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Analysis of parainfluenza virus-5 hemagglutinin-neuraminidase protein mutants that are blocked in internalization and degradation

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ABSTRACT

The PIV-5 hemagglutinin-neuraminidase (HN) protein is a multifunctional protein with sialic acid binding, neuraminidase and fusion promotion activity. HN is internalized by clathrin-mediated endocytosis and degraded. HN lacks internalization signals in its cytoplasmic tail but a single glutamic acid present at residue 37 at the putative transmembrane/ectodomain boundary is critical. We rescued rPIV-5 with mutations E37D or E37K, which have been shown to impair or abolish HN internalization, respectively. These viruses exhibited growth properties similar to wild-type (wt) virus but are impaired for fitness in tissue culture. Biochemical analysis of HN activities showed differences between HN E37D and HN E37K in fusion promotion and incorporation of HN and F into virions. Furthermore, oligomeric analyses indicate that HN E37 mutants perturb the tetrameric organization of HN, probably by destabilizing the dimer-of-dimers interface.

Introduction

Paramyxoviruses are enveloped, negative-stranded RNA viruses that include many clinically and agriculturally important pathogens such as mumps virus, measles virus, Newcastle disease virus (NDV), Hendra virus, and Nipah virus. Cellular entry by paramyxoviruses is mediated by two glycoproteins present at the surface of the virion. For the paramyxovirus parainfluenza virus 5 (PIV-5), these proteins are the fusion protein (F) and the hemagglutinin-neuraminidase protein (HN). F mediates the fusion of the viral membrane with the cellular plasma membrane at neutral pH. Co-expression of the HN protein enhances this fusion process by lowering the activation energy required for F to mediate fusion (Russell et al., 2001). In addition to its fusion promotion activity, HN also functions in binding the virion to its receptor sialic acid on target cells and possesses receptor-destroying activity (neuraminidase activity) that cleaves sialic acid from the surface of both infected cells and virions. This action is thought to prevent the aggregation of budded virions at the surface of infected cells. The viral matrix (M) protein is a peripheral membrane protein that underlies the lipid bilayer and makes contact with the glycoprotein cytoplasmic tails. The PIV-5 ribonucleoprotein is composed of three proteins: nucleocapsid (NP), phosphoprotein (P), and the large polymerase (L), which together act to transcribe and replicate the genome RNA (Lamb and Parks, 2007). Additionally, PIV-5 contains proteins that assist in evasion of host cell immunity: the small hydrophobic protein (SH), which inhibits tumor necrosis factor alpha signaling and prevents apoptosis in infected cells (He et al., 2001; Lin et al., 2003; Wilson et al., 2006) and the V protein that antagonizes interferon synthesis and signaling (Andrejeva et al., 2004; Didcock et al., 1999).

PIV-5 HN is a type II integral membrane protein that consists of a short N-terminal cytoplasmic tail of 17 residues, a hydrophobic domain of 19 residues that acts as both a signal sequence to target HN to the ER membrane and as a stop-transfer transmembrane (TM) domain, a stalk region of 82 residues, and a large globular head (447 residues) that contains both the receptor binding and destroying activities (Hiebert et al., 1985; Parks and Lamb, 1990). HN exists at the surface of virus-infected cells as a tetramer, consisting of two disulfide-linked dimers that are linked through non-covalent interactions (Ng et al., 1989). The crystal structure of the full-length ectodomain of HN has been solved both in the presence and absence of ligand (Yuan et al., 2005). No electron density was identified for the stalk region; however, biophysical data indicates that the stalk adopts a flexible and rod-like α-helical conformation. Additionally, HN head domain expressed with the stalk forms a tetramer, whereas expression of the HN head domain on its own is monomeric. Thus it is thought that the stalk domain stabilizes the head domain oligomer (Yuan et al., 2008). The enzymatically active head region of HN contains the antigenic sites of the protein and has a typical sialidase/neuraminidase fold, a superbarrel with six antiparallel β strands with a centrally located active site. The crystal structure data shows that

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the dimer interface within the head region buries an extensive area between the monomers of 1810 Å^2. In contrast, the interface between the dimer-of-dimers is much smaller, burying only 657 Å^2 and involving 10 residues (Yuan et al., 2005). This suggests that the dimer-of-dimers interface may be easier to perturb. The interaction between the dimer-of-dimers interface is not well conserved among paramyxovirus attachment proteins, and its weaker interaction energy could be a feature of the process of F activation.

