Endocytosis of Chimeric Influenza Virus Hemagglutinin Proteins That Lack a Cytoplasmic Recognition Feature for Coated Pits

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Abstract. The influenza virus A/Japan/305/57 hemagglutinin (HA) can be converted from a protein that is essentially excluded from coated pits into one that is internalized at approximately the rate of uptake of bulk membrane by replacing the HA transmembrane and cytoplasmic sequences with those of either of two other glycoproteins (Roth et al., 1986. *J. Cell Biol.* 102:1271–1283). To identify more precisely the foreign amino acid sequences responsible for this change in HA traffic, DNA sequences encoding the transmembrane (TM) or cytoplasmic (CD) domains of either the G glycoprotein of vesicular stomatitis virus (VSV) or the gC glycoprotein of herpes simplex virus were exchanged for those encoding the analogous regions of wild type HA (HA wt). HA-HA-G and HA-HA-gC, chimeras that contain only a foreign CD, resembled HA wt in having a long residence on the cell surface and were internalized very slowly. HA-HA-gC was indistinguishable from HA in our assays, whereas twice as much HA-HA-G was internalized as was HA wt. However, HA-G-HA, containing only a foreign TM, was internalized as efficiently as was HA-G-G, a chimeric protein with transmembrane and cytoplasmic sequences of VSV G protein. Conditions that blocked internalization through coated pits also inhibited endocytosis of the chimeric proteins. Although the external domains of the chimeras were less well folded than that of the wild type HA, denaturation of the wild type HA external domain by treatment with low pH did not increase the interaction of HA with coated pits. However, mutation of four amino acids in the TM of HA allowed the protein to be internalized, indicating that the property that allows HA to escape endocytosis resides in its TM. These results indicate that possession of a cytoplasmic recognition feature is not required for the internalization of all cell surface proteins and suggest that multiple mechanisms for internalization exist that operate at distinctly different rates.

As part of the process of communicating with their external environment, cells maintain an active traffic of membranes moving between the cell surface and internal organelles. Solutes are absorbed through fluid-phase endocytosis, nutrients and hormones are concentrated and internalized by receptor-mediated endocytosis, and resident proteins of the plasma membrane are removed for degradation in lysosomes. Cellular pathogens, like many viruses, use these endocytic processes to enter the cell. It is clear that there are at least several distinct pathways for this traffic (Watts and Marsh, 1992), but the exact number of pathways and the relations between them are not currently known. However, in cells that are not undergoing major changes in shape, such as those that occur during locomotion or phagocytosis, a major pathway for internalization of lipid and proteins occurs through coated pits.

Coated pits occupy 1 to 2% of the cell surface and internalize every 1 to 2 min, taking in membrane equivalent to 100 to 200% of the plasma membrane each hour (Anderson et al., 1977; Goldstein et al., 1979; Thilo, 1985; Pearse and Crowther, 1987; Naim et al., 1995). There are three classes of cell surface proteins with respect to internalization through coated pits. Certain membrane proteins, such as Thy-1 and the influenza virus hemagglutinin A/Japan/305/57 (HA),[1] are excluded from coated pits (Bretscher et

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1. *Abbreviations used in this paper*: CV-1, African Green Monkey kidney; VSV, vesicular stomatitis virus; HA, influenza virus A/Japan/305/57 hemagglutinin; G, VSV G glycoprotein; gC, herpes simplex virus-1 glycoprotein C; TM, transmembrane domain; CD, cytoplasmic domain; HA wt, HA wild type; HA-HA-G, HA chimera with a G protein cytoplasmic domain; HA-G-HA, HA chimera with a G protein transmembrane domain; HA-G-G, HA chimeric protein with both TM and CD derived from G. Chimeric proteins with gC sequences are named analogously. The names for HAs with amino acid mutations contain the single letter code for the replacement amino acid, and its position in the HA sequence.

al., 1980; Roth et al., 1986; Miettinen et al., 1989; Lemanski et al., 1990, Keller et al., 1992) and are internalized 40 times more slowly than is the bulk of the plasma membrane (Roth et al., 1986; Lazarovits and Roth, 1988). At least some of these excluded proteins are highly mobile on the cell surface (Ishihara et al., 1987; Fire et al., 1991, 1995). Other proteins, such as certain cell surface receptors, appear to cluster in coated pits and are endocytosed at a rate many times faster than is the plasma membrane as a whole (Goldstein et al., 1985). A third group of proteins are internalized more slowly, at close to the rate of internalization of membrane surface by coated pits (Watts and Marsh, 1992). The mechanism by which coated pits achieve this selectivity is not understood precisely. Recent studies of receptor-mediated endocytosis have demonstrated convincingly that the cytoplasmic domains (CD) of many proteins contain one of at least three distinct small epitopes necessary for interaction with coated pits. The best studied of these signals has at least one aromatic residue (Collawn, 1990; Ktistakis et al., 1990; Canfield et al., 1991) and is likely to interact with clathrin-associated proteins (Pearse, 1988; Sorkin and Carpenter, 1993; Sosa et al., 1993; Boll et al., 1995; Ohno et al., 1995). A second type of signal requires a di-leucine (Letourneur & Klausner, 1992; Sandoval and Bakke, 1994) and the third requires a di-lysine motif at the third and fourth positions from the carboxyl terminus (Itin et al., 1995). However, most cell surface proteins are degraded in lysosomes, and not all of them contain cytoplasmic signals of the types currently identified.

Of the few proteins demonstrated to be "excluded" from coated pits, HA is by far the best characterized. To investigate the mechanisms by which cell surface proteins are recognized by coated pits, we have introduced specific changes into the HA protein that will allow (or cause) HA to enter coated pits. In previous experiments, the transmembrane domain (TM) and CD of two different proteins, the vesicular stomatitis virus (VSV) G glycoprotein and the herpes simplex virus-1 (HSV-1) gC glycoprotein, were substituted by genetic engineering for the analogous regions of HA (Roth et al., 1986). The resulting chimeric proteins, HAG and HAgC, were both internalized and were detected by immunocytochemistry and electron microscopy in coated pits. However, more recent work has shown that the VSV G protein internalizes very slowly, although it does contain a cytoplasmic tyrosine in a sequence context similar to that found in internalization signals (Gottlieb et al., 1993; Thomas et al., 1993; Thomas and Roth, 1994). In contrast, the short CD of the gC glycoprotein of herpes simplex I virus is highly basic, does not include an aromatic residue, and does not resemble the CD of any other protein known to be internalized. To investigate which sequences are responsible for allowing the HAG and HAgC proteins to be internalized, we have constructed new chimeric HAs that contain only a single foreign topological domain. These proteins have been expressed in CV-1 cells infected with recombinant SV40 virus vectors and their ability to be internalized has been measured. We find that the VSV G protein TM, when substituted for the HA domain, causes the resulting chimeric protein to be internalized. Mutations of some residues, but not others, in the HA TM also allow the protein to be internalized. The property of HA that normally allows it to be excluded from coated pits resides in its TM sequences.

