The Signal for Clathrin-Mediated Endocytosis of the Paramyxovirus SV5 HN Protein Resides at the Transmembrane Domain–Ectodomain Boundary Region

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The hemagglutinin-neuraminidase (HN) glycoprotein of the paramyxovirus SV5 is internalized from the cell surface via clathrin-coated pits. However, the cytoplasmic domain of SV5 HN does not contain a previously characterized internalization motif. A cell-surface-expressed chimeric protein (APK), consisting of the cytoplasmic tail, transmembrane (TM) domain, and 12 residues of the ectodomain of HN joined to the cytoplasmic protein pyruvate kinase is internalized, indicating that the N-terminal region of HN contains an internalization signal. Although SV5 HN is internalized at a rate similar to that of influenza virus hemagglutinin (HA) mutant Y543, which contains a degenerate tyrosine-based signal in its cytoplasmic tail, the elimination of the majority of the HN cytoplasmic tail, or substitution of the HN TM domain with leucine residues, did not affect the rate of HN internalization. The HN protein of the closely related virus, Newcastle disease virus (NDV), is not internalized from the cell surface. Working under the usual convention that the TM domain consists of the hydrophobic residues bounded by two charged residues, analysis of internalization of mutant and chimeric NDV HN molecules indicates that the first seven SV5 HN ectodomain residues are critical for internalization of HN. A glutamic acid residue (E37) that abuts this presumptive HN TM domain/ectodomain boundary is important for SV5 HN internalization.

INTRODUCTION

The paramyxovirus SV5 hemagglutinin-neuraminidase (HN) is a type II integral membrane glycoprotein (Hiebert et al., 1985). The HN protein is transported to the cell surface through the cellular exocytic pathway, and it is incorporated into the envelopes of progeny virions that bud from the plasma membrane of virus-infected cells. Previously we observed that HN is internalized from the surface of virus-infected cells and is degraded with a t1/2 of 2.5 h (Ng et al., 1989). An examination of the pathway of HN endocytosis, by using immunoelectron microscopy, showed that HN is concentrated in coated pits and then enters the endosomal cellular compartments: colloidal gold-tagged HN accumulated in lysosomes (Leser et al., 1996). If the common presumption is made that the N-terminal 19 hydrophobic residues that are bounded by two charged residues constitutes the transmembrane (TM) domain, then HN has a N-terminal cytoplasmic domain of 17 residues and the ectodomain begins at glutamic acid residue 37, although there are no direct experimental data that support this precise delineation of the three protein domains (Hiebert et al., 1985; Hiebert and Lamb, 1988). As discussed below, the HN 17 residue cytoplasmic tail lacks any of the previously recognized internalization motifs.

Several classes of signals contained within the cytoplasmic domain of membrane proteins specifying clathrin-mediated internalization have been identified. The best-characterized signal is based upon a critical aromatic amino acid, most frequently a tyrosine residue. This signal conforms mostly to one of two subtypes: YXXZ (where Z is a hydrophobic amino acid) or NPXY (Davis et al., 1986; Iacopetta et al., 1988; Jing et al., 1990; Hansen et al., 1992; Kibbey et al., 1998). The LDL receptor cytoplasmic tail internalization signal has been shown to be NPVY (Davis et al., 1986; Iacopetta et al., 1988; Jing et al., 1990; Hansen et al., 1992), and the YTRF sequence of the cytoplasmic domain of the transferrin receptor has been shown to be necessary and sufficient to cause receptor concentration and internalization through coated pits (Iacopetta et al., 1988; Jing et al., 1990; Hansen et al., 1992). The EGF receptor contains both the NPXY and YXXZ signals in its cytoplasmic tail. Another class of internalization signal found in the cytoplasmic tails of some proteins, including CD3y and insulin receptors, is the dileucine motif (LL or LI) (Letourneur and Klausner, 1992; Dietrich et al., 1994; Hamer et al., 1997). A third class of internalization motif, a dilysine motif, appears to be related to the better-characterized ER-retention signal (Jackson et al., 1993; Itin et al., 1995). Endocytosis of transmembrane proteins...
also can be mediated through monoubiquitination (Hicke and Riezman, 1996; Terrell et al., 1998; reviewed in Bonifacino and Weissman, 1998).

Studies on the surface accumulation and internalization of plasma membrane and lysosomal proteins after expression of chimeric proteins containing specific internalization signals indicate that YXXZ and LL signals bind to distinct saturable components (Marks et al., 1996). Furthermore it has been shown that the transferrin receptor (YTRF signal), LDL receptor (NPVY signal), and EGF receptor (multiple signals) do not compete for the same components for internalization (Warren et al., 1998). In addition, individual membrane proteins containing LL signals may not bind to the same components (Heilker et al., 1996; Honing et al., 1998). Thus it seems there are multiple internalization signals that can interact with different components of the internalization machinery.

The influenza A virus hemagglutinin (HA) has proven to be a useful prototype protein for studying the behavior of surface expressed membrane proteins (Roth et al., 1986; Lazarovits and Roth, 1988; Naim et al., 1995). Native HA is expressed on the surface of cells and is highly mobile in the plane of the membrane, yet it is internalized at a rate 40-fold slower than the rate of bulk turnover of membrane proteins (Roth et al., 1986). Mutant HA proteins bearing a tyrosine substitution at position 543 are endocytosed efficiently in a clathrin-dependent manner at a rate of 8–9%/min (Lazarovits et al., 1996). The signal created by the placement of tyrosine at position 543 is most likely a degenerate form of the aromatic amino acid internalization motifs. The substitution of amino acids surrounding the tyrosine of HA Y543, in a pattern dictated by the positional requirements of characterized tyrosine-based signals, can improve the efficiency of endocytosis (Klitstakis et al., 1990).

