Degradation of Mutant Influenza Virus Hemagglutinins Is Influenced by Cytoplasmic Sequences Independent of Internalization Signals*

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A mutant influenza virus hemagglutinin, HA+8, having a carboxyl-terminal extension of 8 amino acids that included 4 aromatic residues, was internalized within 2 min of arriving at the cell surface and was degraded quickly by a process that was inhibited by ammonium chloride. Through second-site mutagenesis, the internalization sequence of HA+8 was found to closely resemble the internalization signals of the transferrin receptor or large mannose 6-phosphate receptor. Comparison of the intracellular traffic of HA+8 and a series of other HA mutants that differed in their rates of internalization revealed a relation between the amount of the protein on the plasma membrane at steady state and the internalization rate that would be predicted if most of each protein recycled to the cell surface. However, there was no simple correlation between the internalization rate and the rate of degradation, indicating that transport to the compartment where degradation occurred was not simply a function of the concentration of the proteins in early endosomes. The internal populations of both HA+8, which was degraded with a t1/2 of 1.9 h, and HA-Y543, which was degraded with a t1/2 of 2.9 h, were found by cell fractionation and density-shift experiments to reside in early endosomes with little accumulation in lysosomes. A fluid-phase marker reached lysosomes 3-4-fold faster than these proteins were degraded. Degradation of these mutant HAs involved a rate-determining step in early endosomes that was sensitive to some feature of the protein that depended upon sequence differences in the cytoplasmic domain unrelated to the internalization signal.

Cells receive nutrients, respond to the extracellular environment, and continually remodel their plasma membranes through the process of endocytosis. The different functions of endocytosis require that internalized proteins and lipids be sorted and delivered to different intracellular destinations. However, with the exception of the initial recognition of some proteins by components of clathrin-coated pits (1), little is currently known about the mechanism by which various membrane proteins are recognized and sorted in the endocytic pathway.

Following internalization, released ligands and fluid rapidly separate from the bulk of endocytosed membrane, most of which returns rapidly to the plasma membrane from which it came (2–4). At least initially, this recycling membrane appears in the form of tubules with limited internal volume (5–8). Sorting of proteins in the fluid phase from membrane bound receptors can be explained by an iterative process of separating recycling membranes, which have a small volume and large surface area, from vesicles traveling farther along the endocytic pathway (3). However, the sorting of membrane-bound proteins is more complex, and currently cannot be explained by a single, simple mechanism.

The majority of plasma membrane proteins recycle after internalization (9, 10), and mutations that drastically inhibit the internalization of receptors have no effect upon the ability of the mutant proteins to recycle (11, 12). In addition, endocytosed receptors (13, 14) and lipids return to the surface at the same rate, suggesting that recycling of membrane proteins to the cell surface is non-selective (4). It is likely that most plasma membrane proteins are degraded in lysosomes (15), an observation most easily explained if transport to lysosomes is also by a nonselective transport pathway. Delivery to lysosomes cannot be completely nonselective, however, since the rates of delivery to lysosomes can differ dramatically among different proteins (16), which suggests that some proteins are specifically sorted to lysosomes. Thus, the relative contributions early in the endocytic pathway of specific recognition systems and of stochastic events that depend primarily upon protein concentration is currently uncertain, and this is particularly true for our understanding of the delivery of proteins to lysosomes.

As an approach to understanding the features by which membrane proteins interact with the cellular sorting machinery in the endocytic pathway, we have used site-directed mutagenesis to construct gain-of-function mutant proteins with altered patterns of intracellular transport. We have introduced changes into a protein, the influenza virus hemagglutinin (HA),1 that is highly mobile in the plasma membrane (17) but is internalized much slower than the rate of bulk internalization of membrane through coated pits. We have identified a series of mutant HAs that are internalized at very different rates and the rate of degradation, indicating that transport to the compartment where degradation occurred was not simply a function of the concentration of the proteins in early endosomes.

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This report is dedicated to David E. Zwart, who initiated this work before his death in December, 1991.

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¶ The abbreviations used are: HA, influenza hemagglutinin; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; endo H, endoglycosidase H; HRP, horseradish peroxidase; Man-6-P/IGF II receptor, the large mannose 6-phosphate/insulin growth factor II receptor; PAGE, polyacrylamide gel electrophoresis; SBTI, soybean trypsin inhibitor; FITC, fluorescein isothiocyanate; Tf, transferrin; TIR, transferrin receptor; HA wt, wild type HA.

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rates. One, HA-Y543, has a single amino acid change in the cytoplasmic domain 4 amino acids from the carboxyl terminus, a substitution of tyrosine for cysteine at position 543 (18). When expressed in amounts that do not saturate the internalization capacity of CV-1 (19) or Madin-Darby canine kidney cells (20), this protein is internalized as rapidly as some well characterized cell surface receptors (21-24) and interacts dynamically with coated pits (17). We have identified several other mutant HAs that are internalized much faster than HA-Y543. One of these, HA-S8, is internalized at 50-70%/min and binds essentially irreversibly to coated pits (25). HA-S8 is present only transiently in the plasma membrane and is degraded rapidly. The amino acid sequence that contributes the internalization signal of HA-S8 is quite similar to those of the mannos 6-phosphate/insulin growth factor II (Man-6-P/IGF II) receptor and the transferrin receptor, two proteins that are not degraded rapidly. To investigate the possibility that HA-S8 contains information specifying rapid degradation, we compared the endocytic transport of HA-Y543, HA-S8, and second-site mutants of both proteins. Our observations suggest that the rapid degradation of HA-S8 is not primarily determined by its rate of internalization and are consistent with the interpretation that a rate-limiting step in degradation of mutant HAs is a specific recognition event that controls their transfer from early endosomes to later endocytic compartments that are probably lysosomes. Changes in the HA cytoplasmic sequences can influence this event independently of the internalization signal.