Materials and Methods

Recombinant DNA Techniques

Chimeric genes, and the HA-G-G^{tyr-} point mutation, were constructed by the two primer method of Zollar and Smith (1985) and genes were subcloned into SV40 vectors and stocks of recombinant viruses prepared as described (Naim and Roth, 1994*a*). HA mutants with changes in the TM were constructed by megaprimer PCR mutagenesis (Sarkar and Sommer, 1990). Enzymes used were from New England Biolabs Inc. (Beverly, MA), Bethesda Research Laboratories (Gaithersburg, MD), or United States Biochem. Corp. (Cleveland, OH) and were used according to instructions provided by the manufacturer.

Cell Culture Techniques

CV-1 fibroblasts were obtained from American Type Culture Collection (Rockville, MD) and passages between 22 and 32 were used for experiments. Cells were maintained in Dulbeccos' Modified Eagles' Medium supplemented with 10% serum plus (Hazleton Biologics, Inc., Lenexa, KS). Immunofluorescence techniques, pulse–chase protocols, and PAGE were as described (Roth et al., 1986; Lazarovits and Roth, 1988).

Assays for Internalization

The major internalization assay employed was as described previously (Lazarovits and Roth, 1988; Naim and Roth, 1994*b*). At 28 h postinfection with recombinant SV40 vectors, cells expressing chimeric proteins were pulse-labeled with 10–30 μCi Trans^{35}S label (ICN Biomedicals, Inc., Irvine, CA) and the proteins were chased to the cell surface. Heat-inactivated rabbit anti-HA serum was allowed to bind to HAs at the cell surface at 4°C, and after unbound antibody was washed away, cells were warmed to 37°C for various intervals. Cells were returned to 4°C, and HAs at the cell surface were cleaved into HA1 and HA2 by trypsin added to culture medium at a final concentration of 50 μg/ml. After 30 min, trypsin was removed by washing and any residual trypsin was inactivated with 10-fold excess soybean trypsin inhibitor (Sigma Immunochemicals, St. Louis, MO). Cells were lysed with 1% NP40, 0.1% SDS, 0.1 unit/ml aprotinin (Boehringer Mannheim Corp., Indianapolis, IN), 50 μg/ml soybean trypsin inhibitor, 50 mM Tris-HCl, pH 8.0, and immunoprecipitated with protein A-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden). Immunoprecipitates were separated by electrophoresis on 12.5% polyacrylamide gels (Laemmli, 1970) and treated for fluorography (Bonner and Laskey, 1974) if quantified by densitometer, or were untreated if quantified by PhosphorImager. The dried gels were either exposed to preflashed XAR-5 film (Kodak, Rochester, NY) at −80°C and quantified by laser scanning densitometry or were exposed to PhosphorImager plates (Molecular Dynamics, Sunnyvale, CA).

For internalization assays using iodinated antibody, monoclonal anti-HA antibody (Fc125, a gift from Dr. Thomas Braciale, University of Virginia) from ascites fluid was concentrated on Protein A columns and then iodinated with Iodobeads (Pierce, Rockford, IL) according to the instructions of the manufacturer. The iodinated antibody was added to culture medium to a final concentration of 2 μg/ml at 37°C for various intervals. Uninfected CV-1 cells were treated in parallel to determine nonspecific binding. For each time point, one half of the samples were incubated for 30 min at 23°C with 0.2 M acetic acid in 150 mM NaCl to remove antibody bound to the cell surface. The remaining samples were left in PBS at 4°C during this incubation. All samples were rinsed twice with fresh incubation buffer and were lysed with immunoprecipitation lysis buffer. ^{125}I radioactivity was measured with a counter (model 1282; Pharmacia LKB Biotechnology). The average nonspecific radioactivity determined from uninfected cells for each treatment was subtracted from the values of the infected cells. The percent internal proteins was determined by dividing the average corrected acid-resistant counts by the average corrected unstripped counts × 100%. Duplicate values that differed by no more than 15% were used for each time point. Under the conditions employed, essentially no internalization of radioactive antibody was observed for the first 2 min after antibody was added to the medium. Internalization data are plotted starting at the end of this lag period.

Treatment of Cells with Hypertonic Medium or Low pH

For experiments measuring the degree of inhibition of endocytosis of chimeric HAs by hypertonic medium, cells were prepared exactly as for the usual internalization assay but were rinsed twice in cold DME containing 0.45 M sucrose for several minutes just before beginning the 37°C internalization chase in the same medium. At the end of the chase, cells were processed as usual. Fluid-phase endocytosis was measured by the internalization of horseradish peroxidase that had been added to the culture medium for 30 or 60 min, as described (Wang et al., 1990).

To examine the effect of denaturing the HA ectodomain on its interaction with coated pits, CV-1 cells were treated with 5 μg/ml of trypsin in serum-free DME for 5–10 min at 37°C. This resulted in cleavage of HA at the cell surface into HA1 and HA2 subunits, which is necessary to allow the protein to undergo the conformational changes at low pH that result in membrane fusion. The medium containing trypsin was removed and cells were rinsed twice for 1 min at 37°C with either DME buffered to pH 7.0 with 10 mM Hepes, or with low pH buffer (PBS containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM MES, pH 4.8–5.0). Cells were then cultured at 37°C for 60 min in DME containing ~25 μg/ml rabbit anti-HA antibody. Cells were cooled on ice and then fixed at room temperature with 3% formaldehyde in PBS, pH 7.0. The fixative was removed and quenched with DME. The samples were then stained with goat anti–rabbit IgG conjugated to FITC (40 μg/ml in PBS; Fisher Biotech, Pittsburgh, PA) to label anti-HA antibody bound to the cell surface. Samples were then permeabilized with 0.1% Triton X-100 in 50 mm Tris, pH 7.5, 150 mM NaCl, 5 mM EGTA, 0.25% gelatin for 15 min at room temperature. Samples were labeled a second time with 40 μg/ml goat anti–rabbit IgG conjugated to RITC (Fisher Biotech) to label any anti-HA that had been internalized. Samples were photographed with a microscope (Axioplan; Carl Zeiss, Inc., Thornwood, NY) using Kodak T-Max 3200 film.