We show here that the paramyxovirus HN cytoplasmic tail is dispensable for internalization and the TM domain exerts no control over internalization. The seven residues in the HN ectodomain that abut the presumptive TM domain are critical for internalization of SV5 HN. In particular, the glutamic acid residue at the presumptive TM domain/ectodomain boundary is important for internalization.

## RESULTS

Internalization of HN is an intrinsic property of the protein and occurs with a rate similar to that of internalization of the surrogate marker HA Y543

In SV5-infected cells the HN glycoprotein is internalized from the cell surface and turned over (1/2 t ∼ 2.5 hr) (Ng et al., 1989). An examination of the pathway of HN endocytosis, by using immunoelectron microscopy, showed that HN is internalized via clathrin-coated pits (Leser et al., 1996), and HN internalization was shown to be independent of antibody binding (Ng et al., 1989; Leser et al., 1996). Although HN is internalized via clathrin-coated pits, HN lacks any of the previously characterized amino acid motifs in its cytoplasmic tail that act as signals to facilitate clathrin-mediated endocytosis. To confirm that internalization of HN is an intrinsic property of HN and not a result of SV5 infection of cells, CV-1 cells were infected with a SV40-recombinant virus that expresses HN. At 44 h p.i., cells were incubated with HN-specific immunoglobulins at 4°C and then incubated at 37°C for 0, 15, 30, or 60 min. After fixation and permeabilization, the cells were stained with an FITC-conjugated secondary antibody (Fig. 1A). At time zero, HN was uniformly distributed over the plasma membrane; the plane of the optical section was such that the membrane over the nucleus was out of the image and appeared unstained. After 15 min at 37°C, the staining became rearranged into a more punctate pattern and some large apparent aggregates were visible that were spread throughout the periphery of the cell. After 30–60 min incubation at 37°C, HN was found predominantly in bodies arranged in a juxtanuclear pattern typical of the late endosomal compartment (Lippincott-Schwartz and Fambrough, 1987), where HN is presumably degraded. We have not rigorously eliminated the possibility that HN is capable of endosome to cell surface recycling before degradation.

To confirm the identity of the subcellular compartment
FIG. 2. The HN internalization signal resides in N-terminal residues 1–48 but not in the HN cytoplasmic tail residues 2–13. (A) A chimeric protein was constructed consisting of the first 48 amino acids of SV5 HN (cytoplasmic tail, TM domain and 12 ectodomain residues) fused to all but the 16 N-terminal amino acids of chicken pyruvate kinase (APK). APK is a membrane bound protein that is expressed at the surface of cells (Hiebret and Lamb, 1988). CV-1 cells were infected with SV40-recombinant virus expressing APK, and at 40 h p.i. the cells were incubated with ovalbumin-Texas Red (ova-TR) to stain endocytic compartments. At 44 h p.i., cells were incubated at 4°C with antibody specific for pyruvate kinase and then incubated at 37°C for varying periods. Cells were fixed with formaldehyde, permeabilized with saponin, and stained with FITC-conjugated secondary antibody. Colocalization with APK staining with ova-TR is shown as yellow. (B) CV-1 cells were infected with a rescued SV5 virus that contains a deletion of HN cytoplasmic tail residues 2–13 (HN Δ2–13). At 16 h p.i., the cells were incubated at 4°C with HN-specific mAb and then incubated at 37°C for varying periods. Cells were fixed with formaldehyde and permeabilized with saponin and stained with FITC-conjugated secondary antibody.
in which internalized HN becomes localized, cells expressing HN or, as a control, cells expressing another type II integral membrane protein influenza A virus neuraminidase (NA) were costained for a lysosomal marker, the lysosomal membrane glycoprotein lamp-1. The juxtanuclear staining pattern of HN was coincident with that for the lysosomal protein lamp-1 as shown by the yellow-to-orange signal in Fig. 1B, suggesting internalized HN is shuttled to lysosomes. In contrast there was little if any overlap in the staining patterns between lamp-1 and NA which remained largely on the surface of the plasma membrane (Fig. 1B).

To obtain information on the rate of HN internalization, we compared the internalization of HN with that of wt HA and the tyrosine-containing mutant HA Y543. Quantification of HA internalization was carried out as described previously (Ktistakis et al., 1990). This assay takes advantage of the fact that HA is synthesized as a precursor (HA0) that can be cleaved at the cell surface by addition of exogenous trypsin. Internalized HA0 is protected from trypsin digestion. Internalization of metabolically labeled HA0 was measured by surface biotinylation. When the rates of internalization for HN, HA, and HA Y543 were compared at 44 h p.i., HN was found to be internalized with an initial rate of ~2–4%/min and HA Y543 was internalized at an initial rate of ~2–3%/min (Fig. 1C). It had been reported previously that SV40-expressed HA Y543 was internalized at ~2–3%/min when examined at 44 h p.i. (Ktistakis et al., 1990). In contrast, HA appeared to be stably expressed on the cell surface with almost no detectable internalization (Fig. 1C). Previously it had been found that when the internalization rate of HA Y543 was measured at 22–30 h p.i. with SV40-recombinant virus, internalization occurred more rapidly (8–9%/min) (Ktistakis et al., 1990; Lazarovits et al., 1996). We directly compared internalization of HA Y543 and SV5 HN at 28 h p.i. and found that they both had internalization rates of 6.5–7.5%/min (Fig. 1C). The reason for the difference in internalization rates for both HA Y543 and HN at the two different times after SV40 infection is not known. In the experiments described below, we have mainly compared the internalization of mutant HN molecules expressed by SV40-recombinant viruses at 44 h p.i. because of the greater radioactive or fluorescent signal obtained with the higher expression level at 44 h p.i. than at 28 h p.i. The faster rate of HA Y543 and HN internalization at 28 h p.i. than at 44 h p.i. does eliminate the possibility that the higher expression level at 44 h p.i. is causing the internalization.

