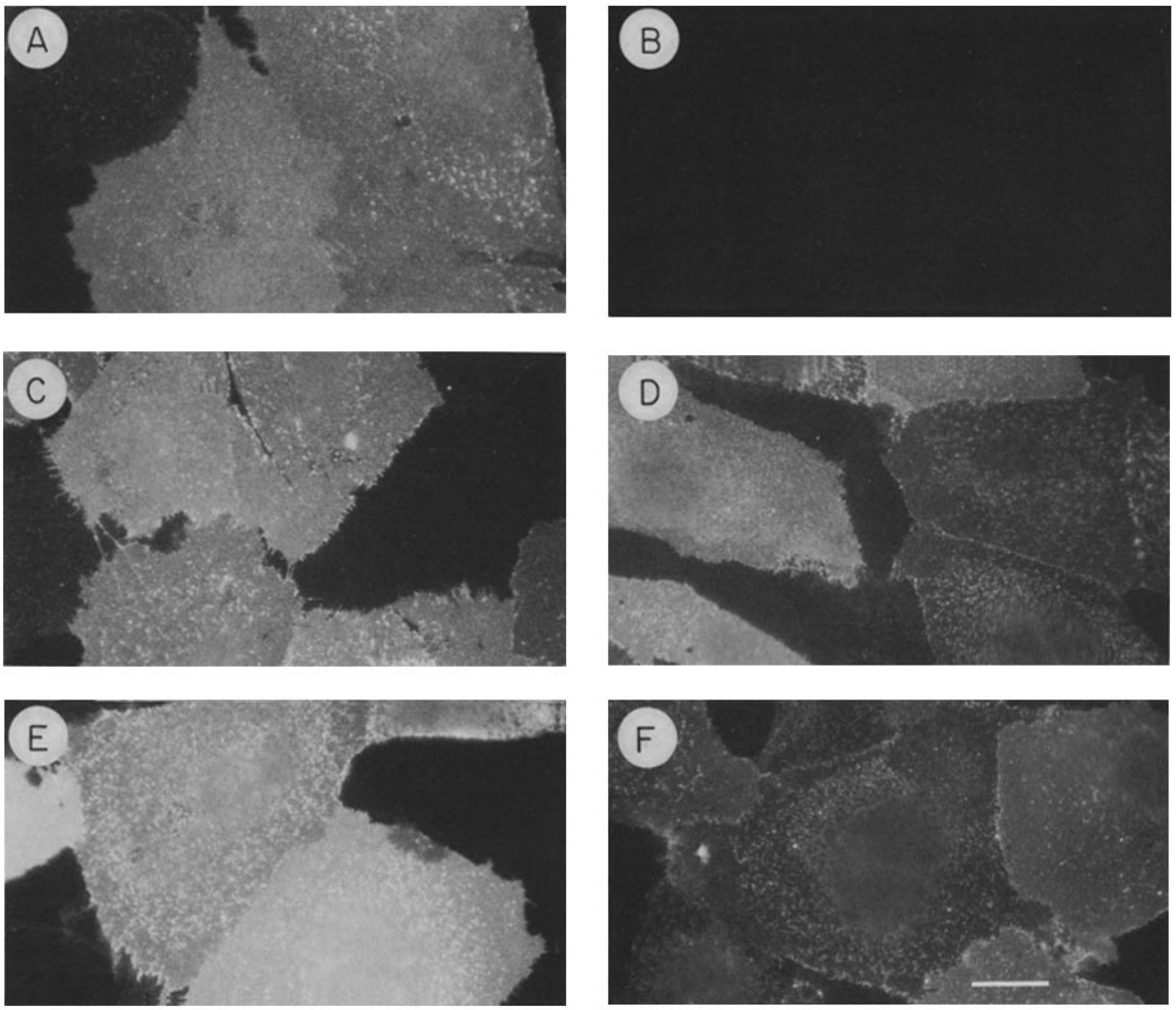


Figure 3. HAG and HAgC express epitopes that are cryptic on the mature HA ectodomain. *A*, *C*, and *E* show cells labeled with a rabbit IgG that recognizes all forms of HA. *B*, *D*, and *F* show cells labeled with the IH-2 antiserum (for specificity, see Table I). (*A* and *B*) HA. (*C* and *D*) HAG. (*E* and *F*) HAgC. The exposure time was the same for all photographs. Bar, 6 μm.