Photobleach recovery experiments were performed as previously described (Fire et al., 1991, 1995) on living cells that had been treated with trypsin and low pH buffer as described above.

Results

Construction of Chimeric HAs

Although crystallographic analysis has established the three-dimensional structure of a large proteolytic fragment that includes almost all of the external domain of HA (Wilson et al., 1981), the structures of the other two topological domains of the protein have not been solved. The boundaries of the TM region of the HA can be predicted by a hydropathy program (Kyte and Doolittle, 1982) and by comparison to sequences of HAs from other influenza virus strains. We have used such an analysis to predict that glutamine 510 is the last polar residue of the external domain (in other HA strains this amino acid is aspartic acid) and that asparagine 538 is one of the first polar residues of the CD (other HA strains have an arginine 538). Based on these assumptions, we employed oligonucleotide-directed mutagenesis (Zollar and Smith, 1985) to substitute DNA sequences encoding topological domains of G protein or of gC for those encoding the analogous regions of HA wt. The carboxy-terminal sequences for the resulting chimeric HA proteins are presented in Fig. 1. The proteins HA-G-G and HA-gC-gC differ from the original HAG and HAgC proteins (Roth et al., 1986) in that the position joining the HA ectodomain sequences to the foreign TM in the newer proteins is between the last polar residue of HA and the first hydrophobic foreign residue. The original chimeric proteins were created by fusions that introduced foreign sequences into the HA ectodomain. We have not observed significant differences in stability or membrane traffic between the original chimeras HAG and HAgC and the new proteins HA-G-G and HA-gC-gC, indicating that the foreign sequences in the ectodomains of HAG and HAgC played no role in allowing them to be internalized.

Protein	Ecto	Transmembrane	Cytoplasmic
HA wt	MGVYQ	ILAIYATVAGSLSLSIMMAGISFWMCS	NGSLQCRICI
HA-HA-gC	MGVYQ	ILAIYATVAGSLSLSIMMAGISFWMCS	RTSQRQRHRR
HA-HA-G	MGVYQ	ILAIYATVAGSLSLSIMMAGISFWMCS	RVGIHLCIKLKHTKKRQIYTDIEMNRLGK
HA-G-G	MGVYK	SSIASFFFIIGLIIGLFLVL	RVGIHLCIKLKHTKKRQIYTDIEMNRLGK
HA-G-HA	MGVYK	SSIASFFFIIGLIIGLFLVL	NGSLQCRICI
HA-gC-gC	MGVYE	WVGIGIGVLAAGVLVVTAIVYVV	RTSQRQRHRR
HA-gC-HA	MGVYE	WVGIGIGVLAAGVLVVTAIVYVV	NGSLQCRICI

Figure 1. Amino acid sequences of chimeric HAs. The amino acid sequences of the transmembrane and cytoplasmic regions of chimeric glycoproteins are predicted from nucleic acid sequences. The sequences are aligned at the junction between the ectodomains and TMs, at the last polar amino acid of the extracellular domain. The three-part names of the proteins give the origin of the external–transmembrane–cytoplasmic domains.

Each chimeric protein was independently constructed and fully characterized at least twice to insure that our experimental results were not influenced by second-site mutations occurring during subcloning steps. Each of the recombinant HA genes was inserted in place of the late transcriptional unit of SV40 under control of the SV40 late promoter. Stocks of recombinant viruses were prepared that produced comparable levels of HA protein synthesis in infected CV-1 monkey cells. We have reported the results of an extensive comparison of the biosynthesis and the physical properties of the chimeric and HA wt (Lazarovits et al., 1990). Independently constructed isolates of each of our mutants gave identical results in all assays.

Transmembrane Sequences of Either G or gC Allow Internalization of Chimeric HAs

To determine if the chimeric proteins were internalized and at what rate, HAs at the surface of living cells were bound by anti-HA antibodies at 4°C and the cells were warmed to 37°C to allow endocytosis to resume (Lazarovits and Roth, 1988; Ktistakis et al., 1990; Naim and Roth, 1994*b*). Cells were chased for various periods at 37°C to allow internalization of cell surface proteins, and the extent of internalization was measured by the proportion of protein that became inaccessible to extracellular trypsin added at 4°C at the end of the chase. The results of densitometric scanning of autoradiographs from representative experiments are graphed as a function of time in Fig. 2. Curves were fitted to the experimental data using second-order polynomials (Cricket Software, Malvern, PA) and the internalization rates shown were taken from the initial slope of the curve. As a positive control in our assay, we included the HA-Y543 mutant HA, which is internalized at a rate of 8%/min, a rate similar to that reported for Fc receptors in CHO cells (Miettinen et al., 1989). The initial rate of internalization observed for the chimeras HA-G-G (1.8%/min) and HA-gC-gC (2.4%/min) agreed quite well with the rates measured for the original HAG and HAgC mutants by cell surface iodination (Roth et al., 1986). The HA-G-HA protein (2.1%/min) was internalized as effi-

