A Mutation in the Stalk of the Newcastle Disease Virus Hemagglutinin-Neuraminidase (HN) Protein Prevents Triggering of the F Protein despite Allowing Efficient HN-F Complex Formation

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Newcastle disease virus (NDV)-induced membrane fusion requires formation of a complex between the hemagglutinin-neuraminidase (HN) and fusion (F) proteins. Substitutions for NDV HN stalk residues A89, L90, and L94 block fusion by modulating formation of the HN-F complex. Here, we demonstrate that a nearby L97A substitution, though previously shown to block fusion, allows efficient HN-F complex formation and likely acts by preventing changes in the HN stalk required for triggering of the bound F protein.

Newcastle disease virus (NDV) is a member of the negative-stranded, RNA-containing *Paramyxoviridae* (1). Similar to most paramyxoviruses, NDV-induced membrane fusion requires an interaction between the hemagglutinin-neuraminidase (HN) attachment and fusion (F) proteins (1).

The ectodomain of HN consists of a stalk supporting a terminal globular head (1). Whereas the globular domain mediates binding to sialic acid receptors, catalyzes neuraminidase activity (NA), and contains all the antigenic sites (2, 3), the stalk mediates the F interaction (4–7). Mutations in NDV HN stalk residues A89, L90, and L94 specifically modulate fusion with no effect on hemadsorption (HAD), NA, or antigenic structure (8). For these mutations, the extent of fusion correlates directly with the amount of the HN-F complex detectable at the cell surface (9), consistent with these residues contributing to the F interaction. This is supported by the surface exposure of all three residues in the crystal structure of the tetrameric NDV HN ectodomain (10).

However, in this structure these residues appear to be shielded from contacting F by the base of the globular head. This has led to the hypothesis that receptor binding may induce a conformational change that shifts the heads up to expose the F-interactive domain (11). This is consistent with the idea, based on ER retention (12) and attachment-deficient mutants (13), that HN does not interact with F until triggered to do so by receptor binding (9), though evidence to the contrary has recently been published (14).

Whereas the F protein must undergo a dramatic conformational rearrangement to trigger fusion (1), it is difficult to envision this taking place while F is still HN bound, suggesting that the HN-F complex may be transient. We sought to identify HN stalk mutations that would allow formation of the complex, but block fusion triggering, reasoning that such mutations might make it possible to capture an intermediate in the fusion-triggering cascade, which could serve as a reagent to probe the structure of the complex.

HN stalk residues R83 and L97 are also surface exposed (10) and R83N and L97A mutations also specifically inhibit fusion (15, 16). Although the fusion deficiency of R83N-mutated HN correlated with loss of the ability of the protein to interact with F (16), the F-interactive capability of L97A-mutated HN was not previously determined. Thus, we have characterized the effect of A and

T substitutions for residues R83 and L97. Also, reasoning that the triggering cascade is transmitted from the receptor binding site in the head to the F-interactive domain in the stalk, we have determined the effect of alanine substitutions for additional residues immediately membrane-distal to L97 for their effect on HN function. A95R and -S substitutions were previously shown to have little or no effect on fusion (8), and an L96A-mutated protein retained about 1/3 of wild-type (wt) activity (15). Therefore, these residues were not investigated.

Using NDV HN in pBluescript SK(+) (Stratagene Cloning Systems, La Jolla, CA) and the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), we prepared HN mutants with substitutions of R83A or -T, L97A or -T, N98A, T99A, or E100A. The presence of each mutation was confirmed by sequencing.

Wild-type and mutated HN proteins were expressed in BHK-21F cells, using the vaccinia virus-T7 RNA polymerase expression system (17). Cells were seeded in six-well plates at 4×10^5 cells/well 1 day prior to transfection and assays were performed at 24 h posttransfection. Cell surface expression (CSE) was determined by the University of Massachusetts Medical School Flow Cytometry Laboratory, using a mixture of conformational HN-specific monoclonal antibodies (MAbs) (18–21). Receptor binding and NA were determined, respectively, by HAD of guinea pig erythrocytes (Bio-Link Laboratories, Liverpool, NY) (8) and cleavage of 2-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (MUN) (22). Fusion was quantitated using a content-mixing assay (8).

Each of the mutated proteins is expressed at the cell surface at levels ranging from 71 to 109% of wt (Fig. 1A). HAD activity at 4°C was only minimally affected, with levels ranging from 79 to 109% of wt (Fig. 1B). Although NA was diminished for E100A-mutated

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FIG 1 Functional characterization of HN stalk mutants. CSE (A), HAd (4°C) and NA (B), and fusion-promoting activity (C) were determined for each of the mutants. Data were corrected for background obtained with vector alone and normalized to the value obtained with wt HN, which was set at 100%. Averages $\pm$ standard deviations from three independent experiments are shown.

HN (33% of wt), each of the other mutants exhibited $>60\%$ of wt activity (Fig. 1B).