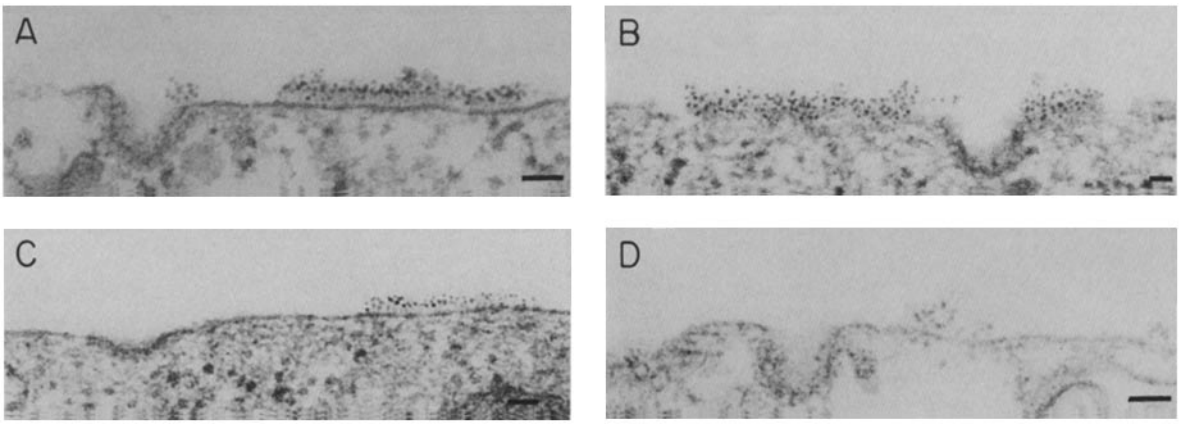


Figure 4. HA is rarely observed in coated pits. Cells expressing HA were fixed 36 h after infection and labeled with anti-HA IgG and ferritin-conjugated second antibody, then processed for electron microscopy. Ferritin was often observed quite near, but not inside, coated pits, even on cells (*A* and *B*) that were heavily labeled over their surfaces. Bar, 50 nm.

to take place. No differences were observed between samples fixed before labeling and samples labeled at 0°C (but not warmed to 37°C) before fixation. As shown in Fig. 4, coated pits on cells expressing wild-type HA usually contained no ferritin-conjugated antibody. Even in examples in which considerable HA was labeled at adjacent cell surfaces little ferritin was found in coated structures (Fig. 4, *A–C*). In these experiments, cells expressing HA351 were indistinguishable from

