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Quantitative Basic Residue Requirements in the Cleavage-Activation Site of the Fusion Glycoprotein as a Determinant of Virulence for Newcastle Disease Virus

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Newcastle disease virus exhibits a wide range of pathogenicity and virulence which, as with all paramyxoviruses, is directly related to the cleavability of a precursor (F_0) of the fusion glycoprotein by cellular proteases. Sequence analyses of the cleavage site of several virulent and avirulent isolates of the Newcastle disease virus serotype reveal a correlation between virulence or pathogenicity and a high content of basic amino acid residues at the cleavage site. A similar correlation has been seen for other paramyxoviruses.

Paramyxoviruses are a group of negative-stranded RNA-containing enveloped viruses (8), of which Newcastle disease virus (NDV) is unique in that the NDV serotype is composed of hundreds of isolates encompassing a wide range of virulence and pathogenicity (18). Paramyxovirions possess two types of glycoprotein spikes protruding from the cellularly derived envelope. The hemagglutinin-neuraminidase (HN) and fusion (F) glycoproteins are responsible for, respectively, attachment of the virus to cell surface receptors and fusion of the viral and cellular membranes (15). The latter is produced as a precursor molecule (F_0), which must be proteolytically cleaved, producing two disulfide-linked polypeptides, F_1 and F_2 (11, 15). The cleavage activation of F_0 is catalyzed by a host-cell protease(s) with trypsinlike activity (5). The new amino terminus of F_1 is extremely hydrophobic and functions as the insertion peptide, promoting fusion of the viral and cellular envelopes (14).

There is a strict correlation between the cleavability of F_0 in tissue culture cells and the virulence and host cell range of NDV isolates (10, 11). The F_0 of virulent isolates is cleaved, and thus, the virus will form plaques in many cell types, including cultured chicken embryo cells. The F_0 of avirulent isolates, on the other hand, is cleaved in relatively few cell types, such as cells of the chorioallantoic membrane of embryonated hen eggs. These viruses will not form plaques in cultured chicken embryo cells, unless an exogenous protease, such as trypsin, is present in the agar overlay of the plaque assay.

Using primer extension and dideoxy sequencing, we have compared the deduced amino acid sequences around the proposed cleavage site of the F_0 glycoprotein of a number of virulent and avirulent isolates of NDV. During the preparation of this manuscript, a report (17) appeared concerning the role of basic amino acids in the region preceding the F_2/F_1 cleavage site in five different isolates of NDV, as well as two isolates used in this study. Their findings, and the data presented here, suggest a requirement for several basic amino acids at the cleavage site of F_0 for proteolytic cleavage and activation in many cell types.

Virulent isolates of NDV used in this study include AV (Australia-Victoria, 1932), F (NJ-Roakin, 1946), HP (Israel-HP, 1953), IM (Italy-Milano, 1945), IS (Iowa-Salsbury, 1949), L (L-Kansas, 1948), and RO (California RO, 1944). Avirulent isolates include B (B1-Hitchner [Blacksburg]), EF (England F [ARS], 1949), N (NJ-LaSota, 1946), U (Ulster), and W (Wisconsin-Appleton, 1950). Virus was grown in the allantoic sac of 10-day-old embryonated hen eggs at 37°C (3). Virions were purified and concentrated (6, 19) and used as a source of genomic RNA (16).

A primer (GACTACTTTGCTCACCC) encompassing nucleotides 316 to 332 of the F glycoprotein sequence of the AV isolate (9) was purchased from the DNA Synthesis Facility at the University of Massachusetts Medical School. This region was chosen for the primer because it lies about 70 nucleotides upstream from the proposed cleavage site and only 18 nucleotides upstream from an Arg-Arg sequence which has been proposed as a possible second cleavage site (9). The primer was purified by electrophoresis in a 20% polyacrylamide–7 M urea gel, followed by chromatography with a SEP-PAK C_{18} cartridge (Waters Associates, Milford, Mass.).