Despite the lack of a significant modulation of CSE, HAd, or NA, several of the mutated proteins were markedly diminished in the ability to trigger fusion (Fig. 1C and Fig. 2). While R83A-mutated HN promotes approximately 20% of wt fusion, the R83T, L97A, and L97T proteins all promote fusion at $<6\%$ of the level of wt HN. The phenotype of the L97A protein is similar to that reported previously (15), though at that time fusion was re-

FIG 2 Syncytium formation in monolayers coexpressing wt HN or HN stalk mutants and the wt NDV F protein. The extent of syncytium formation is shown in monolayers expressing wt F with vector control (vec), wt HN, or HN carrying a R83A, R83T, L97A, L97T, N98A, T99A, or E100A mutation. Monolayers were fixed with methanol and stained with Giemsa.

ported as 18% of wt, as determined by counting nuclei in syncytia in Cos-7 cells. The A mutations of residues N98, T99, and E100 all promoted significant levels of fusion (Fig. 1C and Fig. 2) and were not considered further.

Since the fusion deficiency resulting from dimer interface mutations in the globular domain correlates with an apparent disruption of a second sialic acid binding site, detectable by unstable HAd at 37°C (23), we also measured HAd at this temperature. For all the fusion-deficient mutants, HAd relative to that of the wt protein was actually more efficient at the higher temperature than it was in the cold (data not shown). Thus, we conclude that the diminished fusion promoted by these mutants is not due to an effect on either sialic acid binding site.

Having confirmed that three additional amino acid substitutions, R83T, L97A, and L97T, result in a specific decrease in fusion, we next determined the ability of the mutated proteins to take part in the complex with F. This was accomplished using a cell surface coimmunoprecipitation (co-IP)-Western blot assay (23). Briefly, wt or mutated HN was coexpressed on the surfaces of BHK-21F cells with a cleavage site mutant form of F (csmF), surface proteins were biotinylated, the cells were lysed, and HN was immunoprecipitated through its interaction with csmF using an F-specific MAb and detected by Western blotting using an MAb to a linear epitope in HN (21).

Figure 3 shows that the R83T substitution also markedly diminishes the ability of HN to interact with F (Fig. 3). The amount of co-IP is comparable to that of the negative control, A89Q-mutated HN (8). L97T-mutated HN coimmunoprecipitates more efficiently than either of these mutated proteins but still at a lower level than wt HN. However, L97A-mutated HN interacts with the F protein to an extent comparable to that of wt HN (Fig. 3). Efficient expression of HN and F was verified in each sample (Fig. 3).

Taken together, these findings indicate that L97A-mutated HN associates efficiently with F but does not trigger it to promote

FIG 3 Ability of the HN stalk mutants to interact with csmF. (Top) Amount of HN brought down in a cell surface co-IP with antibody to the F protein, as detected by a biotinylated co-IP-Western blot assay. The negative control A89Q-mutated HN interacts with csmF only weakly (8). Total cell surface HN (middle) and csmF (bottom) are also shown.

fusion. Thus, the mutation blocks a step in the fusion cascade subsequent to HN-F complex formation and may act by preventing the rearrangements in HN necessary for fusion triggering. This mutation is analogous to the I98A mutation in the stalk of the measles virus (MV) hemagglutinin (H), which also blocks fusion triggering and increases the avidity of the H-F complex (24, 25). This is significant, as it is thought that while the MV H-F complex forms intracellularly prior to receptor binding, the NDV HN-F complex appears to initially form at the cell surface upon receptor binding (reviewed in reference 9). The identification of a trigger that acts similarly in both viral attachment proteins indicates a commonality in paramyxovirus fusion triggering models in the two viruses and is consistent with the stalk domains of both MV H and NDV HN playing an active role in fusion triggering.

Finally, whereas L97A-mutated NDV HN appears to allow HN-F complex formation but prevents the complex dissociation that would presumably be integral to fusion triggering, it may make it possible to capture an intermediate in the fusion-triggering cascade that could be useful in understanding the structure of the HN-F complex.

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REFERENCES

- Lamb RA, Parks GD. 2007. Paramyxoviridae: the viruses and their replication, p 1449–1496. In Knipe DM, Howley PM (ed), *Fields virology*, 5th ed. Wolters Kluwer/Lippincott Williams & Wilkins, Philadelphia, PA.
- Thompson SD, Laver WG, Murti KB, Portner A. 1988. Isolation of a biologically active soluble form of the hemagglutinin-neuraminidase protein of Sendai virus. *J. Virol.* 62:4653–4660.
- Mirza AM, Sheehan JP, Hardy LW, Glickman RL, Iorio RM. 1993. Structure and function of a membrane anchor-less form of the hemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Biol. Chem.* 268:21425–21431.
- Deng R, Wang Z, Mirza AM, Iorio RM. 1995. Localization of a domain on the paramyxovirus attachment protein required for the promotion of cellular fusion by its homologous fusion protein spike. *Virology* 209:457–469.
- Tsurudome M, Kawano M, Yuasa T, Tabata N, Nishio M, Komada H, Ito Y. 1995. Identification of regions on the hemagglutinin-

neuraminidase protein of human parainfluenza virus type 2 important for promoting cell fusion. *Virology* 213:190–203.