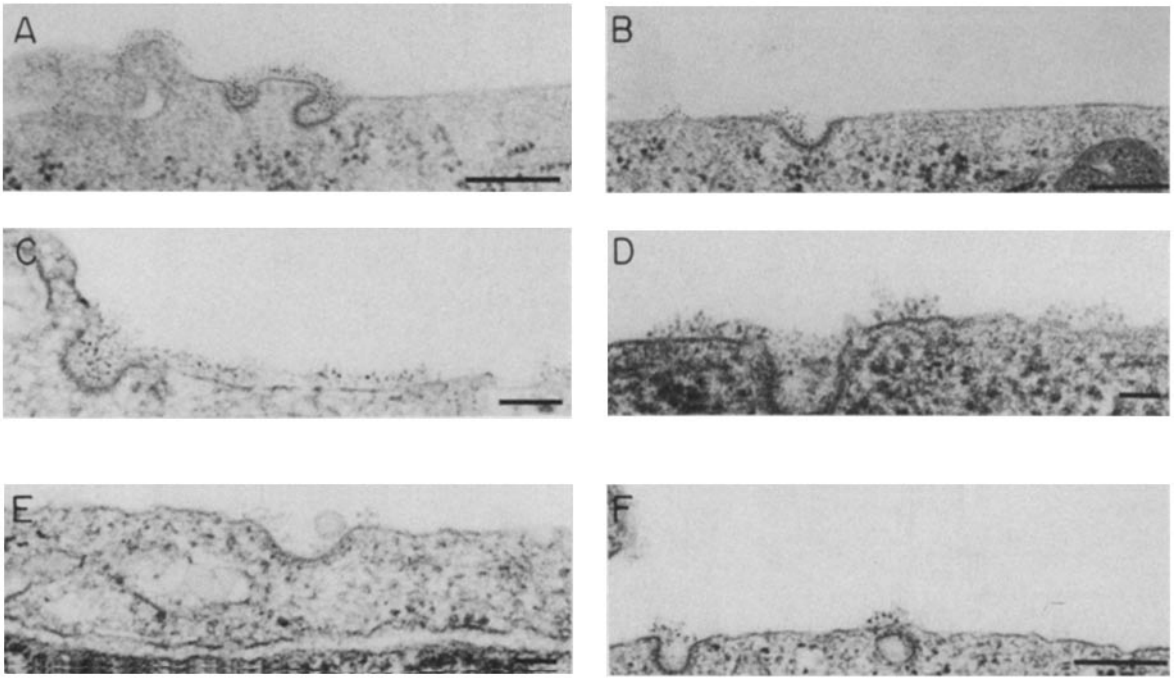


Figure 5. HAG and HAgC preferentially collect in coated pits. Cells expressing HAG (*A–D*) or HAgC (*E* and *F*) were fixed and immunospecifically labeled 36 h after infection. Coated pits on these cells usually contained ferritin, regardless of whether most of the cell membrane was labeled (*C* and *D*) or lacked much label (*A*, *B*, *E*, and *F*). Bar, 50 nm.

cells expressing wild-type HA (not shown). By contrast, coated pits on cells expressing the chimeric proteins were well labeled. As shown in Fig. 5, coated pits on cells expressing HAG were often labeled with ferritin even when nearby areas of the plasma membrane were not (Fig. 5, *A* and *B*), suggesting that HAG concentrated preferentially in coated pits. The surface distribution of HAgC resembled that of HAG. Fig. 5, *E* and *F* present examples of cells expressing HAgC where ferritin is seen concentrated in coated pits but is absent from the surrounding plasma membrane.

The chimeric HAs were clearly observed to accumulate in coated pits whereas the wild-type HA appeared to be excluded from those structures. To quantitate this difference in surface distribution, we calculated the percentage of coated pits containing ferritin for cells expressing each of these HAs. This procedure gave a very conservative estimate of the extent to which location in coated pits was favored for the chimeras in comparison to HA. Coated pits on cells expressing HAG or HAgC were often filled with ferritin, but such heavily labeled pits were rare on cells that expressed comparable levels of wild-type HA. Less than 10% of the coated pits on cells expressing wild-type HA scored as unambiguously positive for ferritin. Fig. 4*D* shows a coated pit that might contain a few ferritin particles and could not be scored with certainty. If examples similar to this were scored as positive, the upper limit of coated pits on cells expressing HA, which might have contained a few ferritin particles, was 35%. By contrast, on cells expressing HAG ~90% were observed to be well labeled with ferritin. The remaining 10% could not be scored unambiguously. In parallel experiments, ~75% of coated pits on cells expressing HAgC definitely contained ferritin.

When cells labeled at 0°C were warmed to 37°C for 90 s before fixation, ferritin-labeled HAG was observed located in what appeared to be small vesicles inside cells; such vesicles were more prevalent when cells expressing either HAgC or HAG were fixed after a 10-min incubation at 37°C (data not shown). Endocytosis of labeled cell surface receptors into vesicles of the size and morphology of those we observed has been reported to occur over a similar time scale (17, 29, 44). Thus it appeared that both HAG and HAgC were preferentially located in coated pits as a first step in the internalization of those proteins.

Chloroquine Causes HAG and HAgC to Concentrate at an Intracellular Site

Agents that raise intracellular pH have been shown to inhibit delivery of many proteins to lysosomes and the recycling of many receptors to the cell surface, causing an accumulation of internalized proteins in endosomes (6, 23, 25, 43, 62). Some of these agents, such as monensin, significantly affect the exocytic pathway as well (1, 65). However, we have observed that moderate levels of chloroquine have little effect on the exocytosis of wild-type HA, HAG, or HAgC (unpublished results). Thus if HAG and HAgC were preferentially entering the endocytic pathway through coated pits, the presence of chloroquine should result in a significant intracellular accumulation of HAG and HAgC, but the cellular distribution of HA should remain unaffected. At an early period of infection with SV40 vectors when wild-type or chimeric HAs were beginning to accumulate at the cell surface, chloroquine was added to the cell culture medium, and the cells were then examined by immunofluorescent staining. Chloroquine treatment had little effect on the cellular location of HA or HA351; even after 12 h of treatment HA was observed predominately at the cell surface (Fig. 6*A*). Thus, the pattern of fluorescence in chloroquine-treated cells expressing HA was indistinguish-