EXPERIMENTAL PROCEDURES

Construction of Recombinant HAs—Enzymes from New England Bio-labs (Beverly, MA), Life Technologies, Inc., or United States Biochemicals according to instruction protocols were used. Plasmid DNAs were pulse-labeled at 100 μCi/ml and are used as substrates for transfection. US Biochemicals (Cleveland, OH) provided the EcoRI restriction enzyme. The cDNA encoding the A/Japan/305/57 (H2) HA contains a translational reading frame for 8 codons before the next termination occurs. A SalI to BamHI fragment bearing the HA-S8 mutation was subcloned into the pSK5VE vector (27). Recombinant SV40 virus stocks were prepared in CV-1 cells as described (28, 29). For immunofluorescence or biochemical experiments, CV-1 cells were infected and incubated at 37 °C for 24-36 h depending on the experiment. The infection was ended by treatment with 5% EM grade trypsin in HEPES buffer, pH 7.4, at 18°C for 2 min each at 4°C, and cells were incubated in a shallow 37°C water bath for 2 min each at 4°C. The cells were washed with phosphate-buffered saline (heparanized) three times. 18–24 h after infection, the medium was replaced with serum-free DMEM containing 10% serum, and the cells were used for experiments.

To measure the rate of appearance of HAs at the cell surface, the rate at which HA0 was cleaved into its disulfide-bonded subunits, HA1 and HA2, was measured. Cells were pulse-labeled for 5 min with 0.6 mCi of Tran35S-label/ml and chased for 30 min in serum-free DMEM. The rate of degradation of HA proteins, cells were pulse-labeled with 1 mCi of Tran35S-label/ml and chased for intervals of 3-7 h in a CO2 incubator in DMEM containing 10% serum. Duplicate samples were processed for each time point. For each sample, the amount of HA present was determined by immunoprecipitation, polyacrylamide gel electrophoresis (PAGE), and phosphorimaging as described (32, 33). To measure the rate of appearance of HA0 at the cell surface, the rate at which HA0 was cleaved into its disulfide-bonded subunits, HA1 and HA2, was measured. Cells were pulse-labeled with 1 mCi of Tran35S-label/ml and chased for 30 min at 37 °C in DMEM to allow all of the protein to trimerize.

The cells were then incubated at 18°C for 2 h, to allow proteins to accumulate in the trans Golgi network. Cells were then shifted to 37 °C for 12 min, an interval sufficient for the first proteins leaving the Golgi to arrive at the plasma membrane, and then were placed on ice and treated with medium containing trypsin at 100 μg/ml for 3 min to cleave HA at the cell surface into HA1 and HA2. The trypsin solution was removed, and the cells were treated with DMEM containing SBTI at 100 μg/ml at 4°C to inhibit the trypsin. After removal of the SBTI solution, one cell sample was lysed and immunoprecipitated and the amount of HAs that had arrived at the cell surface was determined as described above. DMEM at 37 °C was added. Under conditions where trypsin was prevented from entering the cells, there was no detectable signal from FITC in the red channel. The amount of HAs arriving at the cell surface during the 5-min chase into HA1 and HA2. The procedure was repeated for a series of 5-min chases at 37 °C conducted in the same manner. For each period of trypsinization, a control sample was treated again with trypsin or trypsin inhibitor but was allowed to proceed through all subsequent incubations at 4°C and 37 °C to demonstrate that trypsin had been completely inhibited by the SBTI treatment between each successive chase. At the end of the series of 37 °C chases, the samples were lysed and immunoprecipitated, and the amount of HAs arriving at the cell surface during the experiment was determined by PAGE and densitometry.

Internalization Assay—At 36 h after infection, cells were labeled with 0.6 μCi of Tran35S-label/ml for 15 min and chased for 80 min at 37 °C. Anti-HA antibody was allowed to bind to HA present on the surface for 1 h on ice. Unbound antibody was removed by five washes in DMEM of 2 min each at 4°C, and cells were incubated in a shallow 37 °C water bath containing 3% BSA in DMEM containing 50 mM Hepes at pH 7.3. After the chase, the medium was replaced with DMEM at 4°C containing trypsin at 100 μg/ml to cleave HAAs at the cell surface. After 1.5 h, the medium containing trypsin was removed and residual trypsin was inactivated by addition of DMEM containing SBTI at 100 μg/ml. Cells were lysed, and HAs that had initially bound antibodies to the cell surface were recovered by precipitation with protein A-Sepharose. PAGE and densitometry were employed to determine the amount of internalized (undeaved HA0) present at each time point.
Location of HA+8 in Endocytic Membranes—Cells expressing HA+8 or other HA mutants were labeled with 1–5 mCi/ml Tran35S-label for 15 min and chased for intervals varying from 2.5 to 5 h, depending upon the experiment. For some samples, 100 μg/ml leupeptin was included in the last 2.5 h of chase. To identify early endosomes, in some samples horseradish peroxidase (HRP) at a final concentration of 10 mg/ml was included in the medium at 37 °C for 5 min. Cells were chilled on ice and homogenized with a stainless steel homogenizer and centrifuged at 1200 × g for 10 min. The supernatant from the sample was mixed with a Percoll solution (final concentrations 17% Percoll, 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.25 mM sucrose, 4 mg/ml bovine serum albumin) layered over a 2.5 mM sucrose cushion and centrifuged at 25,000 × g for 1 h. Gradients were collected as fractions from the top with a Beckman Auto Densi-flow IIIC. Fractions were split and assayed for HRP activity (34) and β-galactosidase activity (35), and the HA present was precipitated and quantified by PAGE and phosphorimaging.