Primer extension was performed as described by Air (1) with the following modifications: reactions contained 2 μg of virion RNA and 100 ng of primer annealed for 6 min at 70°C, 7.5 U of reverse transcriptase (Life Sciences, St. Petersburg, Fla.) was added, the reaction was incubated for 60 min at 42°C, and the 30-min chase was omitted. Reaction products were analyzed on 8% polyacrylamide–7 M urea sequencing gels. For better resolution of some regions, 40% formamide was added to the gel mixture.

The nucleotide sequence in the region of the proteolytic cleavage site of F_0 was determined for six virulent and five avirulent isolates and compared with the corresponding sequence in NDV-AV (9) (Table 1). Also included is the published sequence of the F_0 of the virulent Beaudette C (BE) isolate (4). A blank space indicates nucleotide homology with NDV-AV. The deduced amino acid sequences are shown in a similar manner in Table 2. The five amino acids preceding the new F_1 amino terminus produced by cleavage are underlined. In all of the virulent isolates, this pentapeptide always consists of four basic amino acids with an intervening glutamine residue (Arg-Arg-Gln-$^{Arg}_{Lys}$-Arg). In

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TABLE 1. Comparison of nucleotide sequences among virulent and avirulent NDV isolates

NDV isolates	Nucleotide sequence differences[a]																					
Virulent		375								400									425			
AV[b]	ACT	ACA	TCC	GGA	GGA	AGG	AGA	CAG	AAA	CGC↓	TTT	ATA	GGT	GCT	ATT	ATC	GGC	AGT	GTA	GCT	CTT	GGG
RO			T		G				GG				C	A	C			G	T			
HP			T						G					C					G			
IM		T							G													
IS			T		G								C	C		T		G	C			
F			T		G								C	C		T		G	G			
L			T		G								C	C		T		G	G			
BE[c]			T		G								C	C		T		G	G			
Avirulent																						
N			T		G	G			GGG		C		C			T		G	G			
B			T		G	G			GGG		C		C			T		G	G			
W			T		G	G			GGG		C		C			T		G	G			
U			T			G	A		GG		C		C									
EF			T		G	G			GGG		C		C			T						

[a] Arrow points to cleavage site. Blank spaces indicate homology with NDV-AV.
[b] Nucleotide sequence as determined by McGinnes and Morrison (9).
[c] Nucleotide sequence as determined by Chambers et al. (4).

contrast, in the five avirulent isolates examined here, a neutral amino acid, glycine, is found in place of the basic arginine residues at positions 1 and 4 of the pentapeptide (Gly-Arg-Gln-Gly-Arg). This suggests that a high content of basic residues in the cleavage site may be at least one of the requirements for NDV to form plaques in most cell types, including chicken embryo fibroblasts. It is also noteworthy that the hydrophobic nature of the new amino terminus formed by the proteolytic cleavage is extremely highly conserved in all virulent NDV isolates, in each case the new amino-terminal residue being a phenylalanine. All avirulent isolates have a leucine residue in this position which, although different from the virulent isolates, is a relatively conservative change, maintaining the hydrophobic nature of the terminus of the insertion peptide.

TABLE 2. Comparison of deduced amino acid sequences among virulent and avirulent NDV isolates

NDV isolates	Amino acid sequence differences[a]																					
Virulent						112				116									125			
AV[b]	T	T	S	G	G	R	R	Q	K	R↓	F	I	G	A	I	I	G	S	V	A	L	G
RO									R						L			G				
HP									R													
IM									R													
IS																		V				
F																		G				
L																		G				
BE[c]																		G				
Avirulent																						
N						G			G		L							G				
B						G			G		L							G				
W						G			G		L							G				
U						G	K		G		L											
EF						G			G		L											

[a] Arrow points to cleavage site. Blank spaces indicate homology with NDV-AV.
[b] See Table 1, footnote b.
[c] See Table 1, footnote c.