- Tanabayashi K, Compans RW. 1996. Functional interaction of paramyxovirus glycoproteins: identification of a domain in Sendai virus HN which promotes cell fusion. *J. Virol.* 70:6112–6118.
- Deng R, Mirza AM, Mahon PJ, Iorio RM. 1997. Functional chimeric HN glycoproteins derived from Newcastle disease virus and human parainfluenza virus-3. *Arch. Virol. Suppl.* 13:115–130.
- Melanson VR, Iorio RM. 2004. Amino acid substitutions in the F-specific domain in the stalk of the Newcastle disease virus HN protein modulate fusion and interfere with its interaction with the F protein. *J. Virol.* 78:13053–13061.
- Iorio RM, Melanson VR, Mahon PJ. 2009. Glycoprotein interactions in paramyxovirus fusion. *Future Virol.* 4:335–351.
- Yuan P, Swanson KA, Leser GP, Paterson RG, Lamb RA, Jardetzky TS. 2011. Structure of the Newcastle disease virus hemagglutinin-neuraminidase (HN) ectodomain reveals a four-helix bundle stalk. *Proc. Natl. Acad. Sci. U. S. A.* 108:14920–14925.
- Bose S, Zokarkar A, Welch BD, Leser GP, Jardetzky TS, Lamb RA. 2012. Fusion activation by a headless parainfluenza virus 5 hemagglutinin-neuraminidase stalk suggests a modular mechanism for triggering. *Proc. Natl. Acad. Sci. U. S. A.* 109:E2625–E2634.
- Paterson RG, Johnson ML, Lamb RA. 1997. Paramyxovirus fusion (F) protein and hemagglutinin-neuraminidase (HN) protein interactions: Intracellular retention of F and HN does not affect transport of the homotypic HN or F protein. *Virology* 237:1–9.
- Li J, Quinlan E, Mirza A, Iorio RM. 2004. Mutated form of the Newcastle disease virus hemagglutinin-neuraminidase interacts with the homologous fusion protein despite deficiencies in both receptor recognition and fusion promotion. *J. Virol.* 78:5299–5310.
- Porotto M, Salah ZW, Gui L, DeVito I, Jurgens EM, Lu H, Yokoyama CC, Palermo LM, Lee KK, Moscona A. 2012. Regulation of paramyxovirus fusion activation: the hemagglutinin-neuraminidase protein stabilizes the fusion protein in a pretriggered state. *J. Virol.* 86:12838–12848.
- Stone-Hulslander J, Morrison TG. 1999. Mutational analysis of heptad repeats in the membrane-proximal region of the Newcastle disease virus HN protein. *J. Virol.* 73:3630–3637.
- Melanson VR, Iorio RM. 2006. Addition of N-glycans in the stalk of the Newcastle disease virus HN protein blocks its interaction with the F protein and prevents fusion. *J. Virol.* 80:623–633.
- Fuerst TR, Niles EG, Studier FW, Moss B. 1986. Eucaryotic transient expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc. Natl. Acad. Sci. USA* 83:8122–8126.
- Iorio RM, Bratt MA. 1983. Monoclonal antibodies to Newcastle disease virus: delineation of four epitopes on the HN glycoprotein. *J. Virol.* 48:440–450.
- Iorio RM, Borgman JB, Glickman RL, Bratt MA. 1986. Genetic variation within a neutralizing domain on the haemagglutinin-neuraminidase glycoprotein of Newcastle disease virus. *J. Gen. Virol.* 67:1393–1403.
- Iorio RM, Glickman RL, Riel AM, Sheehan JP, Bratt MA. 1989. Functional and neutralization profile of seven overlapping antigenic sites on the HN glycoprotein of Newcastle disease virus: monoclonal antibodies to some sites prevent viral attachment. *Virus Res.* 13:245–262.
- Iorio RM, Syddall RJ, Sheehan JP, Bratt MA, Glickman RL, Riel AM. 1991. Neutralization map of the HN glycoprotein of Newcastle disease virus: domains recognized by monoclonal antibodies that prevent receptor recognition activity. *J. Virol.* 65:4999–5006.
- Tappert M, Smith DF, Air GM. 2011. Fixation of oligosaccharides to a surface may increase the susceptibility to human parainfluenza virus 1, 2 or 3 hemagglutinin-neuraminidase. *J. Virol.* 85:12146–12159.
- Mahon PJ, Mirza AM, Iorio RM. 2011. Role of the two sialic acid binding sites on the NDV HN protein in triggering the interaction with the F protein required for the promotion of fusion. *J. Virol.* 85:12079–12082.
- Corey EA, Iorio RM. 2007. Mutations in the stalk of the measles virus hemagglutinin protein decrease fusion but do not interfere with the virus-specific interaction with the homologous fusion protein. *J. Virol.* 81:9900–9910.
- Brindley MA, Takeda M, Plattet P, Plemper RK. 2012. Triggering the measles virus membrane fusion machinery. *Proc. Natl. Acad. Sci. U. S. A.* 109:E3018–E3027.