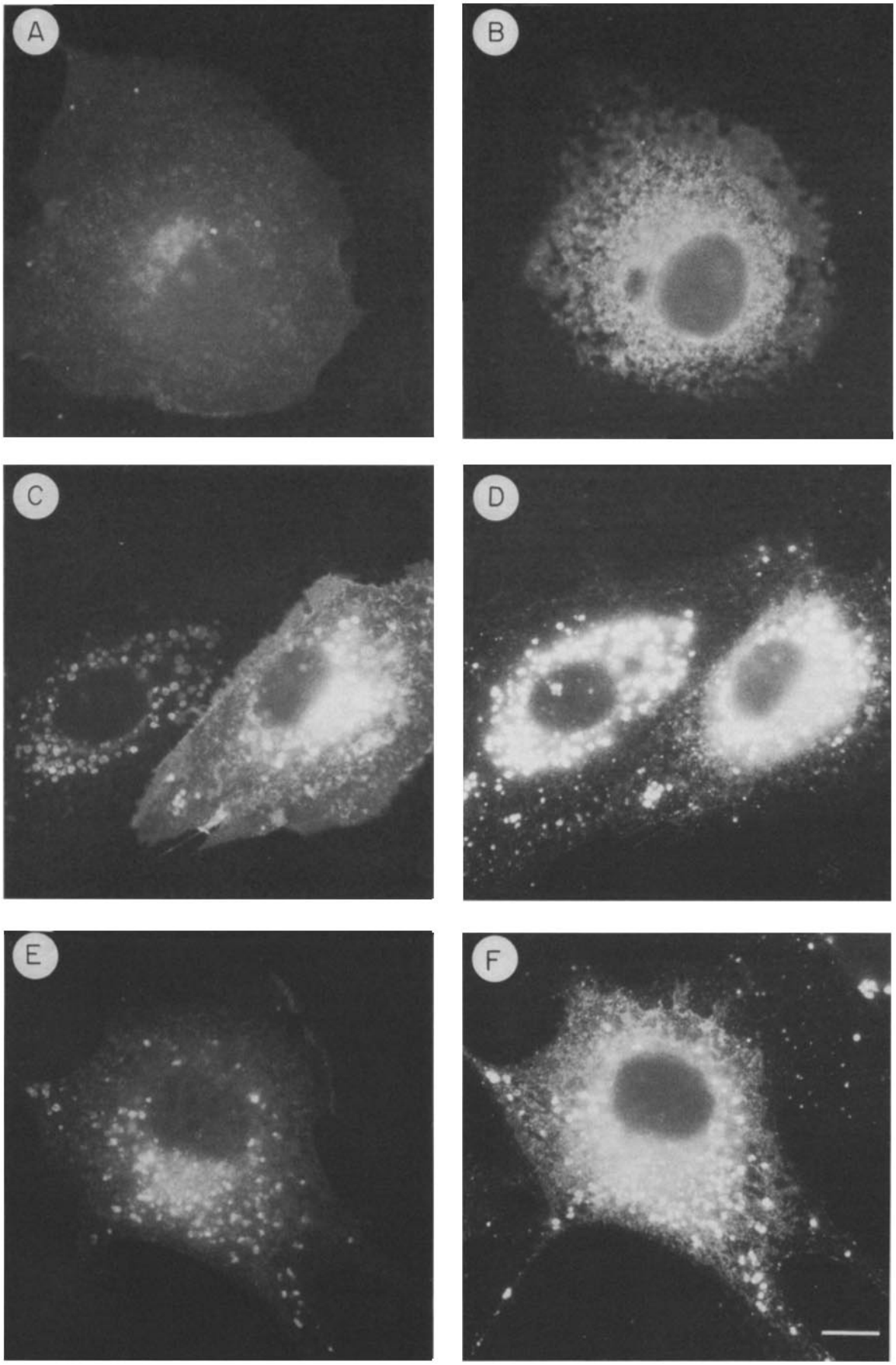


Figure 6. HAG and HAgC concentrate in vesicles in cells treated with chloroquine, but HA does not. After 12 h of treatment with 25 μM chloroquine, cells expressing HA (*A* and *B*), HAG (*C* and *D*), or HAgC (*E* and *F*) were fixed and stained by indirect immunofluorescence. *A*, *C*, and *E* show cells labeled with a monoclonal antibody that recognizes only mature undenatured HA, and an anti-mouse fluorescein isothiocyanate–conjugated second antibody. *B*, *D*, and *F* show the same cells as on the left labeled with IH-2 antiserum and with a tetramethylrhodamine isothiocyanate–conjugated second antibody. Bar, 10.3 μm.