The HN internalization signal is contained in its first N-terminal 48 residues

To localize a signal governing HN internalization, we examined the fate of a cell-surface-expressed chimeric protein, APK, which contains the N-terminal 48 residues of HN, (cytoplasmic tail, TM domain, and the first 12 residues of the HN ectodomain) fused to all of the cytoplasmic protein pyruvate kinase except its 16 N-terminal residues (Fig. 2A) (Hiebert and Lamb, 1988). CV-1 cells infected with SV40-recombinant virus expressing APK at 40 h p.i. were incubated with ovalbumin-Texas Red (ova-TR) as a marker for fluid phase internalization. At 44 h p.i. cells were incubated with APK-specific antibody at 4°C, and then endocytosis was permitted to occur at 37°C for 0, 30, 60 or 90 min. After fixation and permeabilization, the cells were stained with an FITC-conjugated secondary antibody (Fig. 2A). At time zero APK was observed on the cell surface in a punctate pattern, but after 30 min incubation APK staining was localized to vesicles in the periphery of the cell with some APK starting to accumulate in bodies around the nucleus. After 60 and 90 min at 37°C, APK was found in bodies surrounding the nucleus, coincident with the location of ovalbumin-Texas Red (yellow staining indicates coincidence of APK and ova-TR). After 90-min incubation, APK staining was considerably reduced, presumably because APK was being degraded. Thus as pyruvate kinase would not be expected to contain any signals related to the exocytic or endocytic pathways, the data suggest that the information sufficient to cause internalization resides within the first 48 amino acids of HN.

HN cytoplasmic tail residues 2–13 are not required for internalization

To assess the role of the HN cytoplasmic tail residues in mediating internalization, we used a rescued SV5 (rSV5) virus that contained a deletion of residues 2–13 in the HN cytoplasmic tail. The rSV5 HN Δ2–13 virus was obtained by manipulation of an infectious cDNA clone of the SV5 genome (He et al., 1997). Although HN Δ2–13 still contains the initiation methionine residue and four other residues including the hydrophobic residues VLF in the predicted cytoplasmic tail, it has been found previously that potential internalization signals that are adjacent to the lipid bilayer are not active (Jadot et al., 1992; Prill et al., 1993), presumably due to steric hindrance of the signal with effector molecules. Deletions in the HN cytoplasmic tail greater than Δ2–13 were not useful due to the deleterious effects of the deletions on intracellular transport of HN. CV-1 cells were infected with rSV5 HN Δ2–13, and at 16 h p.i. cells were reacted with HN-specific antibodies at 4°C and then incubated at 37°C for 60 min to allow internalization to proceed before the cells were fixed, permeabilized, and reacted with an FITC-conjugated secondary antibody. As shown in Fig. 2B, at time zero HN Δ2–13 was distributed diffusely over the plasma membrane, whereas after 60 min at 37°C, HN Δ2–13 was localized in vesicles surrounding the nucleus indicative of localization to the late endocytic/lysosomal
Thus HN residues 2–13 are not required for HN internalization.

HN containing a poly-leucine TM domain is internalized

The TM domain of several proteins has been implicated in the retention of Golgi-resident proteins (Munro, 1995) and the sorting of membrane proteins between cellular compartments (Banfield et al., 1994; Rayner and Pelham, 1997; Yang et al., 1997). The TM domain has not been identified as containing signals mediating internalization; however, specific substitutions in the TM domain of HA allow its passive incorporation into coated pits (Lazarovits et al., 1996). We investigated the role of the HN TM domain sequence in mediating internalization. A mutant HN protein was expressed that had the HN TM domain substituted with 19 leucine residues (HNleu) (Fig. 3A) working under the assumption that a stretch of 19 leucine residues would contain neither a positive nor negative signal for endocytosis. The kinetics of resistance of HNleu carbohydrate chains to digestion by endoglycosidase H (endo H), indicative of transport to the medial Golgi apparatus, had a t_{1/2} ~ 76 min as compared with a t_{1/2} ~ 90 min for wt HN. However, when the level of plasma membrane expression of HNleu was analyzed by flow cytometry using a conformation-specific mAb (HN4b) (Ng et al., 1989), it was found to be only 60% of the level of wt HN. Nonetheless, chemical cross-linking of the cell-surface-expressed HN molecules indicated that HNleu formed dimers and tetrabers in proportions similar to wt HN (data not shown). The ability of cells infected with SV40-HNleu recombinant virus to internalize antibody bound to surface expressed HNleu protein was analyzed as described for wt HN (Fig. 3B). The fluorescence staining observed was initially diffuse over the cell surface; however, after 60 min at 37°C, most of the protein showed a staining pattern consistent with localization of HNleu to the late endosomal compartment. The rate of internalization of HNleu was measured using the biotinylation assay detailed above. HNleu was found to be endocytosed at a rate similar to that of wt HN (Fig. 3C). HN containing a 22-leucine residue TM domain also was examined, and it also was found to be internalized at a rate similar to wt HN (data not shown).