Despite the fact that PIV-5 HN is a major spike glycoprotein of the budded virion, in virus-infected cells PIV-5 HN is extensively internalized from the cell surface whereas F is not internalized and is stably expressed at the cell surface (Ng et al., 1989). Furthermore, it has been shown that HN is internalized by clathrin-coated pits and enters the endocytic pathway (Leser et al., 1996). When HN was expressed from cDNA using an SV40-recombinant virus it was found that the rate of HN turnover from the cell surface was 6.5–7.0%/min, which is faster than the bulk membrane turnover and comparable with the rate of other endocytosed receptors. Analysis by electron microscopy showed that PIV-5 HN colocalizes with transferrin and gold-conjugated bovine serum albumin, markers for early endosomal/late endosomal compartments and lysosomal compartments, respectively (Leser et al., 1996). Furthermore it was shown by fluorescence microscopy that HN colocalized with lamp-1, indicating that the lysosome is the subcellular compartment where internalized HN becomes localized (Leser et al., 1996).

The observation that PIV-5 HN is internalized from the cell surface was surprising, as the protein lacks a canonical internalization signal in its cytoplasmic tail. The 17 residue cytoplasmic tail of HN does not contain a tyrosine-based internalization sequence, nor does it have other known internalization signals such as a di-leucine, a di-lysine, or a site for monoubiquitination (Bonifacino and Weissman, 1998; Chen et al., 1990; Collawn et al., 1990; Hamer et al., 1997; Itin et al., 1995; Letourneur and Klausner, 1992; Trowbridge et al., 1993). Mutagenesis experiments showed the cytoplasmic tail of HN is dispensable for internalization (Leser et al., 1999). Further, a single glutamic acid (E37), the first residue of the ectodomain at the putative TM/ectodomain boundary, was shown to be critical for HN internalization (Leser et al., 1999). A series of E37 HN mutants were constructed and expressed in cells. One of these mutants, E37D, had a rate of internalization and overall extent of internalization that was significantly less than that of wild-type (wt) HN. Other mutants, such as E37K, were not internalized above background levels. Reversing the first seven amino acids of the ectodomain was also examined, as was substituting leucine for the glutamic acid at position 37 and glutamic acid for the leucine at position 43; all of these combinations diminished HN internalization. Thus it was concluded that the presence of a glutamic acid at position 37 was critical for PIV-5 HN internalization (Leser et al., 1999).

We have examined the role of internalization mutants on the lifecycle of PIV-5 through the rescue of recombinant PIV-5 containing the mutations HN E37D or E37K. These rescued viruses were greatly impaired or defective for HN internalization during cellular infection. We describe the growth characteristics of these viruses together with an analysis of the enzymatic and receptor binding activities of the mutated HN proteins. The ability of these E37 mutants to promote F mediated fusion is also described, and related to findings regarding the oligomeric organization and stabilities of these proteins.