RESULTS

HA+8, Containing 8 Additional Amino Acids at the Carboxyl Terminus, Exhibits Altered Intracellular Transport—In the course of investigating the effects of introducing tyrosines into the carboxyl terminus of HA, we noticed that a unique NdeI restriction endonuclease recognition site at the HA terminus codon provided us with a simple method of changing the HA translational reading frame to extend the coding region for an additional 8 carboxyl-terminal amino acids with the sequence YDYKSFYN (see “Experimental Procedures” for details). This sequence resembles closely the internalization signals identified for the Man-6-P/IGFII receptor and the transferrin receptor (1). Two independently derived mutant HA genes containing this frameshift were isolated, and the proteins they encoded were expressed from recombinant SV40 virus vectors in CV-1 cells. These proteins proved to behave identically in all assays, excluding the possibility that an undetected second-site mutation could be responsible for the observations described in this report. Results with each isolate have been combined, and the proteins are referred to collectively as HA+8.

Routine examination by immunofluorescence microscopy of CV-1 monkey fibroblasts infected for 24 h with a recombinant SV40 virus expressing HA+8 indicated that the changes in the HA+8 cytoplasmic domain had a profound effect on the steady-state distribution of the protein. Very little HA+8 was observed on the cell surface, with considerable punctate labeling of small vesicles (data not shown). After longer periods of infection when protein expression levels had risen dramatically, a significant amount of HA+8 was detected on the cell surface, an expression pattern similar to that observed for lysosomal membrane proteins expressed from recombinant vectors (36–38). To determine if the vesicles containing HA+8 were derived from the endocytic pathway, cells expressing HA+8 or a second mutant HA that we have previously characterized, HA-Y543, were treated with cycloheximide for 2 or 6 h and followed to the leave the exocytic pathway and then the cells were prepared for immunofluorescence (Fig. 1a). HA-Y543 has an internalization signal and undergoes internalization and recycling, with 60–80% of the protein at the cell surface and 20–40% internal at steady-state (18, 19, 31). As expected, HA-Y543 was present both at the cell surface and in intracellular vesicles distributed throughout the cytoplasm, and this pattern of localization did not change during the 6-h treatment with cycloheximide, although the amount of HA-Y543 detected was observed to decrease (Fig. 1a, panel B). In contrast, HA+8 exhibited very little surface labeling after 2 h of treatment with cycloheximide, but was present in vesicles located throughout the cytoplasm (Fig. 1a, panel C). After 6 h in cycloheximide, this pattern had changed. Significantly less HA+8 was detected and most of the protein was located in vesicles close to the nucleus (Fig. 1a, panel D). The loss of HA+8 from the cells was inhibited when 100 μM chloroquine was included in the medium during the final 3 h of the 6-h treatment with cycloheximide (data not shown). Clearly, HA+8 was either failing to reach the plasma membrane or was being efficiently removed from the plasma membrane by endocytosis, and appeared to be degraded faster than HA-Y543 by a process sensitive to chloroquine.

To determine if the punctate staining of cells expressing HA+8 indicated that the protein mainly localized to endocytic vesicles, cells expressing either HA+8 or HA-Y543 were treated with cycloheximide for 2 or 6 h, and for the final 20 min of treatment with the drug, Tf-FITC was included in the medium to label cellular compartments containing the TfR (Fig. 1b, panels A, C, E, and G). The cells were then fixed, and the HA proteins were localized with anti-HA antibody and a second antibody conjugated to Texas Red (panels B, D, F, and H). In Fig. 1b, paired images collected with FITC fluorescence to locate transferrin bound to its receptor (panels A, C, E, and G) and Texas Red fluorescence to locate HAs in the same cell (panels B, D, F, and H) are shown. After 2 h of drug treatment, there was extensive co-localization of both HA mutant proteins and the TfR. After 6 h in cycloheximide, HA-Y543 (panel D) continued to share compartments labeled with Tf-FITC (panel C), but many cells expressing HA+8 showed essentially no co-localization, as shown in panels G (TfR) and H (HA+8). In other cells there was some co-localization (data not shown), but much less than seen after 2 h of cycloheximide treatment. A simple interpretation of these results is that in the first hours after its synthesis, HA+8 resided in early endosomes that were in contact with recycling TfR. After several hours HA+8 moved to other compartments not occupied by the TfR, and was degraded.

HA+8 Is a Properly Folded Trimeric HA—The results of these immunofluorescence experiments were consistent with two possibilities; either HA+8 was quantitatively transported to the cell surface and very rapidly internalized into early endosomes containing TfR, or only a portion of HA+8 ever reached the cell surface and the rest traveled by an intracellular route to early endosomes. Ultimately HA+8 moved out of endosomes to acidic, degradative organelles that are probably lysosomes. To determine the intracellular route of HA+8 transport and also whether the additional sequences of the HA+8 cytoplasmic domain had influenced folding of the protein in some way that made it unstable, we investigated HA+8 bio-synthesis by pulse-chase protocols. The rate of folding of HA+8 into protease-resistant trimers was measured by two previously published assays (32, 39, 40) and compared to that of HA-Y543 and HA wt measured in parallel (Table I). Previously we and others have shown that the HA external structure is tolerant of changes in the cytoplasmic domain (19, 28), but is affected by changes in the transmembrane domain (27, 32). We detected no difference in the rate or extent of folding of HA+8 as compared to the HA wt, indicating that any effect of the HA+8 cytoplasmic sequences was limited to the cytoplasmic portion of the protein.