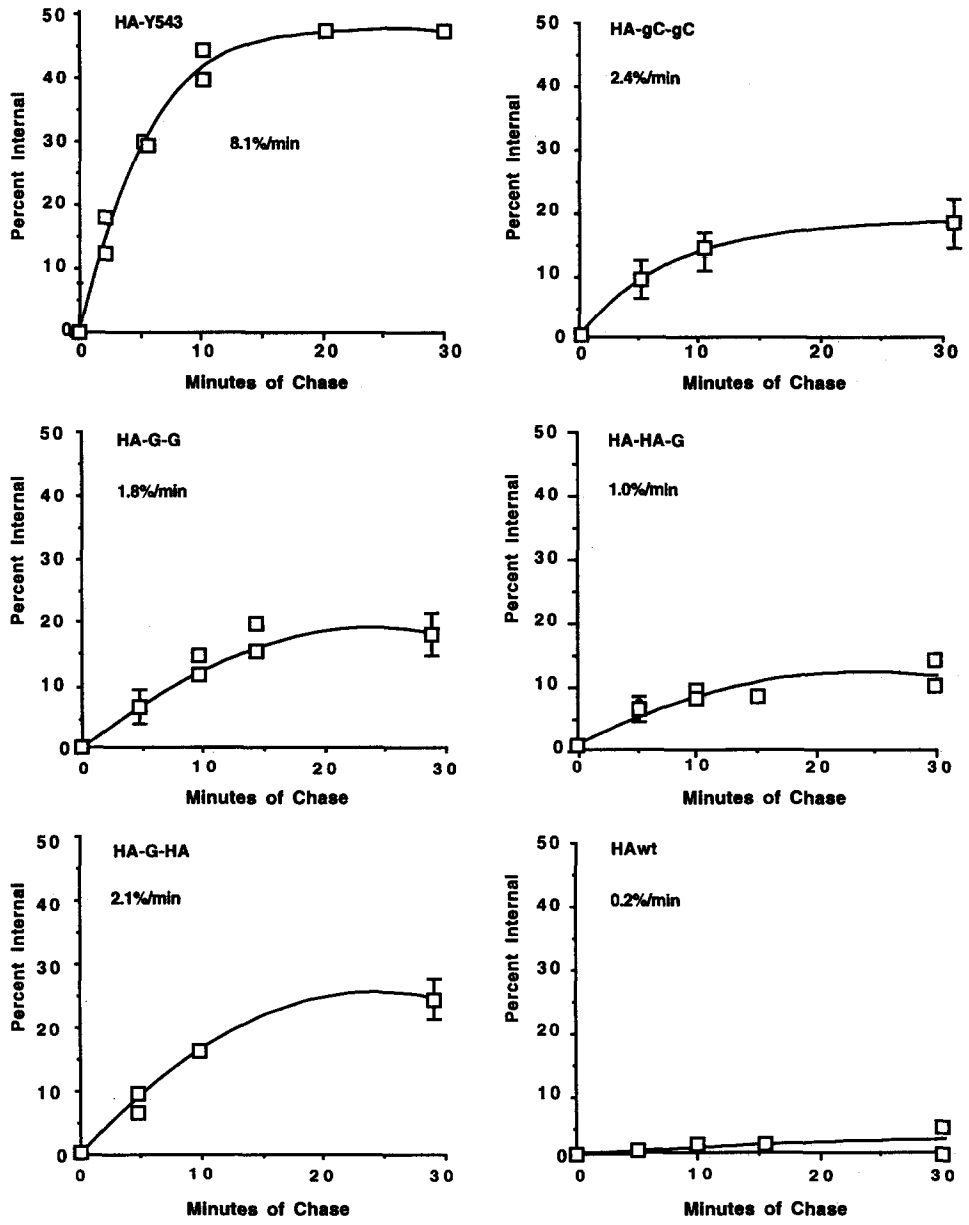


Figure 2. Rates of internalization of HA wt, HA-Y543, and chimeric HAs. Internalization of ^{35}S-labeled proteins was measured as described in Materials and Methods, using laser scanning densitometry. For each protein, the percentage of protein inaccessible to extracellular trypsin is plotted as a function of the interval at which cells were shifted to 37°C. Curves were fitted to the experimental data using second-order polynomials and used to derive the internalization rates shown. For duplicate experiments, both data sets are shown. Error bars indicate standard deviations for averages of three or more experiments. For data points with single symbols, the error bars are smaller than the symbol or duplicate values are superimposed.

ciently as was HA-G-G, and the HA-HA-G protein was internalized at half the rate of the other chimeric proteins. This slow rate, however, was much faster than the rate of internalization of HA wt (0.2%/min). The HA-gC-HA protein was internalized quite slowly (see Fig. 7), and thus the gC TM sequences, in the presence of the HA CD, did not affect HA to the same extent as did the G TM sequence in HA-G-HA. The HA-HA-gC protein was internalized as slowly as was HA wt (not shown). Internalization of all of the proteins appeared to reach a maximum rate by 10 min (Fig. 2).

The internalization assay used for the experiments shown in Fig. 2 employed trypsin to cleave protein at the cell surface at the end of the assay. However, the HA-G-HA protein had previously been shown to be protease sensitive (Lazarovits et al., 1990), in contrast to most other HA mutants, which are very resistant to digestion except at the HA1-HA2 cleavage site. To determine the internalization of HA-G-HA by a method independent of trypsin, we added ^{125}I-labeled monoclonal anti-HA to cell culture medium at 37°C and measured the internalization of antibody as a function of time. A representative experiment is shown in Fig. 3, comparing internalization of the antibody by cells expressing HA-G-HA and cells expressing the HA-Y543 mutant. Internalization of HA-Y543 measured by this method, 7%/min, was slightly slower than measured by the assay shown in Fig. 2 and internalization of HA-G-HA, 3%/min, was slightly faster. However, HA-G-HA internalized slowly by both assays, at less than half the initial rate of HA-Y543.

The internalization of chimeric HAs shown in Figs. 2 and 3 appeared to reach a maximum after 10 to 15 min, as is commonly observed for proteins that recycle to the plasma membrane after internalization. If one assumes

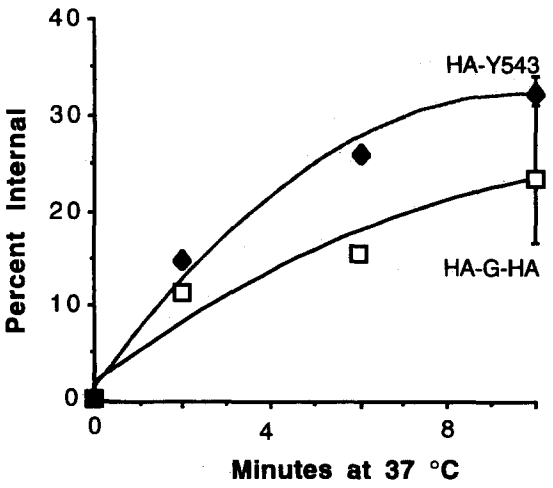


Figure 3. Internalization of ^{125}I-labeled monoclonal anti-HA antibodies by cells expressing HA-G-HA or HA-Y543. The percentage of antibody that was internalized, as represented by the fraction of the total radioactive antibody bound that was acid-insensitive, is plotted as a function of chase time at 37°C. Curves were fitted to the data using second-order polynomials.