The first seven amino acids of the HN ectodomain causes internalization of a reporter protein that is normally stably expressed at the cell surface

As APK that contains the N-terminal 48 residues of HN was internalized (Fig. 2A) and as neither the HN cytoplasmic tail residues 2–13 (Fig. 2B) nor the TM domain residues (Fig. 3) are required for internalization, it seemed possible that the residues of HN that abut the presumptive TM domain and remained in APK may mediate internalization. A reporter protein was sought that is not internalized from the cell surface yet could tolerate protein engineering without protein malfolding occurring. The HN protein of NDV, a virus closely related to SV5 fulfilled these criteria, and chimeras were constructed (Fig. 4A). The NDV HN chimeric protein SSS^{7}N contained the cytoplasmic tail, TM domain, and the first seven amino acids (S^{7}) of the ectodomain of SV5 HN joined to the ectodomain of NDV HN (amino acids 56–570), working again on the assumption that the NDV ectodomain begins with the first charged residue after the hydrophobic TM domain. In the NDV HN chimeric protein NNS^{7}N, the seven NDV HN ectodomain residues adjacent to the TM domain were substituted with the S^{7} region from SV5 HN. The protein NDV NleuN has the NDV TM domain substituted with 19 leucine residues. The NDV chimeric protein NleuS^{7}N is NDV HN with the 22-residue TM domain substituted with 19 leucine residues and the first seven residues of the NDV ectodomain substituted with the S^{7} region of the SV5 HN ectodomain. The chimeric proteins were expressed in CV-1 cells using SV40-re-
combinant viruses. All of the chimeric proteins reacted with three conformation-specific NDV HN mAbs in immunoprecipitation reactions to comparable extents, suggesting normal protein folding, and all the chimeras formed equivalent amounts of disulfide linked dimers and tetramers suggesting normal oligomerization (data not shown). The rate at which chimeric HN proteins acquired endo H resistance, indicative of transport of the proteins to the medial Golgi apparatus has been found repeatedly to be the most reliable indicator of native protein folding and exit from the ER. As shown in Figs. 4B and 4C, the four NDV chimeric HN molecules were all transported to the medial Golgi apparatus fairly equivalently.

The chimeric proteins were assessed for their ability to be internalized from the surface of CV-1 cells by examination of the subcellular localization of surface bound antibody after incubation at 37°C (Fig. 5), and the kinetics of internalization was determined using the biotinylation assay (Fig. 6). After 60 min at 37°C, the fluorescence staining pattern of NDV HN differed little from the distribution observed without incubation (Fig. 5, NDV HN) and the protein was spread diffusely over the surface of the cell. In the biotinylation assay, wt NDV HN showed very little, if any, internalization (Fig. 6). In contrast the staining pattern of the chimeric protein SSS7N after 60 min incubation at 37°C showed a staining pattern that was condensed and clustered around the nucleus in a pattern similar to that observed for wt SV5 HN (Fig. 1). The biotinylation internalization assay showed a rate of ~3%/min, and the percent internal at steady state was similar to that found for wt SV5 HN (Fig. 6). Chimeric protein NNS7N appeared not to be internalized in either the immunofluorescence assay or in the biotinylation assay (Figs. 5 and 6, NNS7N). This result was unanticipated as the data obtained to date appeared to be suggesting that the S7 ectodomain residues contained the internalization signal. However, it was considered possible that the NDV HN TM domain might contain a signal that causes exclusion of NDV HN from coated pits as has been suggested for HA (Lazarovits et al., 1996). Furthermore it is always possible when dealing with chimeric proteins that a substitution may cause local non-native structure. Thus given that SV5 HN containing 19 leucine residues as a TM domain was internalized (Fig. 3), two derivatives of NDV HN were constructed, NleuN and NleuS7N (Fig. 4A). NDV HN NleuN was not internalized above background levels (Figs. 5 and 6). In contrast in the immuno-fluorescence analysis after 60 min at 37°C, NDV HN NleuS7N showed a staining pattern characteristic of the late endosomal/lysosomal cellular compartment (Fig. 5, NleuS7N). In the biotinylation assay, NDV HN NleuS7N was internalized at an initial rate of ~3–4%/min and had a steady-state level of internalized HN comparable with that of SV5 wt HN (Fig. 6, NleuS7N). To demonstrate that NDV HN NleuS7N was internalized