To determine if transport from the ER to the Golgi complex was affected by the HA+8 mutation, we measured the rate at which HA+8 and the HA wt and HA-Y543 controls acquired resistance to digestion with endo H (Table I). Processing of the oligosaccharides of HA+8 to the complex form resistant to endo H was delayed compared to the very rapid processing of the two control proteins, but was essentially complete, as greater than 95% of the protein was processed after a 1-h chase. Thus, the mutation in HA+8 affected either the rate of its transport to the Golgi apparatus or the rate of processing of its oligosaccharides, but did not prevent the protein from leaving the ER.
To determine the rate at which HA+8 moved from the Golgi complex to the cell surface and the fate of the protein once it arrived there, we measured the rate at which pulse-labeled HA+8 could be chased to a location in which it could be cleaved into its HA1 and HA2 subunits by trypsin added to the extracellular medium (32, 41). HA+8, HA wt, and HA-Y543 were chased at 37°C for times between 20 and 200 min in medium containing trypsin (Fig. 2, top panels). HA+8 gained access to

TABLE I

| Protein       | $t_{1/2}$ of formation of trimers $^b$ | $t_{1/2}$ of acquiring endo H resistance $^c$ | Extent of endo H resistance $^d$ |
|---------------|----------------------------------------|---------------------------------------------|--------------------------------|
| HA wt         | 6                                      | 13                                          | >95                           |
| HA-Y543      | 6                                      | 13                                          | >95                           |
| HA+8          | 6                                      | 13                                          | >95                           |

$^a$ Rate is %/min.

$^b$ $t_{1/2}$ values are in minutes. Trimer formation was assayed by sucrose gradient centrifugation.

$^c$ $t_{1/2}$ values are in minutes.

$^d$ Extent of resistance to endo H is the percentage of each protein resistant to digestion with endoglycosidase H after a chase of 2 h at 37°C.

Fig. 1. Immunofluorescent labeling of HA-Y543 and HA+8. Panel a. Cells expressing HA-Y543 (panels A and B) or HA+8 (panels C and D) were treated with cycloheximide for 2 h (panels A and C) or 6 h (panels B and D). Cells were fixed, permeabilized, and labeled by indirect immunofluorescence with rabbit anti-HA antibodies. B, Tf-FITC was added to the culture medium for the last 20 min of the interval of drug treatment (which was 2 h for upper panels A, B, E, and F and 6 h for lower panels C, D, G, and H). Cells were then fixed, permeabilized, and labeled with anti-HA antibodies and a second antibody conjugated to Texas Red. Cells are shown as paired images, collected simultaneously with a dual-channel laser-scanning confocal microscope fitted with filters for FITC and Texas Red fluorescence. Panels A (TfR) and B (HA-Y543), after 2 h in cycloheximide. Panels E (TfR) and F (HA+8), after 2 h in cycloheximide. Panels C (TfR) and D (HA-Y543), after 6 h in cycloheximide. Panels G (TfR) and H (HA+8), after 6 h in cycloheximide.
trypsin present continually in the chase medium at a rate of 1.3%/min, slightly slower than the rate of either of the two control proteins (Fig. 2, graphs).

This observation is consistent with the interpretation that the slower acquisition of oligosaccharides resistant to endo H by HA18 compared to the two other HAs was due to a delay in transport to the Golgi apparatus. When trypsin was present continually, over 95% of each of the three proteins was cleaved into HA1 and HA2 after a chase of 80 min. However, when trypsin was added at 4 °C only at the end of a 80-min chase, less than 10% of HA+8 was cleaved and greater than 90% had reached a compartment inaccessible to extracellular trypsin (Fig. 2, panel’s marked 4 °C). In contrast, >95% of HA wt remained accessible to trypsin at the cell surface and, at steady state, 70% of HA-Y543 was external and 30% internal (graphs, Fig. 2). The percent of HA+8 detected at the cell surface by this assay was entirely consistent with the immunofluorescence results, suggesting that HA+8 was only transiently at the cell surface. However, since trypsin in the medium at 37 °C was internalized and was present in the early endosome at a concentration equal to that in the extracellular medium, this assay did not distinguish between the internalization of HA+8 that had been delivered to the plasma membrane and a potential intracellular pathway transporting HA+8 to early endosomes in which trypsin was active. To distinguish between these possibilities, we employed a pulse-chase protocol to measure both the rate and the extent to which radioactive HA+8 reached the cell surface and came into contact with trypsin added to the extracellular medium at 4 °C. At this temperature, fluid phase endocytosis is blocked (42) and trypsin was not internalized.

To ascertain that we could detect the transient presence of HA+8 at the cell surface, the majority of a cohort of pulse-labeled HA+8 was trapped in the trans Golgi network by being chased for 120 min at 18 °C (41, 43), then released to the cell surface in a series of brief chases at 37 °C. Cells were first incubated at 37 °C for 12 min, and then treated with trypsin at 4 °C to cleave HA+8 at the plasma membrane into HA1 and HA2. The medium containing trypsin was removed, and residual trypsin was inactivated with SBTI. A series of six 5-min chases was then initiated with fresh DMEM at 37 °C to allow more HA+8 to reach the cell surface. After each 5-min incubation at 37 °C, the cells were returned to 4 °C and HA+8 present on the surface was cleaved by trypsin, which was then inactivated with SBTI and removed by washing in DMEM. For each chase period, samples were placed on ice for measurement of the amount of HA+8 that had become accessible to trypsin and the chase was continued with the remaining samples. Using this procedure, active trypsin never entered the cells, eliminating the possibility that internalized trypsin gained access to, and cleaved, internal HA+8. This was verified by control samples (see “Experimental Procedures”). After the final cycle, the proteins were immunoprecipitated and the portion of pulse-labeled proteins that had been cleaved into HA1 and HA2 subunits was determined by PAGE and quantitative autoradiography (Fig. 3). Greater than 65% of HA+8 reached the cell surface from the trans Golgi after a chase of 42 min. Longer chases were not possible due to the condition of the cells. Parallel samples incubated at 37 °C with trypsin