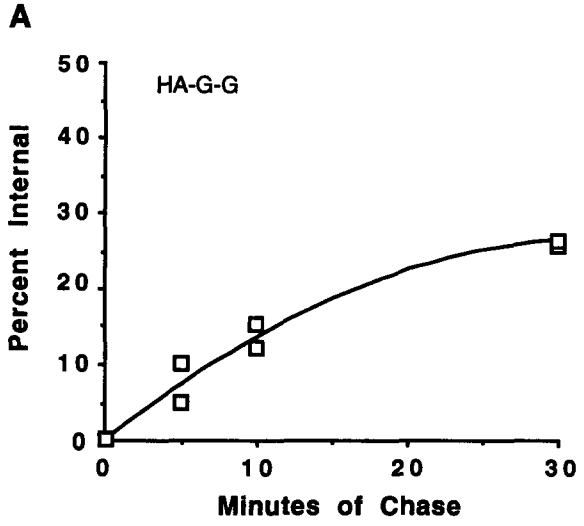
that each of the proteins recycled back to the cell surface after internalization, so that the plateau values of the internalization curves shown in Fig. 3 represent the steady-state internal fraction of the cell surface population of each protein, then one can estimate a recycling rate for each protein using the relationship that the recycling rate is equal to the internalization rate multiplied by the fraction of protein at the plasma membrane divided by the internal fraction. The recycling rates for all of the chimeric proteins and HA-Y543 were quite similar, averaging 8%/min with a standard deviation of 1.3%/min. This is the same recycling rate calculated for several HA mutants that contain internalization signals and bind with high affinity to coated pits (Zwart et al., 1996). It is difficult to directly measure the recycling of the chimeric HA's because they have a small internal fraction (the plateau values shown in Fig. 2) and do not release their antibody ligands after externalization. As an alternative for confirming that the entire population of a chimeric HA was internalized and recycling, we blocked protein synthesis with 500 μM cycloheximide and measured the internalization of ^{125}I-labeled anti-HA antibody by cells expressing HA-G-HA after 1 or 4 h in the drug. If HA-G-HA failed to recycle efficiently, any HA-G-HA capable of internalizing at the rate shown in Fig. 2 would have been removed from the cell surface by 4 h after protein synthesis had ceased, and no additional internalization would be detected at the second time point. In fact, we observed that 10% of HA-G-HA was internalized in 5 min after 1 h in cycloheximide and 6.8% was internalized in 5 min after 4 h in cycloheximide. The amount of antibody bound to cells expressing HA-G-HA at the 4-h time point was 47% that bound at the 1-h time point. These observations are consistent with continued slow internalization of HA-G-HA with recycling to the cell surface and a degradation rate that is much slower than the internalization rate.

The Single Tyrosine in the Cytoplasmic Domain of HA-G-G Is Not Required for Internalization

The observation that HA-G-HA was internalized as efficiently as HA-G-G suggested that the G TM had a dominant effect on HA causing or allowing the protein to be internalized. However, the fact that HA-HA-G had a significantly greater rate of internalization than did HA wt or HA-HA-gC could be interpreted as indicating that the G CD did contain some information for internalization and that HA-G-HA and HA-G-G were being internalized by separate mechanisms. Since the CD of G contains a single tyrosine, and cytoplasmic tyrosines have been shown to be important for recognition by coated pits, we changed the tyrosine in the CD of HA-G-G to serine and measured the effect of this mutation on the rate of internalization of the mutant protein HA-G-G^{tyr-} (Fig. 4). The change of the G protein cytoplasmic tyrosine to serine had no significant effect on internalization of HA-G-G.

Internalization of HA-G-HA and HA-gC-HA Is Blocked by Hypertonic Medium

The original HAG and HAgC chimeras were observed in coated pits by immunocytochemistry and electron microscopy (Roth et al., 1986). Since HA-G-G and HA-G-HA were internalized at the same rate as HAG and apparently shared a recognition mechanism dependent upon the presence of G TM sequences in the chimeric protein, it seemed likely that both these chimeras were also internalized through coated pits. However, alternative pathways of endocytosis have been reported (Daukus and Zigmond, 1985; Moya et al., 1985; Sandvig et al., 1987). To determine whether an alternative endocytic pathway existed in CV-1 cells and the extent to which the internalization of HA-G-G and HA-G-HA might occur by such a pathway, we measured the extent to which endocytosis was blocked in CV-1 cells treated with hypertonic medium or cytochalasin D. Medium that is 0.45 M hypertonic prevents the formation of normal clathrin lattices (Heuser and Anderson, 1989) and has been reported to block receptor-mediated but not fluid-phase endocytosis in leukocytes (Daukus and Zigmond, 1986). In fibroblasts, hypertonic medium also blocks most of fluid-phase internalization (Heuser and Anderson, 1989). Cytochalasin D has been reported to inhibit nonclathrin-dependent internalization (Sandvig and van Deurs, 1990) but not internalization through coated pits. Since cells appear to differ in the extent to which nonclathrin-mediated endocytosis occurs, we determined the effect of hypertonic medium and cytochalasin D on CV-1 cells by measuring the effect of these agents on fluid-phase endocytosis of horseradish peroxidase. In three experiments, hypertonic medium inhibited 91.3 ± 0.5% of fluid-phase endocytosis in CV-1 cells, indicating the presence of a minor internalization pathway that was presumably clathrin-independent. We obtained qualitatively similar results using FITC-dextran and fluorescence microscopy (data not shown). In contrast to the results with hypertonic medium, which were similar to previous reports in other cell types, we observed that treatment with 10 μg/ml of cytochalasin D increased fluid-phase internalization in CV-1 cells more than twofold (a mean of 230.7 ± 15.4% of control in three experiments). Thus, cytochalasin D could not



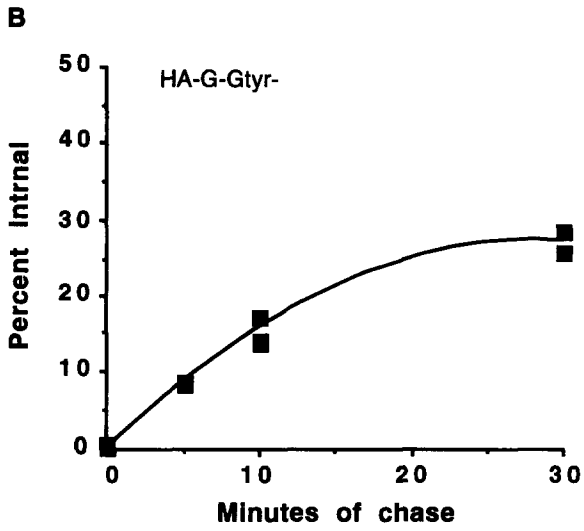


Figure 4. The rates of internalization of HA-G-G and HA-G-G^{tyr-} are compared. Internalization was measured and the data plotted as for Fig. 2. Values from duplicate experiments are shown.