Results

HN internalization in virus-infected cells

Previous work has demonstrated that in PIV-5-infected cells, HN is internalized from the cell surface and degraded by lysosomes (Ng et al., 1989). Analysis of HN expressed from a recombinant SV40 virus indicated that internalization of HN was an intrinsic property of the HN protein, not requiring expression of other PIV-5 proteins (Leser et al., 1996). It was also found that mutation of the charged residue (E37) that defines the ectodomain/TM domain boundary largely abolished internalization (Leser et al., 1996, 1999). To determine if the HN E37 mutants affected internalization in PIV-5-infected cells, HN E37D and HN E37K mutations were recovered in PIV-5 virus using a previously described reverse genetics system (He et al., 1997; Waning et al., 2002). To detect HN degradation, CV-1 cells were infected with wt PIV-5 or with mutant HN E37D or HN E37K viruses. The virus-infected cells were metabolically labeled with [^{35}S]-TranS-label in a pulse chase protocol followed by immunoprecipitation with a mix of monoclonal antibodies and analysis of polypeptides by SDS-PAGE. Two of the antibodies used for immunoprecipitation (HN-1b and HN-4b) have been shown previously to be conformation specific (Ng et al., 1989). Thus, samples taken at 0 time display lower signals than those samples taken after 1 h because the conformational epitopes had not fully matured (Fig. 1).

Cells infected with wt virus displayed robust fragmentation of HN, with degradation products (40 kD and 34 kD) detectable as early as 2 h in the chase period (Fig. 1). By 5 h post-label, approximately 61% of the detectable HN protein was present as degradation products. In contrast, cells infected with either PIV-5 HN E37D or HN E37K viruses did not display high levels of HN degradation. For HN E37D virus-infected cells, degradation products were barely detectable at 3 h post-label and comprised only 2.7% of detectable HN at 5 h post-label. Results for HN E37K virus-infected cells were similar with degradation products comprising less than 1% of all detectable HN at the 5 h time point. The small difference in HN E37D and HN E37K degradation mirrors earlier findings in that the block of HN E37D internalization is not absolutely complete as compared with HN E37K internalization, which is indistinguishable from the bulk membrane turnover (Leser et al., 1999). This analysis shows that mutations at residue E37 in HN block internalization and degradation of the protein in the context of virus-infected cells.

To assess further the stability of HN E37D and HN E37K at the cell surface protein synthesis was inhibited in virus-infected cells at 18 h p.i. by treatment with cycloheximide to prevent transport of newly synthesized HN to the cell surface. At 0 or 4 h after cycloheximide treatment, cells were fixed and labeled using either HN-1b to detect HN or F1a to detect the F protein, and stained using an Alexa Fluor 488-conjugated secondary antibody. At the $t=0$ time point, HN staining was widespread at the surface of the infected cells and there was no detectable staining above background for the mock infected cells (Fig. 2). After 4 h of cycloheximide treatment there was less HN present on the surface of the infected cells, though HN protein was detectable (Fig. 2). This is consistent with the immunoprecipitation experiments shown above that indicated that about half of wt HN protein in PIV-5-infected cells was still present in its full-length form at 4 h post-labeling (Fig. 1). This is also consistent with the data obtained in flow cytometry experiments (Fig. 3). In contrast, cells infected with PIV-5 HN E37D or HN E37K virus did not display a decrease in HN staining (Fig. 2). This indicates HN E37D and HN E37K proteins are stably expressed at the surface of infected cells for at least 4 h. PIV-5 F protein was stably expressed at the cell surface confirming earlier data (Leser et al., 1996; Ng et al., 1989). This experiment also demonstrates that the stability of HN protein on the cell surface does not affect the stability of F on the cell surface when HN and F are present together in virus-infected cells.

The surface expression level of HN E37D and HN E37K in PIV-5-infected cells was also analyzed by flow cytometry. CV-1 cells were infected for 18 h and treated with cycloheximide as described above. Following cycloheximide treatment for either 0 or 4 h, cells were fixed and stained for either HN or F proteins using either a polyclonal HN antibody or mAb F1a and then analyzed by flow cytometry. The results were expressed as a percentage of the mean fluorescence intensity of the wt HN staining at $t=0$. Fig. 3 shows that at the 0 h time point there is ~50% more HN E37D and E37K present on the surface on virus-infected cells when compared to wt virus-infected