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**Fig. 2. Distribution of HA proteins as a function of time after synthesis.** Pulse-labeled protein was chased for the times indicated with trypsin in the medium during the chase at 37 °C or trypsin added at 4 °C after the chase. HA1 and HA2 are the tryptic cleavage products of HA0. Percent accessible = (HA1 + HA2)/(HA0 + HA1 + HA2). The graphs in the lower panel present the results of densitometry of proper exposures of the autoradiograms shown in the upper panels. The percent of total protein accessible when trypsin was present at 37 °C (triangles) is a measure of the amount that left exocytic compartments during the chase. The percent accessible when trypsin was present at 4 °C (squares) is a measure of the protein present at the plasma membrane at the end of the chase period shown. The percent of each protein present in the endocytic pathway (circles) is calculated from the difference between the percent accessible when trypsin was present at 37 °C and the percent accessible when trypsin was present at 4 °C.
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The rate taken from the slope of the curves in Fig. 3, 1.3%/min, is the same regardless of whether the trypsin was added repeatedly at 4 °C or was present continuously at 37 °C. The rate of internalization-incompetent HA wt, CV-1 cells expressing each of these proteins were pulse-labeled 36 h after infection with recombinant virus vectors and the radioactive proteins were chased to the plasma membrane using the strategy described for HA-Y543. HA +8 bound by antibodies at the cell surface began to be deaved into HA1 and HA2 subunits even in the absence of trypsin in the medium, beginning 8 min after a shift to 37 °C (data not shown). This cleavage probably represented delivery of HA +8 to a compartment containing an endogenous protease and is probably responsible for the slow decrease in the amount of HA +8 that scores as internal in our assay after 8 min (Fig. 4).

HA +8 Is Internalized through Coated Pits—Although the steady-state concentration of HA +8 in the plasma membrane was low, which made immunocytochemistry difficult, we observed by electron microscopy that coated pits on cells that were expressing HA +8 could be labeled with anti-HA antibodies and protein A-colloidal gold (data not shown). Several lines of evidence suggest that fibroblasts such as CV-1 cells may not have a significant clathrin-independent internalization pathway (44–46). Nevertheless, to confirm that HA +8 was internalized through coated pits, we compared the degree to which internalization of that protein could be inhibited by hypertonic media, which blocks clathrin-mediated internalization (46–48), or by cytochalasin, which has been reported to prevent non-clathrin-mediated internalization in Vero cells (49). Cytochalasin treatment had no effect on internalization of either HA-Y543 or HA +8, whereas hypertonic medium inhibited the internalization of HA-Y543 by 95% and HA +8 by 85%. These findings are in accord with our previous demonstration that treatments that block internalization of clathrin-coated pits were effective in inhibiting internalization of transferrin receptors and HA-Y543 in CV-1 cells (17). Taken together, our data suggest that internalization through coated pits accounted for the majority of endocytosis of HA +8.

The Internalization Signal of HA +8 Is YKSF—To identify the amino acids in HA +8 responsible for its extremely rapid internalization, we constructed second site mutants of HA +8. All of these mutants were efficiently transported to the cell surface and were well folded, as measured by resistance to degradation by trypsin. The rate of internalization of each mutant was measured and results of a representative experiment are shown in Fig. 5, and the sequences of mutants that define the internalization signal of HA +8 are shown in Table II. The 2 tyrosines at positions 548 and 554 were relatively unimportant for internalization of HA +8. Single changes of Y548 (Fig. 5) or Y554 (data not shown) to serine decreased internalization of the mutated HA +8s by about 20%. These inhibitory effects might be due to structural changes, because the double mutant HA +8-S548,S554 was internalized essen-
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3 times as fast as HA+8 (Table II and Fig. 5). Three changes in HA+8 were found to severely inhibit internalization. The substitution of Tyr-550 to serine in HA+8-S550, or of Phe-553 to alanine in HA+8-S548,A543,S554, reduced the rate of internalization to 2–3%/min (Table II). Truncation of the extended carboxyl-terminal sequence of HA+8 to the 4 amino acids YDYK reduced endocytosis of the mutant HA+4 to 1%/min. Thus, the HA+8 internalization signal appeared to be similar to other sequences that have been identified as important for internalization, with the most important part of the signal residing in the sequence YKSF. This sequence resembles the core of the transferrin receptor internalization signal, which is YTRF, and that of the Man-6-P/IGF II receptor, which is YSKV.