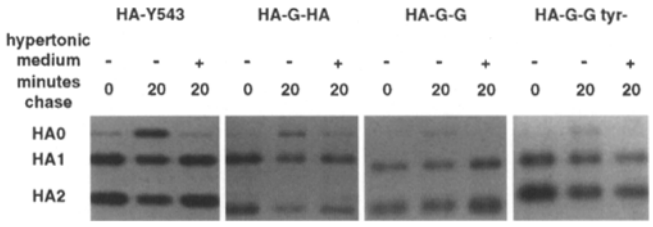


Figure 5. Internalization of HA chimeras with foreign TMs is blocked by hypermolar medium. Anti-HA antibody was added at 4°C to living cells expressing chimeric HAs and the cells were then held at 4°C or chased for 20 min at 37°C in normal medium or medium containing 0.45 M sucrose. At the end of the chase, cells were treated with cold medium containing trypsin to cleave HAs at the cell surface into HA1 and HA2 subunits. The proteins were immunoprecipitated with protein-A Sepharose and analyzed by PAGE. Endocytosis of the proteins is measured by the increase in the uncleaved HA0 polypeptide as a function of chase at 37°C.

be used as an inhibitor of nonclathrin-mediated endocytosis in CV-1 cells.

To determine the extent to which the slow internalization of chimeric HAs was inhibited by hypertonic medium, cells expressing HA wt or the chimeric HAs were pulse-labeled with ^{35}S amino acids and the proteins were chased to the cell surface. Anti-HA antibody was bound to the proteins at the cell surface at 4°C and the cells were then shifted for 0 or 20 min at 37°C in normal culture medium or in medium containing 0.45 M sucrose. The cells were returned to the cold and treated with trypsin, and HAs were isolated by immunoprecipitation and analyzed by PAGE (Fig. 5). Treatment with hypertonic medium completely blocked the internalization of the HA-Y543 mutant HA, a protein that we have observed to enter coated pits, and also inhibited the internalization of the chimeric HA proteins. Approximately 10% of HA-G-HA continued to be internalized in hypertonic conditions, suggesting that the mutant protein was internalized by both pathways but that the pathway sensitive to hypertonic conditions was the principal route of internalization. Thus, the slow internalization of the chimeric proteins did not appear to be due to internalization by a minor pathway unrelated to coated pits.

Disruption of the HA External Domain Does Not Cause Internalization

The proteins that were internalized best, HA-G-G, HA-G-HA, and HA-gC-gC, were those that had the most extensive changes in the folding of their external domains (Roth et al., 1986; Lazarovits et al., 1990). Thus, one mechanism by which the foreign TM might cause HA to be internalized might be through inducing changes in the HA external domain. To determine whether the structural integrity of the external domain of HA was required to keep the protein from being internalized, we purposely denatured HA at the surface of living CV-1 cells and measured the effects of this treatment on the interaction of the protein with coated pits. To denature the external domain of HA, we treated CV-1 cells expressing either HA or the HA-Y543 mutant with low pH to induce the proteins to undergo the large conformational change that activates them as fusion proteins (White and Wilson, 1987; Bullough et al., 1994). The lateral mobility of HA wt and HA-Y543 after acid treatment was measured by fluorescence photobleaching recovery, a technique that detects the dynamic interactions of more slowly internalized proteins with coated pits as a decrease in their diffusion coefficients (Fire et al., 1991, 1995). We have shown that the interaction of HA-Y543 with clathrin coats causes a two- to threefold reduction in the lateral mobility of the mutant compared to the HA wt (Fire et al., 1991). In cells treated with a pH 4.9 buffer for 15 min and then returned to pH 7.0 for mobility measurements, the difference between the lateral mobility of the HA wt and the HA-Y543 mutant remained unchanged (Table I). Thus, HA wt was not induced to interact with coated pits after pH-induced denaturation, nor did this conformational change prevent the HA-Y543 protein from interacting dynamically with coated pits. Qualitatively similar results were obtained by immunofluores-

Table I. Effects of Denaturation on Lateral Mobility of HA and HA-Y543

	D		Rf	
	Acid denatured	Untreated	Acid denatured	Untreated
HA	11.2 ± 1.2	11.7 ± 1.0	78 ± 5	86 ± 3
HA-Y543	5.2 ± 0.6	4.3 ± 0.6	82 ± 3	82 ± 4

D is the lateral mobility coefficient with units of 10^{-10} cm^2/s. R_f is the mobile fraction given in percent of labeled protein. Cells expressing HA or HA-Y543 were treated with pH 4.9 buffer for 15 min (White and Wilson, 1987) to denature the external domains of the proteins, or with pH 7.0 buffer to maintain native structure. Lateral mobility measurements were then made at pH 7.0 at 37°C as previously described (Fire et al., 1991). Values represent more than 30 independent measurements and are given with standard errors.

cence, in which cells expressing HA wt that had been treated with low pH were not observed to internalize anti-HA antibodies, whereas cells expressing HA-Y543 treated at low pH continued to internalize antibody (Fig. 6). The lack of surface labeling of acid treated cells expressing HA-Y543, compared to cells not acid treated (not shown), suggests that after internalization, acid treated HA-Y543 proteins were not recycling efficiently to the cell surface.

Specific Mutations in the HA TM Allow the Protein to Be Internalized

Replacing the HA TM with that of the G protein allowed HA to be internalized slowly, at approximately the rate of uptake of membrane lipid by coated pits in fibroblasts. One explanation for this observation would be the loss of some property of the HA TM that normally allows HA to avoid passive incorporation into coated pits. If so, then specific mutations in the HA TM might also change this feature, allowing HA to be internalized. Thus, we systematically mutated the HA TM by changing blocks of amino acids to alanine and measured the internalization rates of the mutant proteins. Many mutations had no effect on HA internalization (Naim and Roth, manuscript in preparation); however, mutation of four of the five amino acids at the position where the TM enters the outer leaflet of the lipid bilayer (positions 511, 512, 514, and 515) resulted in a protein that was internalized slowly, at 1.8%/min (Fig. 7). This rate was faster than internalization of the HA-gC-HA protein, which was internalized as slowly as the HA wt protein in the same experiment. Thus, the ability of the HA protein to be highly mobile on the cell surface but