able from that in cells expressing either HA, HAG, or HAgC in the absence of chloroquine. By contrast, chloroquine caused a progressive loss of HAG or HAgC from the plasma membrane and a concomitant appearance of large, intracellular vesicles that stained with antibodies to HA. In cultures expressing HAG, these vesicles were observed after only 3 h in medium containing chloroquine; by 6 h of treatment vesicles were observed in most cells (results not shown). Cells expressing HAgC displayed similar patterns of fluorescence although the accumulation of vesicles was slower (results not shown). After 12 h of treatment with chloroquine >90% of cells expressing either HAG (Fig. 6, *C* and *D*) or HAgC (Fig. 6, *E* and *F*) had significantly reduced surface staining and contained numerous large vesicles that react with anti-HA antibodies. It was important to establish that the fluorescent vesicles observed in cells treated with chloroquine were not derived from the Golgi apparatus or some other compartment of the exocytic pathway. Thus, in a control experiment cycloheximide at a level sufficient to abolish protein synthesis in CV-1 cells (100 μg/ml) was added to cells expressing HA or the chimeric proteins 3 h before the addition of chloroquine. Under these conditions the fluorescence was reduced in intensity but the pattern was not changed (data not shown); the main perturbation of transport of HAG and HAgC occurred during endocytosis.

To determine whether HAG or HAgC in intracellular vesicles represented denatured proteins cleared from the plasma membrane, cells were stained using a monoclonal antibody that reacts only with mature terminally glycosylated HA. This antibody recognizes HA at the cell surface but does not recognize denatured HA or unfolded HA in the endoplasmic reticulum (unpublished results). To locate any HAG or HAgC that did not react with the monoclonal antibody, the cells were double labeled with polyclonal antibody IH-2. Fig. 6, *A*, *C*, and *E* present cells stained with a goat anti-mouse IgG that reacts with the monoclonal antibody, whereas *B*, *D*, and *E* display the same cells stained with an anti-rabbit conjugate that reacts with IH-2. HAG and HAgC in the large vesicles observed in chloroquine-treated cells were labeled with the monoclonal antibody that recognizes only the fully processed, mature wild-type HA (Fig. 6, *C* and *E*). Thus, HAG and HAgC molecules with a native conformation were internalized, indicating that endocytosis of the chimeras involved more than the clearance of denatured proteins from the cell surface.

In the absence of chloroquine the pattern of immunofluorescent staining of HAG resembled that of cells expressing wild-type HA. For both wild-type HA and HAG most of the protein was observed to be at the cell surface. Thus, we could find no indication that internalized HAG was collecting at an intracellular site. Nor did we observe increased degradation of HAG, which would suggest that internalized protein was being delivered to lysosomes. When HA and HAG were metabolically labeled with [^{35}S]methionine for 15 min at 36 h after infection, no difference was observed in the amount of protein immunoprecipitated immediately after the labeling period and the amount precipitated after the cells had been maintained in culture for an additional 20 h (data not shown). These observations are consistent with the possibility that HAG was returning to the cell surface quite rapidly after endocytosis.

HAG and HAgC Are Rapidly Internalized

To determine the rate of internalization of HAG and HAgC, we measured the rate at which HAG and HAgC labeled at the cell surface became inaccessible to protease added to cell culture medium. The surfaces of cells expressing HA, HA351, HAG, or HAgC were labeled with ^{125}I at 0°C. When trypsin was added to the cell culture medium at this point, the labeled HAs were quantitatively processed into HA_1 and HA_2 subunits because no internalization had occurred. HAG and HAgC can be detected in coated pits at this temperature (Fig. 5), and thus clustering in endocytic pits at the cell surface does not render these HAs inaccessible to extracellular trypsin. However, when labeled CV-1 cells expressing HAG or HAgC were incubated at 37°C for 5 or 10 min to allow endocytosis to occur, subsequent trypsin treatment at 0°C did not cleave all of the chimeric glycoproteins. In parallel experiments, no uncleaved HA or HA351 was observed even after chases of 60 min at 37°C (not shown). The results of one such experiment with HA and HAG are shown in Fig. 7. As shown in the autoradiograph of the upper gel, wild-type HA was not protected from trypsin cleavage after 10 min chase at 37°C, although by 120 min a trypsin-resistant, 69-kD polypeptide representing uncleaved HA was observed. By contrast, the trypsin-resistant HAG 71-kD band appeared after a 5-min chase at 37°C. In similar experiments, a trypsin resistant