Degradation of Internalization-competent HA Mutants Is Not Directly Proportional to the Internalization Rate—The immunofluorescence experiments shown in Fig. 1 suggested that HA+8 might be degraded rapidly. Proteins that are efficiently internalized, such as the transferrin receptor and polyimmunoglobulin receptor, are not necessarily degraded rapidly but instead are continually recycled back to the plasma membrane. Other proteins, such as P-selectin (16) and epidermal growth factor (EGF) receptors bound to EGF (24), also recycle to some extent but are rapidly sorted to lysosomes by a specific mechanism. Since HA was not designed by nature to be internalized, rapid degradation of HA+8 might occur due to some change in HA structure induced by the environment of endosomes (such as low pH). Alternatively, in addition to its internalization signal, HA+8 might have gained a second signal that specifies delivery to an endocytic proteolytic compartment. If the first possibility were true, then mutant HAs should be degraded as a function of their concentration in early endosomes. If the second possibility were true, the concentration of protein in endosomes would not necessarily be related to degradation. To investigate these possibilities, we compared the steady-state distribution, the internalization and the degradation rates for three rapidly internalized mutant HAs and two more slowly internalized ones. HA+8, HA+8-S548,S554, and a HA mutant that lacks the 8-amino acid cytoplasmic extension, HA-Y543,F546 (31), were internalized at 55–60%/min. HA-Y543 was internalized at about 5%/min in these experiments, and HA+4 was internalized at 1%/min (Table II), which probably represents nonspecific uptake by coated pits. For each of these proteins, the rate of degradation was determined by pulse-chase experiments (Fig. 6, Table II). Both HA-S548,S554 and HA+8 were degraded very rapidly, with a t1/2 of less than 2.0 h. In contrast, HA-Y543,F546 and HA-Y543 were degraded significantly slower than the other two proteins. HA+4 was degraded quite slowly. By t test, the probability that the t1/2 of degradation of HA+8 differed from that of HA-Y543 or HA-Y543,F546 was 99% or 93%, respectively. HA-Y543, which was internalized 10 times slower than HA-Y543,F546, was degraded at a rate similar to HA-Y543,F546. We did not observe a simple correlation between internalization rate and degradation rate for these proteins; thus, their degradation was not solely a function of how fast they entered into early endosomes.

The Internal Population of HA+8 Is in Early Endosomes—By immunofluorescence, 2 h after synthesis HA+8 extensively co-localized with TfR, indicating that it was present in early endosomes. However, the rate of degradation of HA+8, although fast, was 2–4-fold slower than required for a fluid-phase marker, HRP, to reach lysosomes (data not shown and see discussion). This difference could occur through a lag between delivery of HA+8 to lysosomes and its degradation, or because HA+8 has one or more slow steps in transport through endocytic membranes. To distinguish between these possibilities, we determined the amount of HA+8 in early or late endocytic membranes at steady state. Cells expressing two mutants that were degraded at different rates, HA+8 or HA-Y543, were pulse-labeled, and the proteins were chased out of the exocytic pathway. We then prepared a post-nuclear membrane fraction by low speed centrifugation to remove much of the plasma membrane, and analyzed this fraction by rate zonal centrifugation on Percoll gradients. To locate early endosomes on the gradient, some cells were allowed to internalize HRP added to the medium at 37 °C for 5 min. To determine the degree to which HAs at the plasma membrane contaminated other fractions, some samples were treated with trypsin at 4 °C to cleave surface HAs into HA1 and HA2 subunits before cells were homogenized. After collecting the gradients in fractions, HRP activity and endogenous β-galactosidase activity was measured for a portion of each fraction to locate early endo-

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**Fig. 5. Endocytosis of second-site mutants of HA+8.** Internalization assays were performed, and data plotted, as described for figure 4.

**Table II**

| HA type          | Cytoplasmic sequence | Endocytosis rate | Percent at surface | t1/2 of degradation |
|------------------|----------------------|------------------|--------------------|---------------------|
| HA wt            | NGSLQCRICI           | 0.01             | 99 ± 2             | n = 3               |
| HA-Y543          | ······Y···           | 0.05             | 75 ± 2             | n = 3               |
| HA-Y543,F546     | ······Y···F···       | 0.55             | 12 ± 4             | n = 3               |
| HA+8             | NGSLQCRICIYDYKSFYN   | 0.60             | 10 ± 4             | n = 3               |
| HA-S548,S554     | ······S····S··       | 0.55             | 11 ± 4             | n = 3               |
| HA+4             | ········Y····*       | 0.81             | 93 ± 0.4            | n = 5               |
| HA-S548,A553,S554| ······S····A··       | 0.02             | 92 ± 2             | n = 4               |
| HA-S550          | ········Y····F···    | 0.03             | 93 ± 2             | n = 4               |

*The endocytosis rate is the fraction of the surface population internalized per minute.

*bPercent surface is the percent of pulse-labeled HA protein at the cell surface after a 2-h chase, an interval sufficient to allow all of the protein to leave exocytic compartments.

*The t1/2 for degradation is given in hours.

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*a Asterisk indicates a stop codon.
somess and lysosomes, respectively. HAs were immunoprecipitated from other portions of each fraction. Fig. 7a shows the distribution of HRP activity, marking vesicles into which it had access during a 5-min pulse at 37 °C (early endosomes), and of β-galactosidase activity, indicating the location of lysosomes. Fig. 7b shows the distribution of HA+8, HA-Y543, and the β-galactosidase activity present in fractions of each gradient. There is slightly more HA-Y543 in lighter fractions than HA+8, but control experiments in which HA-Y543 at the cell surface had been cleaved by trypsin indicated that some of this might be contamination of early endosomes with plasma membrane. Since relatively little HA+8 was resident on the cell surface, contamination of early endosomes with plasma membranes contributed little to the amount of HA+8 recovered from gradient fractions containing early endosomes. Most of both proteins was found in the fractions containing early endosomes, but more HA+8 than HA-Y543 was recovered from denser fractions that partially overlapped with the main peak of β-galactosidase activity. To determine if the HA+8 present in less dense gradient fractions actually resided inside early endosomes, and not in membranes co-migrating with them, cells expressing HA+8 were allowed to internalize HRP for 5 min before they were chilled and a post-nuclear membrane preparation was prepared. One half of this preparation was reacted with diaminobenzidine and H$_2$O$_2$, which results in the deposition of a dense reaction product inside any vesicles containing HRP (51, 52). This reaction product has two effects. A large proportion of proteins inside vesicles containing HRP become insoluble and cannot be immunoprecipitated (2), and vesicles containing the reaction product migrate faster on velocity gradients (52). After the reaction with diaminobenzidine, both unreacted and reacted fractions were analyzed by centrifugation on Percoll gradients. The HRP reaction significantly reduced the amount of HA+8 recovered by immunoprecipitation. Almost all of HA+8 remaining that could be immunoprecipitated was shifted from lighter to denser fractions of the gradient (Fig. 7c). These results indicated that the main peak of HA+8 in light fractions was within vesicles that had access to HRP during a 5-min chase. A small peak of HA+8 was present in membranes denser than early endosomes, but less dense than lysosomes, even after the DAB density shift. This might represent HA+8 in late endosomes. These results indicated that there was little accumulation of HA+8 or HA-Y543 in lysosomes and that most of HA+8 at steady state was present in early endosomes.