The Arg-Arg sequence just downstream from the primer appears not to function as a general cleavage site, as was suggested by McGinnes and Morrison (9), because it is present in avirulent isolates B, N, and W (data not shown), as well as virulent isolates AV (9), BE (4), and F (data not shown). Thus, it cannot account for the difference in susceptibility to cleavage of the F glycoproteins of virulent and avirulent isolates.

The amino acid sequences of the fusion glycoproteins of several paramyxoviruses have now been determined. These include Sendai virus (2), measles virus (13), parainfluenza virus type 3 (16), respiratory syncytial virus (7), simian virus 5 (12), and two virulent isolates of NDV (4, 9). Looking for regions of homology, many of these researchers have made comparisons of the fusion glycoprotein sequences of different paramyxoviruses, including the putative proteolytic cleavage activation site. The location of the new amino terminus formed upon proteolytic digestion was proposed on the basis of a high degree of conservation of the region in a number of paramyxoviruses and the specific inhibition of paramyxovirus replication by oligopeptides with amino acid sequences similar to that terminus (14). Subsequently, Spriggs and co-workers (16) identified the amino terminus of F_1 of parainfluenza virus type 3 by direct amino acid sequencing. Table 3 shows the amino acid sequences for several paramyxoviruses for five residues on either side of the cleavage site. The amino terminus of F_1 is indeed extremely highly conserved, terminating in phenylalanine for all of these viruses except the avirulent NDV isolates. Comparison of the F_2 carboxy-terminal pentapeptide sequences reveals what appears to be a relationship between the structure of this region and the ability of the virus to grow in many cell types. All members of the group have an arginine as the ultimate residue at the F_2 carboxy terminus. The other residues in this pentapeptide vary in the different viruses. Those that have four (measles and virulent NDV isolates) or five (respiratory syncytial virus and simian virus 5) basic residues in this region are cleaved in many cell types. For parainfluenza virus type 3, with only three basic residues in this stretch, the protein is only partially cleaved in several cell types (16). The F glycoproteins of Sendai virus

TABLE 3. Comparison of paramyxovirus fusion protein cleavage site sequences

Paramyxovirus and reference	Amino acid sequence at carboxyterminus of F_2	No. of basic residues at carboxyterminus of F_2	Amino acid sequence at aminoterminus of F_1	F_0 cleaved in many cell types
Simian virus 5 (12)	R R R R R	5	F A G V V	Yes
Respiratory synctial virus (7)	K R K R R	5	F L G F L	Yes
Measles virus (13)	R R H K R	4	F A G V V	Yes
Virulent NDV isolates	R R Q K R	4	F I G A I	Yes
Parainfluenza virus type 3 (16)	P R T K R	3	F F G G V	Partial
Avirulent NDV isolates	G R Q G R	2	L I G A I	No
Sendai virus (2)	V P Q S R	1	F F G A V	No

and the avirulent NDV isolates which, respectively, have only one and two basic residues in this region are not cleaved in most cell types, including cultured chicken embryo cells without exogenous protease. Thus, the NDV serotype includes isolates which represent both extremes of the paramyxovirus group, those with high basic residue content in the F_2 carboxy terminus and a wide range of cell specificities and those with low basic residue content and an inability to form plaques in cultured chicken embryo cells and many other cell types. The results presented here and those mentioned earlier (17) demonstrate the importance of pairs of basic amino acid residues at the cleavage site for proteolytic activation of the fusion protein.

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ADDENDUM

A mutant of the AV isolate (C. H. Madansky and M. A. Bratt, J. Virol. **26**:724–729, 1978) has three basic residues at the cleavage site. Virions have a partially cleaved F_0 (C. H. Madansky and M. A. Bratt, J. Virol. **40**:691–702, 1981) but still require trypsin to form plaques in chicken embryo cells.

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