![FIG. 4. Intracellular transport of mutant and chimeric NDV HN molecules to the medial Golgi apparatus. (A) A series of mutant and chimeric NDV HN proteins was constructed. SSS7N is NDV HN with its cytoplasmic tail, TM domain, and the first seven amino acids of the ectodomain substituted with the equivalent cytoplasmic tail, TM domain and the first seven ectodomain residues of SV5 HN. NNS7N is NDV HN with the first seven residues of the NDV ectodomain substituted with the first seven residues of the SV5 HN ectodomain. NleuN is NDV HN with the 22-residue TM domain substituted with 19 leucine residues. NleuS7N is NDV HN with the 22-residue TM domain substituted with 19 leucine residues and the first seven residues of the NDV ectodomain substituted with the first seven residues of the SV5 HN ectodomain. (B and C) Kinetics of resistance of HN carbohydrate chains to digestion by endoglycosidase H. SV40-recombinant virus-infected cells at 44 h p.i. were pulse-labeled with [35S]-Promix label for 10 min, and the cells incubated in chase medium for varying periods (min). HN proteins were immunoprecipitated, digested with endoglycosidase H and polypeptides analyzed by SDS–PAGE.](https://example.com/figure4.png)
via clathrin-coated pits, SV40-recombinant virus-infected CV-1 cells expressing NDV HN NleuS'N were examined by immunoelectron microscopy. Cells were surface labeled at 4°C with NDV HN-specific antibodies followed by secondary immunoglobulins conjugated to 10 nm colloidal gold particles. After 15 min at 37°C, the mutant protein was clustered in coated pits (Fig. 6, inset), and some large open vesicles having the morphology of early endosomes also were labeled with gold particles (results not shown). In contrast, in parallel experiments performed on cells expressing wt NDV HN, no clustering in coated pits or internal labeling of the cells was observed (data not shown). Thus the reporter molecule NDV HN NleuS'N is internalized through clathrin-coated pits, and the data suggest that in the context of an appropriate TM domain the S' residues of the SV5 HN ectodomain govern internalization.

A glutamic acid residue at the HN TM domain/ectodomain boundary is critical for internalization

As the S' region of SV5 HN is involved in signaling internalization, it was of interest to define further the signal in the context of SV5 HN. It has been suggested that exposure of hydrophobic residues to the cytosol, or conversely polar residues to the lipid bilayer, enables interactions that allow membrane protein internalization (Lazarovits et al., 1996). Thus we examined if there was a role of the glutamic acid residue abutting the presumptive TM domain in SV5 HN internalization, especially as this residue is common to both SV5 and NDV HN proteins. The SV5 HN E37 was substituted with D and K (charged), L (hydrophobic), or Q (polar) residues (Fig. 7), and the mutants were expressed using SV40-recombinant viruses. The SV5 HN E37 mutant proteins were found to acquire endo H resistance at a rate slightly faster than wt HN, and the level of HN surface expression (examined by flow cytometry) and oligomerization (examined by chemical cross-linking) were found to be comparable with wt HN (results not shown). The ability of the HN E37 mutants to be internalized from the plasma membrane of CV-1 cells was measured by using surface biotinylation as described above. HN E37D was found to be internalized but with an initial rate that was reduced from that found for wt SV5 HN, and the amount of HN E37D internal at steady state also was reduced as compared with wt SV5 HN (Fig. 7). HN E37K, HN E37L, and HN E37Q if internalized at all, were at levels barely above background. The effect of reversing the stretch of amino

NDV HN wt, mutant, and chimeric cDNAs and at 44 h p.i. NDV HN-specific mAbs were bound to the cells at 4°C. After incubation of the cells at 37°C for 60 min the cells were fixed with formaldehyde and permeabilized with saponin. NDV HN antibodies were stained using a FITC-labeled secondary antibody.
acids from residues 37 to 43 in HN (HNrev) also was tested, and it was found that internalization of HNrev from the cell surface was greatly diminished as compared with wt SV5 HN (Fig. 7, HNrev). When leucine was substituted for the glutamate at position 37 and glutamate for lysine at position 43, in effect moving the glutamate residue downstream relative to the TM domain, internalization occurred at a rate and extent very similar to that found for the HNrev mutant (results not shown). Thus a glutamic acid residue at the presumptive TM domain/ectodomain boundary appears critical for efficient internalization of SV5 HN.

FIG. 6. Kinetics of internalization of NDV HN mutant and chimeric proteins. The wt, mutant, and chimeric NDV proteins (SSS'N, NNS'N, NleuN, and NleuS'N) were assayed for their ability to be internalized from the cell surface. CV-1 cells were infected with SV40-recombinant viruses expressing the wt, mutant, and chimeric NDV HN cDNAs, and at 44 h p.i. cells were metabolically labeled and HN internalization assayed by cell surface biotinylation. The amount of HN found to be internal was determined by the fraction sequestered from the action of the membrane impermeable reducing agent, MESNa. Inset: Morphological examination by immunoelectron microscopy of NDV HN NleuS'N internalized from the cell surface was performed as described previously (Leser et al., 1996). NDV HN NleuS'N was concentrated within coated pits (arrows) and internalized through these specialized membrane structures (inset; bar, 0.2 μm).
FIG. 7. Substitution of the charged residue at the HN TM domain/ectodomain boundary affects the rate of internalization. SV5 HN residue E37 was substituted with D, K, L, or Q residues, (HN E37D, HN E37K, HN E37L, and HN E37Q), respectively. In addition, seven ectodomain residues, ESLITQK, that abut the TM domain were substituted with the residues in the reverse order, KQITLSE (HNrev). The altered HN cDNAs were expressed in CV-1 cells by using SV40-recombinant viruses, and at 44 h p.i. cells were metabolically labeled and internalization assays performed on biotinylated cell surface molecules.
Previously we had shown that HN is internalized from the cell surface of SV5-infected cells via clathrin-coated pits with a rate of 4–6%/min (Leser et al., 1996). SV5 HN is turned over with a $t_{1/2} \sim 2.5$ h, and discrete degradation products (M, $\sim 27$ kDa and $34$ kDa) are observed (Ng et al., 1989; Leser et al., 1996). Although HN colocalized with markers for lysosomes, where it is assumed to be degraded, we have not eliminated the possibility of one or more rounds of endosome to cell-surface recycling occurs before degradation. Examination of the rate of internalization of HN expressed from SV40-recombinant virus at 28 h p.i., by using the surface biotinylation assay, yielded a rate of 6.5–7.5%/min. This rate is comparable with that of several endocytosed cellular receptors (reviewed in Trowbridge et al., 1993) and much faster than the rate of basal membrane turnover, although these estimates are widely disparate [0.06%/min (or less) to $\sim 2$/min] (Steinman et al., 1983; Draye et al., 1988; Bretscher, 1992; Almond and Eidels, 1994). The rate of internalization of both HN and HA Y543 when expressed by SV40-recombinant viruses was slower at 44 h p.i. than that found when HN is expressed transiently: the rapid rate of HN internalization observed at 28 h p.i. is at an expression level lower than that found when HN is expressed transiently using a plasmid based vector, pCAGGS, that uses a $\beta$-actin promoter (unpublished observations).