Early endosomes include recycling membranes carrying proteins back to the plasma membrane and vesicles, sometimes called sorting endosomes (4), that will deliver material to late endosomes. At least some of the HA+8 was able to recycle to the cell surface from early endosomes, although we could not measure the rate at which this occurred. To demonstrate recycling directly, we treated cells expressing HA+8 and uninfected cells with cycloheximide and chased long enough for all protein to leave the exocytic pathway, then at hourly intervals added anti-HA antibody to the culture medium for 30 min at 37 °C. The cells were then fixed, and the antibody was located by indirect immunofluorescence. Only cells expressing HA+8 took up the anti-HA antibody, and they did so even after the cells had been in cycloheximide for 4 h, a period much longer than would be required to sequester the whole population of HA+8, if the protein had not been able to return to the cell surface (data not shown). Therefore, a portion of the HA+8 present in early endosomes must have been in contact with recycling membranes.

Although we did not see an accumulation of HA+8 in lysosomes by cell fractionation, the immunofluorescence experiments suggested that HA+8 exited early endosomes before it was degraded. To eliminate the possibility that HA+8 was degraded outside the endocytic pathway (for instance, at the plasma membrane), pulse-chase experiments were performed on cells expressing HA+8 that were treated with ammonium chloride. Degradation of HA+8 was completely blocked under these conditions and after 2 h of treatment, the total amount of labeled HA+8 was more than twice that in untreated cells and the amount of HA+8 recovered from denser membrane fractions had increased from 12% to 19% (data not shown).
DISCUSSION

We have engineered a series of HA mutants that enter the endocytic pathway at extremely different rates. One of these mutants, HA+8, is internalized at one of the fastest rates reported. In a recent report (25), we have shown that HA+8 and another rapidly internalized mutant, HA+8-S548,S554, exhibit stable association with coated pits over the expected 1–2 min lifetime of a clathrin-coated pit in a fibroblast (53) in keeping with their fast internalization. All of the rapidly internalized proteins used in this study, including HA+8, had a steady-state distribution consistent with the simple situation where the concentration of protein at the cell surface, multiplied by the internalization rate, was equal to the concentration inside the cell, multiplied by an externalization, or recycling, rate of 0.08–0.1/min. We confirmed directly for HA+8 and HA-Y543 that the proteins extensively co-localized with the TfR. Most of the internal population of each of these two HA mutants was accessible to HRP taken up for 5 min from the cell culture fluid, consistent with a location for the proteins early in the endocytic pathway. In spite of residing for an extended period in early endosomes, all of the mutant HAs were degraded quickly relative to the rates reported for endocytic receptors or plasma membrane proteins (16), although in all cases the degradation rates were orders of magnitude slower than the measured internalization or the calculated recycling rates. The rate of degradation of HAs, however, was not simply proportional to their internalization rates. This is demonstrated by the rather similar degradation rates of the mutants HA-Y543 and HA-Y543,F546, which differ in internalization rates by 5–10-fold (Ref. 31, and this work), and the significantly slower degradation rate of HA-Y543,F546 as compared to those of HA+8 or HA+8-S548,S554, in spite of the fact that all three of these mutants are internalized at similar rates.

Degradation of these proteins was not due to changes in their external domains. HA+8, like all of the mutant HAs used in this study, was found to be a well folded, trimeric HA that was resistant to proteolysis except for the trypsin-sensitive site that links the HA1 and HA2 subunits. This suggests that the different sequences of the cytoplasmic domains of these proteins directly influenced their degradation rates.

In addition, we have shown the HA+8 mutant was efficiently transported to the cell surface. Although we could not directly demonstrate that all of the protein traveled directly to the cell surface, at least 65% did so, and the rate at which HA+8 reached the cell surface under conditions where extracellular trypsin was not internalized was the same as the rate at which all of the protein came into contact with extracellular trypsin at 37°C. The simplest explanation for these data is that after biosynthesis, all of HA+8 was exported to the plasma membrane and entered the endocytic pathway by the same mechanism as did other HA mutants. Thus, differences in rates of degradation of HA mutants, for example, HA+8 and HA-Y543, were unlikely to be due to different pathways for entering endosomes.