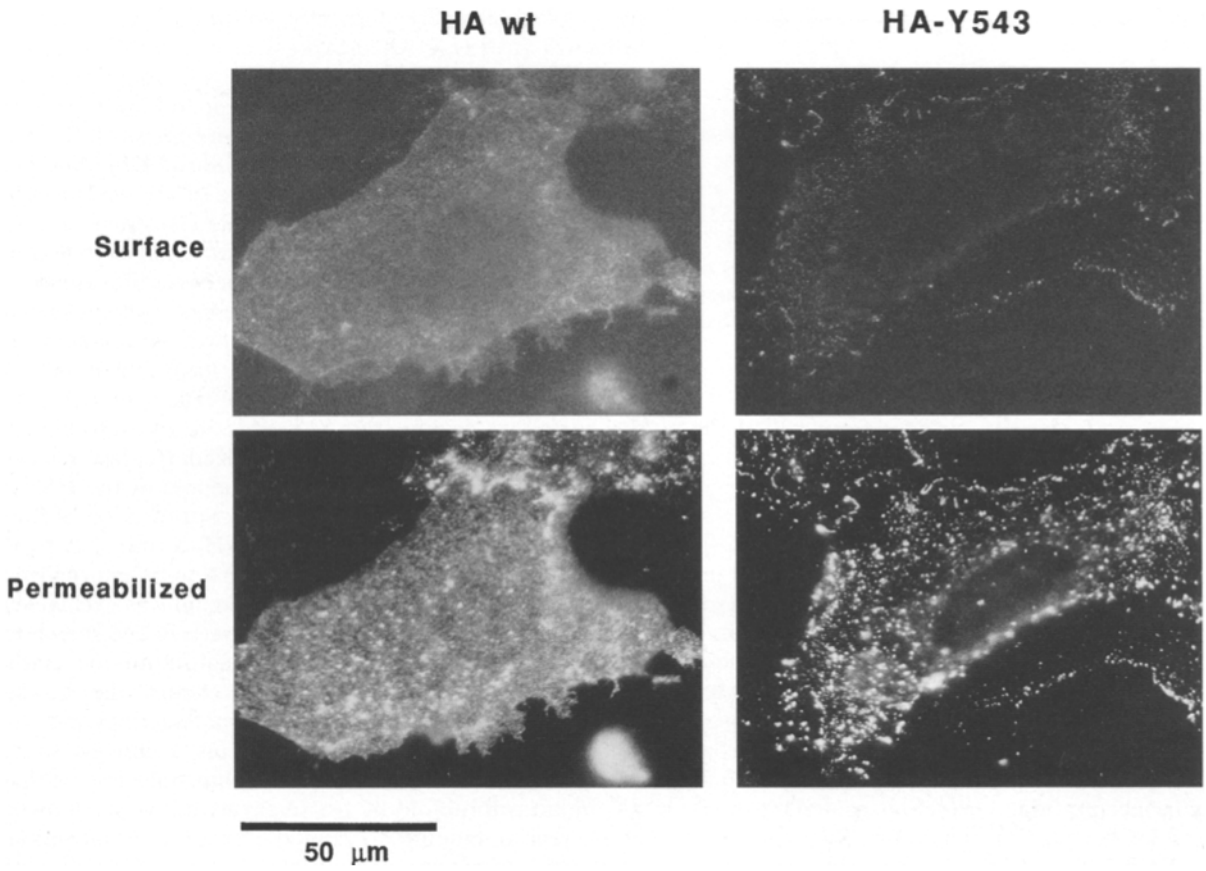


Figure 6. Denaturation of the HA external domain does not allow HA to be internalized. Cells expressing either HA wt or HA-Y543 were treated to cause the HAs to adopt their acid-induced conformation (White and Wilson, 1987). Rabbit anti-HA antibody was added to the cell culture medium for 60 min at 37°C. Cells were fixed and anti-HA present at the cell surface was labeled with goat anti–rabbit antibody conjugated to FITC. Cells were then permeabilized with detergent and internalized anti-HA was labeled with goat anti–rabbit-RITC. Acid denaturation did not induce the internalization of anti-HA by cells expressing HA wt nor did it prevent internalization of antibody by HA-Y543.

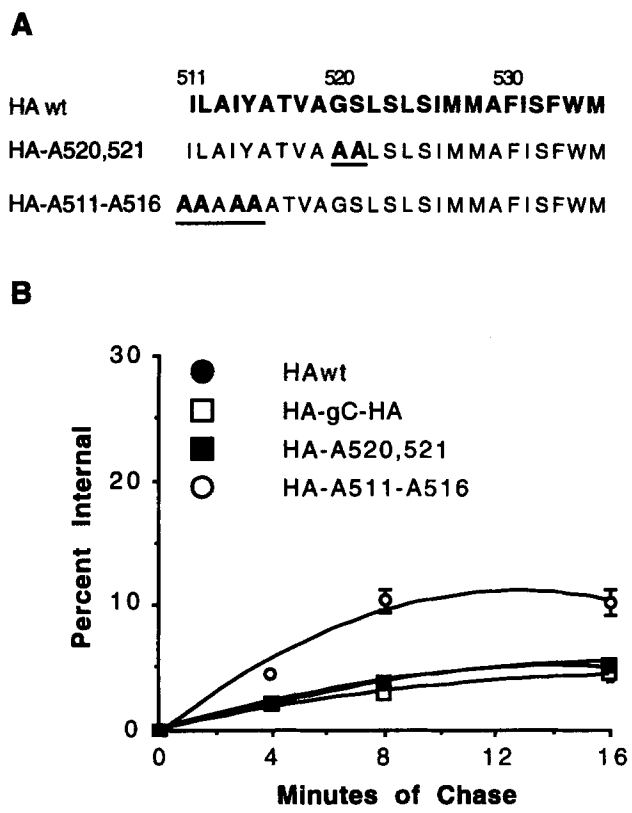


Figure 7. Mutation of four amino acids in the HA TM allows the protein to be slowly internalized. (*A*) The amino acid sequences of the TM of HA wt, HA-A520,521, and HA-A511-515 are shown. (*B*) Internalization of HA wt, HA-gC-HA, HA-A520,521, and HA-A511-515 are compared by the assay described in Fig. 2. Data was collected by phosphoimaging. Values are averages of two experiments. Error bars indicate the range of the two values. For symbols without error bars, the range of values is less than the size of the symbol.

nevertheless to be preferentially excluded from coated pits is due to some property of its TM.