As SV5 HN lacks any previously characterized signal for internalization (YXXZ, NPXY, LL, LI, KK, or addition of ubiquitin), we attempted to elucidate the region in HN containing the signal for internalization. Analysis of cell-surface-expressed chimeric pyruvate kinase (APK), a molecule that contains only 48 N-terminal residues of HN, indicated that APK was internalized like wt HN. As pyruvate kinase is normally localized to the cytoplasm, it would not be expected to contain internalization signals and thus the data indicate that residues 1–48 of HN contain the internalization signal. HN lacking residues 2–13 of the cytoplasmic tail (residues 1–17) was internalized like wt HN molecules, and HN containing a TM domain consisting of 19 leucine residues in place of the normal TM domain (residues 18–36) was internalized like wt HN. Thus these data implicate the HN residues that form the presumptive boundary of the HN TM domain and the HN ectodomain as important for signaling HN internalization.

A suitable reporter molecule was sought that was not internalized yet could withstand protein engineering without protein malfolding occurring. The HN protein of NDV (a virus closely related to SV5), fulfilled these criteria. It was found that when NDV ectodomain residues 56–570 were fused to the cytoplasmic tail, TM domain and first seven ectodomain residues of SV5 (S$^7$), the chimeric molecule, SSS$^7$N, was internalized like wt SV5 HN. However, when the SV5 ectodomain S$^7$ residues were substituted into NDV (NNS$^7$N) internalization was not detected. However, whereas NleuN (NDV HN with a TM domain consisting of 19 leucine residues) was not internalized, a chimeric NDV protein NleuS$^7$N (substitution of the SV5 ectodomain S$^7$ residues into NleuN) was internalized like wt SV5. Further characterization of the SV5 S$^7$ ectodomain residues in the SV5 HN background indicated that glutamic acid residue 37 is critical for rapid internalization. We have not determined the contribution, if any, of other residues in the S$^7$ region. Aspartic acid can substitute for E37 although the rate of internalization was slower than that of wt HN. The position of the glutamic acid residue relative to the junction with the TM domain is also important as reversing the order of the S$^7$ region or moving the glutamic acid residue to position 43 significantly diminished the rate of HN internalization.

Assuming that the delineation of the TM domain boundary is correct, the simplest interpretation of these data is that the SV5 HN S$^7$ residues (37–43) specify internalization but that the signal is only operative in certain TM domain contexts. Thus we consider that whereas the SV5 HN TM domain or 19 leucine residues are permissible contexts, the NDV TM domain is a nonpermissive context. The TM/ectodomain junction region in SV5 HN is much more hydrophobic than that of NDV HN even when the 19 leucine residues are substituted for the NDV HN TM domain. The hydrophobic environment may provide the favorable context for SV5 HN internalization: the interaction of cytoplasmic tyrosine-containing motifs with AP2 is now thought to involve hydrophobic regions rather than a specific conformation (Owen and Evans, 1998). Alternatively, the NDV TM domain may contain a signal that prevents movement of HN into coated pits. A role for TM domains in protein signaling, either by virtue of their length or their sequence, has been identified for the retention of Golgi-resident proteins (Munro, 1995) and the sorting of membrane proteins between cellular compartments (Banfield et al., 1994; Rayner and Pelham, 1997; Yang et al., 1997). However, we have no reason to believe that length of the SV5 HN TM domain is involved in HN internalization as we found that SV5 HN containing 22 leucine residues (the length of the NDV HN TM domain) also is internalized like wt SV5 HN. An example of competing protein signals is found with HA. Native HA is normally excluded from coated pits. Implicated in this process are sequences in the TM domain toward the outer leaflet of the lipid bilayer and amino acid substitutions in the HA TM domain causes HA to enter coated pits passively and to be internalized at a slow rate (Leser et al., 1996).
Furthermore these same HA mutants reduce the presence of these mutant HAs in cholesterol-rich membrane rafts (Lin et al., 1998). In contrast the presence of tyrosine at residue 543 of the HA cytoplasmic tail, which causes HA to be rapidly internalized, is presumably a strong signal and effectively overrides the HA TM domain signal that causes HA exclusion from coated pits.