The pattern of retention in endosomes for a period prior to a slower delivery to lysosomes for degradation has been reported recovered were determined. Data points are the average of two experiments, and individual values differed from the mean by less than 15%. Panel c, density shift of light membranes containing HA+8 demonstrates that the majority of HA+8 resides in early endosomes. Cells expressing HA+8 were allowed to internalize HRP for 5 min, and then homogenized and reacted with diaminobenzidine and H₂O₂ to shift the migration of vesicles containing HRP. Membranes were centrifuged on Percoll gradients, and HA+8 was collected from gradient fractions by immunoprecipitation.
for several hormone receptors. After binding ligand, β-adrenergic receptors shift from a predominantly surface population to one that is internalized and recycles for over 1 h before degradation is measurable (54). In the absence of EGF, the EGF receptor internalizes slowly and returns to the plasma membrane very rapidly. In the presence of its ligand, the receptor internalizes more rapidly, recycles 2-4-fold slower, and is sorted to lysosomes (12) by a saturable mechanism (55) requiring sequences of the cytoplasmic domain between residues 945 and 991 that are separate from the internalization signal (24). EGF receptors have been observed to collect in multivesicular bodies that are continuous with tubules through which transferrin receptors recycle to the plasma membrane (8). It has been proposed that this is due to a specific sorting event in which the cytoplasmic domain is bound by cellular factorsthat are specific to directly direct proteins to be degraded rapidly after internalization to a specific sorting sequence, and/or by a cytosolic interaction signal just as we observe for mutant HAs.

In addition to the few examples of proteins that seem to have lysosomal targeting sequences, the other mechanism observed to specifically direct proteins to be degraded rapidly after internalization is to cross-link them with antibodies or multivalent ligands (53, 56–58). We have observed that HA-8 can be isolated as higher order complexes from cells lysed with octylglucoside, but not with Triton X-100 (25). However, complexes of HA-8-B548,554, which is degraded at a similar rate, are not detected under the same conditions; thus, at present we cannot relate the ability to form higher-order complexes with the ability of HA-8 to be sorted efficiently to a compartment for degradation. Nevertheless, since all of the HA proteins studied were degraded rapidly relative to the rates reported for other proteins, it is possible that the HA external domain has properties that contribute to rapid degradation, such as the potential to form weakly associated aggregates.

Like the proteins that appear to have distinct degradation sequences, cross-linked proteins are degraded with half-lives that are severalfold longer than the period required to transport HRP (42, 51, 59) or viruses (44, 60) to lysosomes. The degradation rates reported for these proteins are an order of magnitude slower than their internalization rates. At least in the case of the Fc receptor, some recycling of the protein bound to polyclonal antibody has also been reported (61). Thus, it is possible that these proteins, like HA-8, P-selectin and activated EGF receptors, spend a protracted period in early endosomes (perhaps multivesicular bodies) in contact with recycling membranes, and are more slowly sorted to lysosomes.

Sorting of proteins in early endosomes has been proposed to occur through exclusion of proteins from recycling membranes (62). One mechanism for achieving this that is consistent with the observations of this report would be the formation of complexes through cross-linking the cytoplasmic domains of proteins by a cytosolic endosomal "coat" protein (63) that recognizes a specific sorting sequence, and/or by a cytosolic chaperone-like protein that recognizes "denatured" cytosolic sequences. In each case, binding to the coat complex would probably be dynamic, much like the binding of HA mutants to coated pits (17, 25). Under such conditions, proteins dissociating from the endosomal coat would likely re-enter recycling membranes. The extent to which a protein having a cytosolic recognition signal for degradation is limited to endosomes or recycled would therefore depend upon its affinity for elements of the endosomal coat. Given the rates that we and others have measured for internalization, recycling and delivery to lysosomes of rapidly degraded proteins, one would predict that either the affinity of the hypothetical endosomal coat protein for plasma membrane proteins is low, or the amount of an important component of this process is limiting. The possibility that a cellular factor or "garbage collector" forms complexes with HA-8 by binding to its cytoplasmic domain is a current focus of our interest in this glycoprotein.

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46. Heuser, J. E., and Anderson, R. G. W. (1989) J. Cell Biol. 108, 389–400
47. Daukas, G., and Zigmond, S. H. (1985) J. Cell Biol. 101, 1673–1679
48. Hansen S. H., Sandvig, K., and van Deurs, B. (1993) J. Cell Biol. 121, 61–72
49. Sandvig, K., and van Deurs, B. (1990) J. Biol. Chem. 265, 6382–6388
50. Heuser, J. E. (1989) J. Cell Biol. 108, 401–411
51. Courtoy, P. J., Quintart, J., and Baudhuin, M. (1984) J. Cell Biol. 98, 870–876
52. Ajioka, R. S., and Kaplan, J. (1987) J. Cell Biol. 104, 77–85
53. Anderson, R. G. W., Brown, M. S., Beisiegel, U., and Goldstein, J. L. (1982) J. Cell Biol. 93, 523–531
54. Kurz, J. B., and Perkins, J. P. (1992) Mol. Pharmacol. 41, 375–381
55. French, A. R., Sudlow, G. P., Wiley, H. S., and Lauffenberger, D. A. (1994) J. Biol. Chem. 269, 15749–15755
56. Hopkins, C. R., and Trowbridge, I. S. (1983) J. Cell Biol. 97, 508–521
57. Mellman I., and Plutner, H. (1984) J. Cell Biol. 98, 1170–1177
58. Nolan, C. M., Creek, K. E., Grubb, J. H., and Sly, W. S. (1987) J. Cell Biochem. 35, 137–151
59. Wang, R. H., Colbaugh, P. A., Kao, C. Y., Rutledge, E. A., and Draper, R. K. (1990) J. Biol. Chem. 265, 20179–20187
60. Schmid, S. L., Fuchs, R., Male, P., and Mellman, I. (1988) Cell 52, 73–83
61. Hönig, S., Jockusch, B. M., Kreimer, G., Vietel, D., Robenek, H., Engelhardt, W., and Frey, J. (1993) Eur. J. Cell Biol. 55, 48–59
62. Linderman, J. J., and Lauffenberger, D. A. (1988) J. Theor. Biol. 132, 203–245
63. Hopkins, C. R. (1992) Trends Biochem. Sci. 17, 27–32