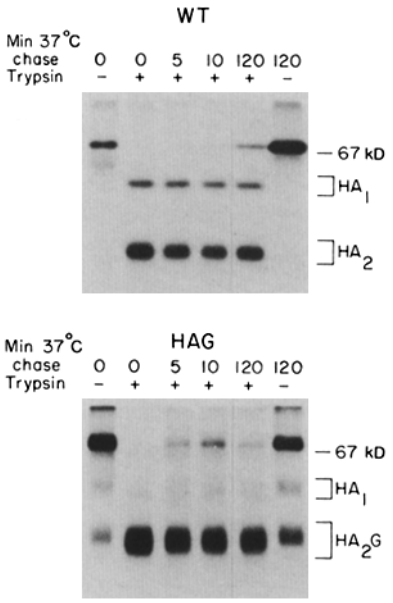


Figure 7. Internalization of chimeric proteins protects them from trypsin cleavage. At 36 h after infection CV-1 cells expressing either chimeric or wild-type HA were labeled with ^{125}I at 0°C, a temperature that does not permit endocytosis (61). After extensive washing, cells were either left on ice or incubated at 37°C for periods as brief as 5 min. The cells were returned to medium at 0°C, and some samples were treated with 100 μg/ml trypsin for 1 h. HA at the cell surface was quantitatively cleaved into HA_1 and HA_2 subunits. HAs that had been internalized, however, would have been inaccessible to the trypsin and remained uncleaved. After processing by immunoprecipitation, PAGE, and autoradiography, uncleaved HA and HAG migrated as bands of 69 or 71 kD, respectively. The cleaved subunits, HA_1 and HA_2 or HA_2G, form the major bands migrating lower in the gels in the lanes marked +. The uncleaved portion of HAG is visible as the 71-kD band in the center two lanes of the lower gel.

Table II. Initial Rates of Internalization of HAs

	5 min at 37°C	10 min at 37°C
HA	0	0
HAG	13 ± 3	16 ± 2
HAgC	7 ± 1	8 ± 0

The table presents the percentage of each molecule that remained uncleaved by trypsin added after a chase at 37°C for the period shown.

HAgC band was also observed after a 5-min chase (not shown).

To determine the amount of HA, HAG, or HAgC that became resistant to trypsin digestion during the chase, the HA, HA_1, and HA_2 bands were cut from gels, and the radioactivity present in the gel slices was measured. Table II presents the initial percentages of HA, HAG, and HAgC that became inaccessible to extracellular trypsin after 5 or 10 min chase at 37°C. During the first 10 min of the chase HA was completely cleaved, indicating that no internalization had occurred. After a long chase of 120 min, uncleaved wild-type HA was detected at a level of 10%. By contrast, ~15% of HAG and ~7% of HAgC became protected after only 5 min. Thus, HAG, and HAgC appeared to be rapidly internalized but at different rates. The percentage of HAG that was inaccessible to trypsin digestion remained constant for the first 30 min of chase and thereafter declined to 2% after 60 min. No similar decline in the amount of protected HAgC was observed. The rate of endocytosis through coated pits is sufficient to internalize 50–150% of the plasma membrane each hour (9, 38, 60). Thus, the rate of internalization of HA of 5% per h represents a significant exclusion of that protein from the endocytic pathway. By contrast, the rate for HAG of 1.5% per min is within the range of rates of internalization measured for cell surface receptors in the absence of ligand (6, 31).

In the bottom of Fig. 7, the outside lanes of the gel contain bands from the immunoprecipitation of samples of HAG that were not treated with trypsin. Nevertheless, bands migrating at the positions of HA_1 and HA_2 can be seen in these lanes. During experiments analyzing the kinetics of exocytosis of HAG and HAgC (to be reported elsewhere), we observed that both of those molecules were cleaved by endogenous proteases on CV-1 cells, whereas there was little endogenous cleavage of wild-type HA. Although the pattern of endogenous cleavage of the various HAs does parallel the pattern of endocytosis, at present we know only that most of this cleavage occurs after HAG and HAgC arrive at the cell surface. Obviously, the proportion of the chimeric proteins that was cleaved before surface labeling would not have scored in our internalization assay, and thus the rates of internalization of HAG and HAgC may be higher than reported above.

Whether labeled by iodination at the cell surface or during synthesis with [^{35}S]methionine, the HA_1 subunits of both HAG and HAgC migrate as diffuse bands on polyacrylamide gels, leading to the impression that less material is present. However, the ratio of ^{125}I radioactivity in HA_1 to that in HA_2 was not significantly different for the chimeric proteins (0.29) than for the wild-type HA (0.25). Thus the HA_1 subunits of HAG and HAgC were not selectively degraded by the trypsin digestion used to generate them. At present, we do not know the cause of this difference in mobility during electrophoresis.