The involvement of the SV5 HN S\(^7\) region in specifying internalization was unanticipated. However, there are an increasing number of reports of protein sorting information residing in ectodomains. The sorting signal specifying the apical delivery of the neurotrophin receptor (p75\(^{NTR}\)) has been localized to the stalk region of the extracellular domain and may require the O-glycosylation of serine/threonine residues (Yeaman et al., 1997). Deletion of either the cytoplasmic or TM domains of the amyloid precursor protein does not alter its basolateral targeting, suggesting that unidentified sorting determinants are contained within the extracellular domain (De Strooper et al., 1995). The luminal stalk region of N-acetylglucosamine transferase I is necessary and sufficient for kin recognition and retention in the Golgi (Nilsson et al., 1996). The glycolipid-anchored prion protein (PrP\(^{30}\)) has information contained within its N-terminal region that provides for its internalization via clathrin pits, and it is thought internalization occurs through an intermediary protein that is in association with coated pits or is internalized through coated pits (Shyng et al., 1995).

For the previously, better-characterized internalization signals, specific interactions between the signals and the internalization machinery have been identified. The FXNXPXY signal of the LDL receptor is thought to have a direct association with the globular domain of the clathrin triskelion (Kibbey et al., 1998), the YXXZ-based internalization motifs are thought to interact directly with the $\mu_2$ chain of AP2 (Boll et al., 1996; Ohno et al., 1996; Rapoport et al., 1997), and peptides containing the dileucine motif bind to the $\beta$ chain of AP1 for sorting at the level of the TGN. Nonetheless, the machinery that interacts with the dileucine signal to facilitate receptor internalization from the plasma membrane has been difficult to identify possibly due to low-affinity protein interactions (Rapoport et al., 1998). Furthermore other atypical cytoplasmically located signals also mediate endocytosis such as the signals found in E- and P-selectin (Setiadi et al., 1995; Chuang et al., 1997) and their interacting partners remain unknown.

HN internalization is not antibody dependent (Ng et al., 1989; Leser et al., 1996), but we cannot rule out self-aggregation of HN through the S\(^7\) domain as a means of driving endocytosis. Alternatively, the HN S\(^7\) region may interact with proteins or lipids at the outer leaflet of the plasma membrane with HN only interacting indirectly with the components of coated pits: e.g., HN could interact with a hypothetical coated pit receptor. The use of an intermediary protein as an adaptor for internalization has been documented in the case of the $\beta_2$-adrenergic receptor that binds $\beta$-arrestin to traffic into the endocytic system (Goodman et al., 1996; Zhang et al., 1997), and $\beta$-arrestin has been shown to interact directly with the globular terminal domain of clathrin (Goodman et al., 1997; Kupnick et al., 1997).

Although we have assumed above that the SV5 HN S\(^7\) region is exposed on the luminal side of the membrane, the boundary between the HN TM domain and the HN ectodomain has not been demonstrated experimentally. The boundary position is based on the location of the charged residue (E37) adjacent to the hydrophobic region. It is possible that the boundary has been incorrectly delineated and E37 resides in the lipid bilayer. Charged residues are found in some TM domains of non-ion channel proteins, and for some subunits of the T-cell receptor complex the charged residue has been shown to be essential for its ER degradation (Bonifacino et al., 1991; Cosson et al., 1991). As hypothesized above, the role of charged residues in a TM domain may be to mediate specific associations with other membrane proteins. Substitution of the SV5 HN S\(^7\) region into NDV HN may have failed to cause internalization because the S\(^7\) region was placed outside of the NDV HN TM domain. Clearly the presence of a negatively charged residue adjacent to a TM domain is not sufficient to initiate internalization as NDV HN, like many other noninternalized proteins, has a glutamic acid at this position. The machinery with which the SV5 HN internalization signal interacts to mediate the interaction with clathrin-coated pits remains to be elucidated.

MATERIALS AND METHODS

Recombinant DNA construction and SV40-recombinant viruses

Recombinant DNA manipulations were carried out according to standard protocols, and specific details are available on request. Nucleotide numbers refer to the published sequences for SV5 HN (Hiebert et al., 1985) and Newcastle disease virus (NDV) HN strain A-V (McGinnies et al., 1987). The point mutations at amino acid E37 in SV5 HN were constructed using the M13mp19 phagemid vector (Paterson and Lamb, 1987) containing HN cDNA as template as described previously (Lamb et al., 1985). The mutations were confirmed by dideoxy chain-terminating DNA sequencing. SV5 HN and NDV HN cDNAs were cloned into pGEM3X in the orientation of the T\(_1\) RNA promoter and used as templates for other constructs described below. The rearrangement of amino acids 37–43 of SV5 HN was performed using USE mutagenesis (Amersham Pharmacia Biotech, Picataway, NJ). The substitution of the SV5 HN TM domain for 19 leucine residues has been previously described (Parks and Pohlmann, 1995). Rescued SV5 (rSV5) virus lacking HN cytoplasmic tail residues 2–13.
was derived using the general scheme described previously (He et al., 1997), and the virus was kindly provided by Dr. Anthony Schmidt (Northwestern University, Evanston, IL).