Discussion

Recent work has convincingly established that efficient internalization of a variety of endocytic receptors and lysosomal membrane proteins depends upon a small secondary structure of the CD that appears to function in the recognition of the protein by coated pits. Three distinct cytoplasmic internalization signals have been identified. Although these signals differ in sequence and in the average rates at which they cause proteins to be internalized, all result in at least a twofold concentration of protein in coated pits relative to the rest of the plasma membrane, assuming that coated pits occupy 1–2% of the surface area (Anderson and Kaplan, 1983). However, the identified signals do not explain all of the internalization of proteins through coated pits. Verrey et al. (1990) replaced the CD of an endocytic receptor (the chicken hepatic lectin) with 11 amino acids of *Xenopus laevis* β-globin lacking any known signals and observed that the chimeric protein was still internalized through coated pits. Wiley and colleagues have observed that mutant EGF receptors unable to engage in high affinity binding to coated pits are nevertheless internalized by a low affinity, high capacity pathway (Lund et al., 1990). In each of these examples, the proteins are capable of being internalized by coated pits but exhibit little concentration by them, being internalized at rates close to the constitutive uptake of membrane.

This second, low affinity mechanism for internalization through coated pits is likely to be biologically important. Receptors for several viruses that require endocytosis for infection have been identified, and few of them are endocytic receptors that are efficiently internalized through coated pits (Marsh and Helenius, 1989). In addition, the majority of plasma membrane proteins are degraded by processes that are inhibited by reagents that raise the pH of lysosomes (Seglin, 1983), suggesting that endocytosis is responsible for clearing aged or damaged proteins from the cell surface. Since in many cell types internalization through clathrin-coated pits is the major pathway of endocytosis (Marsh and Helenius, 1980; Heuser and Anderson, 1989; Cosson et al., 1989), degradation of surface proteins in those cells probably requires an interaction with coated pits.

However, proteins that are internalized through coated pits without being concentrated in them are not just occupying available space. Recently, we have shown that the influenza virus HA, when expressed from a cDNA in the absence of influenza proteins, is highly mobile in the plasma membrane but does not appear to bind to coated pits, even when it is present at 5 million copies/cell (Fire et al., 1991; Naim and Roth, 1995; Fire et al., 1995). Another highly mobile protein, Thy-1 (Ishihara et al., 1987) is also apparently excluded from coated pits (Bretscher et al., 1980; Lemanski et al., 1990). The existence of mobile proteins, like HA, that are internalized an order of magnitude slower than the slowly internalized class of proteins means that the latter proteins must interact with components of the pit in some way. In this report, we have shown that replacement of the transmembrane sequences of HA with the analogous region from the VSV G glycoprotein allowed the chimeric HA to be internalized. Replacement of the HA TM with the analogous sequences of the HSV-1 gC glycoprotein had much less effect. Substitution of four bulky hydrophobic residues in the NH_2-terminal portion of the HA TM with alanine allowed HA to be internalized more rapidly than when the entire domain was exchanged with that of gC. At the positions of the HA TM shown to be important for preventing HA internalization, the analogous gC residues also have large side chains, whereas the G residues at the same positions are small (serines and alanines). Internalization of these proteins is unlikely to be due to changes in their external domains, since HA wt that was induced to unfold by treatment with low pH showed no increased binding to coated pits or internalization. Thus, the interaction responsible for the endocytosis of these proteins probably occurs within the lipid bilayer or in the cytoplasm.

Although we have not rigorously proven that all internalization of HA's with changes in the TM occurs through coated pits, there are several reasons why we believe that this is the case. Our evidence and previous reports (Doxey et al., 1987; Heuser and Anderson, 1989; Cosson et al., 1989) indicate that clathrin-mediated endocytosis is likely

to be responsible for most of the internalization of membrane in this cell type. We have shown that the CV-1 cells used in our experiments have only a minor pathway for fluid-phase internalization that continues to operate in hyperosmotic conditions that inhibited endocytosis of both HA-Y543, a mutant HA known to enter coated pits (Lazarovits and Roth, 1988; Ktistakis et al., 1990), and the chimeric HAs. Thus, the minor, clathrin-independent pathway was not responsible for internalization of the chimera. In addition, the original HAG and HAgC proteins were observed by immunocytochemistry and electron microscopy to enter into coated pits (Roth et al., 1986) and coated vesicles (Roth, unpublished data) in CV-1 cells. In all of our assays, the HA-G-G and HA-gC-gC proteins behaved identically to HAG and HAgC; most importantly, they were internalized at the same rates as the original chimeras.

At present, we do not know whether proteins lacking a cytoplasmic internalization signal for high affinity binding to coated pits interact with a component of the pits within the lipid bilayer or in the cytoplasm. Nor do we know whether the interaction involves a protein component of the pit or membrane lipid. Currently, little is known of the role that specific lipids might play in the function of plasma membrane domains, and one cannot eliminate the possibility that membrane curvature or composition allows some proteins to enter coated pits and excludes others. Whatever the mechanism for internalizing HA proteins having changes in the TM, it seems unlikely that the interaction is due to the presence of a specific amino acid sequence. It is possible that in the HA-G-HA and HA-A511-A515 proteins, the transmembrane sequences cannot fold properly and may act to "denature" the HA transmembrane and cytoplasmic sequences, perhaps exposing hydrophobic residues to the cytosol or polar residues to lipids in the bilayer. If so, those sequences might be recognized by a "chaperon-like" protein much as denatured cytoplasmic proteins are during conditions of stress. An attractive aspect of such a mechanism is that plasma membrane proteins would not need to encode any special information to be recognized by coated pits. Specificity of the interaction would be controlled by some event that changed the structure of the CD and/or TM.

Although the initial interaction between coated pits and endocytic receptors or chimeric proteins such as HA-G-HA must involve different structural features of the glycoproteins, it is still possible that all cell surface proteins are internalized by coated pits through a central mechanism. The dynamic, high affinity binding event in the cytoplasm involving the "tyrosine" recognition feature found on proteins that concentrate in coated pits could serve to collect endocytic receptors into the vicinity of a forming clathrin coat. Commitment to internalization, however, might require a second event, such as the formation of protein complexes large enough to slow their diffusion out of the pit. Chimeric HAs and other proteins lacking cytoplasmic recognition signals for clathrin associated proteins might be incapable of the first event but able to participate in the second. Proteins like the HA wt, unable to participate in either event, would be excluded from the pit.

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