Discussion

Two chimeric HA glycoproteins, HAG and HAgC, containing the large external domain of the HA of influenza virus and foreign transmembrane and cytoplasmic domains, were efficiently expressed at the surface of cells infected with recombinant SV40 vectors. The chimeric HAs retained the biological functions of the wild-type protein. However, by several criteria HAG and HAgC were observed to enter the endocytic pathway, whereas HA was essentially excluded. (*a*) Coated pits on cells expressing the chimeric glycoproteins were much more frequently labeled with anti-HA antibody, and were labeled to a much greater extent, than coated pits on cells expressing wild-type HA. HAG and HAgC appeared to locate preferentially in coated pits; on cells expressing these proteins coated pits were densely labeled even when surrounding areas of membrane contained little anti-HA antibody. (*b*) When cells expressing HA, HAG, or HAgC were treated with levels of chloroquine that allow delivery of proteins to endosomes but that interfere with further transport, the chimeric proteins accumulated in intracellular vesicles. By contrast, chloroquine treatment of cells expressing wild-type HA had no effect on the location of that protein. (*c*) When the surfaces of cells expressing HA, HAG, or HAgC were iodinated at a temperature that prevented endocytosis (61) and the cells were then incubated for 5 min at a temperature which permitted internalization, 13% of HAG and 7% of HAgC became inaccessible to digestion by trypsin. Under the same conditions, HA remained completely sensitive to trypsin even after extended periods of incubation at a temperature permissive for endocytosis. The rate at which HAG and HAgC acquired protection from trypsin was similar to the rates of internalization reported for many cell surface receptors (6, 25, 57), and a simple interpretation of this result is that internalization of the chimeric glycoproteins protected them from trypsin in the culture medium.

The rate of internalization of HAG would be sufficient for the uptake of the entire surface population of that protein within 1 h. Yet in the absence of chloroquine treatment, there was no observable accumulation of HAG in vesicles within the cell and the vast majority of HAG was present at the plasma membrane. Moreover, the rate of degradation of [^{35}S]methionine-labeled HAG was slow, similar to that of wild-type HA (the half-life of HAgC was noticeably shorter). Thus, internalized HAG did not appear to be targeted to lysosomes. It is reasonable to assume that HAG was recycling to the cell surface. We conclude that the chimeric glycoproteins HAG and HAgC are specifically routed into the endocytic pathway and that HAG, at least, mimics a cell surface receptor in recycling to the cell surface.

We do not know whether changes in the ectodomains of HAgC and HAG result in their internalization, perhaps through increased clustering of those molecules, or whether the transmembrane and/or cytoplasmic domains of the G protein and the gC protein contain features that direct the HA ectodomain into coated pits. However, several recent observations suggest that the latter possibility is the more likely. VSV G protein has been reported to be internalized and to recycle when implanted into the cell surface by the fusion of the viral envelope with the plasma membrane (40, 46–48). In recent experiments in which G protein was ex-

pressed from cloned DNA in transfected cells, chloroquine treatment caused the accumulation of G in vesicles (Gottlieb, T., M. Rindler, and D. D. Sabatini, personal communication). Thus, in the case of HAG an activity of G protein appears to be transferred to the chimera. For HAgC the situation is less clear, since the HSV-1 gC protein expressed in the absence of other HSV-1 viral proteins did not appear to reach the cell surface (61) and endocytosis of gC has not been reported.

The HA glycoprotein, whether expressed from SV40 vectors during lytic infection of CV-1 cells (18) or from bovine papilloma virus vectors in continuous mouse cell lines (54), has a long residence time on the cell surface (Copeland, C., A. Helenius, I. Mellman, and J. Hearing, personal communication). In CV-1 cells <5% of HA was internalized per hour, whereas internalization of plasma membrane through coated pits occurs at a rate 10 to 15 times greater (2, 38, 59). Thus HA was excluded from internalization by coated pits. The failure of HA to enter the endocytic pathway could not be caused by HA binding to sialic acid residues on cell surface glycoproteins which were themselves excluded from coated pits. HAG and HAgC both contain the HA ectodomain and both bound sialic acid on erythrocytes as well as does HA, and yet they were efficiently internalized. Rather than being prevented from entering coated pits, it is possible that HA lacks features that allow it to be trapped by elements of the pits. The inefficient uptake of HA would thus be due to the relatively brief residence time for HA in the coated pit.