The NDV HN cDNA was described previously (Horvath et al., 1992). The NDV HN chimeric proteins SSS\(^N\), Nleu\(^N\), and Nleu\(^S\)\(^N\) were constructed by four-primer polymerase chain reactions (PCR). The NDV HN NNS\(^N\) construct was made using the USE mutagenesis procedure. Mutants constructed by four-primer PCRs had their entire coding sequence confirmed by sequencing using an ABI 310 sequencer (Applied Biosystems, Foster City, CA). The construction of the anchored chicken pyruvate kinase (APK) containing the cytoplasmic tail, TM domain, and the first 12 amino acids of the SV5 HN ectodomain joined to all but the N-terminal 16 amino acids of pyruvate kinase has been described previously (Hiebert and Lamb, 1988).

SV40-recombinant virus stocks were prepared according to Naim and Roth (1994). SV40 vectors expressing SV5 HN and NDV HN mutant proteins were prepared by subcloning into the SV40 vector pSV103 at the XhoI site (Paterson et al., 1985). SV40 vectors expressing Japan HA (Doyle et al., 1985) and a mutant containing a tyrosine at position 543 (HA Y543) in the cytoplasmic tail of HA (Lazarovits and Roth, 1988) were gifts of Dr. M. Roth (University of Southwestern Texas Medical Center, Dallas, TX). SV40-recombinant virus expressing influenza virus A/Tokyo/3/67 NA was prepared by subcloning the NA insert of NA pGEM3X into pSV103.

Cells and viruses

CV-1 cells were maintained in Dulbecco’s modified Eagle’s medium (DME) containing 10% NU-Serum (Collaborative Research Laboratories, Inc., Bedford, MA). Infection of CV-1 cells with SV40-recombinant virus stocks was performed as described (Naim and Roth, 1994). Cells infected with SV40-recombinant viruses were used at 44 h p.i. unless indicated. Infection of CV-1 cells with SV5 was performed as described previously (Paterson and Lamb, 1993).

Indirect immunofluorescence, flow cytometry, and electron microscopy

Indirect immunofluorescence microscopy on formaldehyde fixed and saponin permeabilized CV-1 cells was carried out essentially as described previously (Ng et al., 1989; Leser et al., 1996). Simultaneous staining with ovalbumin-Texas Red was performed as described previously (Leser et al., 1996). SV5 wild-type (wt) and mutant HN proteins were stained using mAb HN5a or, for double-labeling, a HN-specific rabbit polyclonal antiserum. The lysosomal membrane glycoprotein lamp-1 was detected using a mouse mAb, made available by Dr. M. Fukuda (Burnham Institute, La Jolla, CA). Rabbit anti-serum specific for influenza neuraminidase (NA) was obtained from Dr. R. Webster (St. Jude Children’s Research Hospital, Memphis, TN), and rabbit antibodies specific for chicken pyruvate kinase have been described previously (Hiebert and Lamb, 1988). Wt and mutant NDV HN constructs were detected by using one of four specific mAbs (2B, 3C, 4A, and 14F) provided by Dr. R. Iorio (University of Massachusetts Medical School, Worcester, MA). Preparations were examined and documented using a Zeiss LSM 410 confocal microscope (Carl Zeiss, Inc., Thornwood, NY). Fluorescence intensity of 10,000 cells was measured by flow cytometry (FACS-Caliber, Becton Dickinson, Mountain View, CA). Immunogold labeling and electron microscopy was performed using NDV HN specific mAbs as described previously (Leser et al., 1996).

Immunoprecipitation, endoglycosidase H digestions, and SDS–PAGE

Metabolic labeling with \[^{35}S\]-Promix label (Amersham Life Sciences, Inc, Arlington Heights, IL), immunoprecipitation, endoglycosidase H treatment and SDS–PAGE on 10 or 15% acrylamide gels under reducing conditions were carried out as described previously (Leser et al., 1996). Depending on the protein expressed, cell lysates were incubated with either 100–1,000 μg tissue culture supernatant containing mAb specific for NDV HN or 4 μl of either SV5 HN-specific rabbit polyclonal antiserum or mouse monoclonal ascites fluid HN5a. Gels were analyzed using a Fuji Bio-Imager 1000 and MacBas Software (Fuji Biomedical Instruments, Stamford, CT).

Internalization assays

Analysis of SV5 HN or NDV HN internalization. CV-1 cells infected with SV40-recombinant viruses expressing wt HN or mutant HN proteins were used at 44 h p.i. unless noted. The rate of internalization of SV5 HN was measured using a surface biotinylation assay using thio- cleavable NHS-SS-biotin (Pierce, Rockford, IL) as described previously (Leser et al., 1996). This assay takes advantage of the sequestration of internalized biotinylated proteins from the action of the membrane impermeable reducing agent 2-mercaptoethanesulfonic acid (MESNa, Sigma Chemical Co., St. Louis, MO). Due to the variability inherent in this assay, the results presented are the average of at least three separate experiments unless otherwise noted. Gels were quantified using a Fuji Bio-Imager 1000 and MacBas Software (Fuji Biomedical Instruments, Stamford, CT). Internalization was expressed as the percent of the total biotinylated protein (untreated samples) recovered at each time point that was internalized (MESNa treated samples).

Analysis of HA internalization. The internalization assay for both wt HA and mutant Y543 HA were carried out as described (Lazarovits and Roth, 1988; Ktistakis et al.,
1990; Leser et al., 1996). SV40-recombinant virus-infected cultures were used 44 h p.i. The antibody used was a goat anti-HA (A/Singapore/1/57) (H2 subtype) (NIAID, NIH, Bethesda, MD). Polypeptides were separated on either 10 or 15% SDS–PAGE gels. Radioactivity was quantified as described above.

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