It is important to mention that the three-dimensional structure for HA was solved only for the HA ectodomain, which had been isolated as a bromelain cleavage fragment lacking the 10 carboxy-terminal residues of the ectodomain, the transmembrane domain, and the cytoplasmic domain (68). The 10 amino acids of the ectodomain proximal to the hydrophobic transmembrane domain do not appear to be important for HA structure, since a mutant HA, HA351, which retains only five of these residues, is wild-type by every test at our disposal. The roles played by the transmembrane and cytoplasmic domains of HA in maintaining the structure of the HA ectodomain are not well understood. The effect of alteration or deletion of the HA cytoplasmic domain was recently investigated; removal of this domain doubled the average time required for newly synthesized Japan HA to reach the cell surface but was not demonstrated to have other effect (unpublished results). Substitution of the HA cytoplasmic domain with that of another glycoprotein had no observable effect on HA function (12). The present report extends these observations to HAs containing foreign transmembrane as well as cytoplasmic domains. Although HAgC moves through the exocytic pathway more slowly than HA (21) HAG was transported to the cell surface as rapidly as the wild-type protein (manuscript in preparation) and was turned over with the same, slow kinetics. HAG and HAgC both retained the biological activities of the HA molecule. Thus in major respects two different transmembrane domains could substitute for that of HA. However, this substitution was not complete, since at the cell surface the chimeric proteins were recognized by an antiserum that binds epitopes that are cryptic on terminally glycosylated wild-type HA. HAG and HAgC at the plasma membrane were also recognized by a monoclonal antibody that reacts solely with the mature form of the wild-type HA and does not bind to the denatured glycoprotein. Both antisera recognized the chimeric glycoproteins that accumulated in vesicles in chloroquine treated cells. The fact that no loss of biological function was observed for the chimera, and in the case of HAG, no increase in the rate of degradation, argues against the existence of two separate populations of HAG and HAgC at the cell surface. Thus, the antigenic difference between the chimera and HA could be due to increased flexibility in the chimera, transiently exposing antigenic sites, or might reflect some subtle, more permanent difference in the shape of their ectodomains.

It is attractive to speculate that proteins destined to be internalized might contain some feature that is recognized by elements of coated pits. Goldstein et al. have discussed the possibility that the cytoplasmic domains of cell surface receptors might contain a specialized feature or sequence that is recognized by a component of the clathrin coat of an endocytic pit (22). Pearse and Bretscher have proposed that recognition could occur between the ectodomain of a receptor and that of an adaptor protein which in turn is recognized on the cytoplasmic side of the pit by an element of the clathrin coat (45). Recently Lehrman et al. have reported results suggesting that the cytoplasmic domain of the LDL receptor contains features important for the internalization of that protein (33). Although topographically the glycoproteins described in the present report resemble cell surface receptors such as the LDL receptor, they have much smaller cytoplasmic domains. If there were a highly conserved signal required for efficient localization into coated pits, one might assume that it would be more obvious in a context of <30 amino acids. Although the sequences of the cytoplasmic domains of the gC and G proteins are both highly charged compared to the same region of HA (see Fig. 1), they are quite different from one another and do not resemble the cytoplasmic domains of cell surface receptors known to be internalized through coated pits (13, 14, 42, 66, 70). Interestingly, when analyzed by the hydropathy program of Kyte and Doolittle (32), the transmembrane domains of both gC and G resemble the very hydrophobic and relatively short transmembrane domains of the cell surface receptors. By the same analysis the transmembrane domains of several subtypes of HA are longer and less hydrophobic than those of surface receptors, G, or gC. It is conceivable that transmembrane domains of receptor proteins pack into some ordered array within a coated pit which greatly increases the probability that those proteins will be internalized. In a sense, the macromolecular structure of the pit itself would function to "recognize" those proteins destined for endocytosis.

Our experience indicates that the HA transmembrane or cytoplasmic domain can be substituted with that of another glycoprotein with minimal effect on the HA ectodomain. Using the techniques of site directed mutagenesis and further gene splicing, we will be able to construct a series of chimeric HAs containing progressively fewer foreign amino acids and in this way locate sequences that direct the HA ectodomain into the endocytic pathway. Knowledge of the topology of the interaction that specifies the internalization of a protein will in turn have important implications for understanding the mechanisms controlling endocytosis through coated pits.

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