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Characterization of Pathotype-specific Epitopes of Newcastle Disease Virus Fusion Glycoproteins by Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry and Post-source Decay Sequencing

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Matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS) was used to characterize the F2 polypeptide of the fusion (F) protein of an avirulent isolate (VRI 82-6409) of Newcastle disease virus (NDV) that was previously identified by immunochemical screening as having a variant cleavage activation sequence in its fusion protein precursor (F0). The major glycoform of the intact F2 polypeptide of the VRI 82-6409 isolate was 89 Da smaller than the F2 polypeptide of the avirulent V4 isolate of the Queensland strain of NDV. Analysis of AspN protease digests of the F2 polypeptides by MALDI/TOF-MS, with and without high-performance liquid chromatographic (HPLC) separation, showed this mass difference to be due to a combination of differences in the extents of glycosylation and an amino acid difference in the AspN peptides derived from the *C*-termini of the F2 polypeptides. Accuracies achieved in analysis of the AspN peptides allowed the identification of this amino acid difference as glutamic acid in the VRI 82-6409 isolate compared with glycine in the V4 isolate. Analysis of fragments formed by post-source decay (PSD) of ions of the *C*-terminal AspN peptides localized the difference to the *C*-terminal residues of the respective F2 polypeptides. The present study demonstrated that MALDI/TOF-MS is a highly effective technique for the characterization of NDV variants identified by immunochemical screening of pathotype-specific epitopes at the *C*-termini of their F2 polypeptides. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: matrix-assisted laser desorption/ionization; post-source decay; peptide sequencing; Newcastle disease virus; fusion protein; pathogenicity

INTRODUCTION

Newcastle disease virus (NDV), or avian paramyxovirus 1, strains have been shown to vary widely in pathogenicity with some strains producing high mortalities in poultry flocks whilst others are relatively inocuous.[1] The variation in virulence of NDV strains has been attributed to variations in proteolytic activation of the membrane glycoproteins of the virus that are responsible for attachment to and penetration of susceptible host cells.[2–6] In particular, variations in the fusion (F) protein, which mediates fusion between paramyxoviruses and cell membranes during penetration,[5,7–9] have a marked effect on virulence.

The F proteins are produced as single-chain biosynthetic precursors (F0) which must be cleaved to produce infectivity.[3,5,7–12] This proteolytic cleavage produces F1 and F2 polypeptides that remain linked by a disulphide bond.[3,8,13] A hydrophobic sequence is exposed at the newly generated *N*-terminus on the larger of the two chains (F1 polypeptide).[8,13,14] This hydrophobic sequence is believed to be directly involved in the fusion process.[8,13–15]

In general, the degree of pathogenicity of different strains can be correlated with the susceptibilities of their F0s to proteolytic activation in a broad range of cells.[3–6] Strains of NDV with F0s more susceptible to proteolytic activation have higher virulence due to a

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greater propensity to present the fusogenic sequence at the *N*-termini of their F1 polypeptides. Susceptibilities of the various F0s to cleavage-activation are dependent on the amino acid sequences preceding the latent *N*-termini of the F1 polypeptides.[16–19] This preceding sequence forms the *C*-terminus of the second and smaller of the polypeptides (F2 polypeptide) of the active F proteins. Virulent strains of NDV have pentapeptide motifs preceding the cleavage site that are rich in basic amino acids by virtue of the presence of two pairs of basic amino acids separated by a single glutamine residue (Fig. 1). The corresponding sequences of low-virulence and avirulent strains have the first amino acid of the pairs of basic amino acids replaced by non-basic amino acids, usually glycine (Fig. 1). Attenuation of virulence is apparently due to the limited distribution of proteases able to cleave the motifs with decreased basic character compared with a ubiquitous distribution of proteases for the more basic motifs of highly virulent strains. Such a differential distribution of proteases would account for the greater abilities of the virulent strains to spread through the infected host and cause greater disease as a consequence.[3–6,9,17,18]

The actual course of proteolytic activation involves a primary cleavage at the arginine residue preceding the *N*-terminus of the F1 polypeptide followed by removal of this arginine and other basic amino acids exposed at the *C*-terminus of the F2 polypeptide in the case of virulent strains (Fig. 1).[19,20] Differences in the sequences of F2 polypeptide *C*-termini of active F proteins, particularly between virulent and avirulent strains, have been exploited to develop antipeptide antibodies specific for various pathotypes of NDV.[21] These antibodies promise to form the basis of a practical pathotyping assay for NDV; however, isolates are occasionally encountered that do not react with this first generation of antipeptide antisera.[22,23] It is necessary to determine the structural features of the cleavage sites of such isolates in order to understand their negative reactions. It is feasible to determine the sequences of the F0s of these isolates by genomic sequencing,[16–18,24,25] but this does not reveal information on the actual sites of cleavage or post-cleavage trimming. The antisera employed were designed to react with the fully processed F2 polypeptide *C*-termini (Fig. 1) that would be present on virions isolated from biological specimens.[5,6,9,19,20] Hence definition of the amino acid sequences of F2 polypeptides isolated from negatively reacting NDV strains is required to explain fully their negative reactivities and to be able to produce antisera to accommodate such sequences.

Fast atom bombardment (FAB) mass spectrometry in conjunction with Edman degradation sequencing and amino acid analysis was originally used to characterize the cleavage-activation process of different pathotypes of NDV[19,20] and the F2 polypeptide *C*-termini of some isolates with anomolous immunochemical reactions.[22,23] However, newer, less complex, more versatile and more sensitive mass spectrometric techniques are now available for the analysis of peptides and proteins. In particular, matrix-assisted laser desorption/ionization (MALDI)[26–28] and electrospray ionization[29] have supplanted FAB as a mode of ionization for high-sensitivity analysis of biomolecules.

The study reported here involves evaluation of MALDI time-of-flight mass spectrometry (MALDI/TOF-MS) for the characterization of the F2 polypeptide of a previously undefined avirulent isolate of NDV, VRI 82-6409, with an anomolous immunochemical profile.[22]

EXPERIMENTAL

Viruses

The VRI 82-6409 isolate[22] and the V4 isolate of the Queensland strain[30] of NDV were propagated for 72 h in 10-day-old embryonated chicken eggs and harvested as described previously.[21,22]

Peptide isolation

Intact egg-propagated virions were disrupted by incubation for 16 h at 4 °C followed by 2 h at 22 °C with 2% (w/v) sodium dodecyl sulphate (SDS) and 10 mM dithiothreitol to reduce the disulphide bond linking

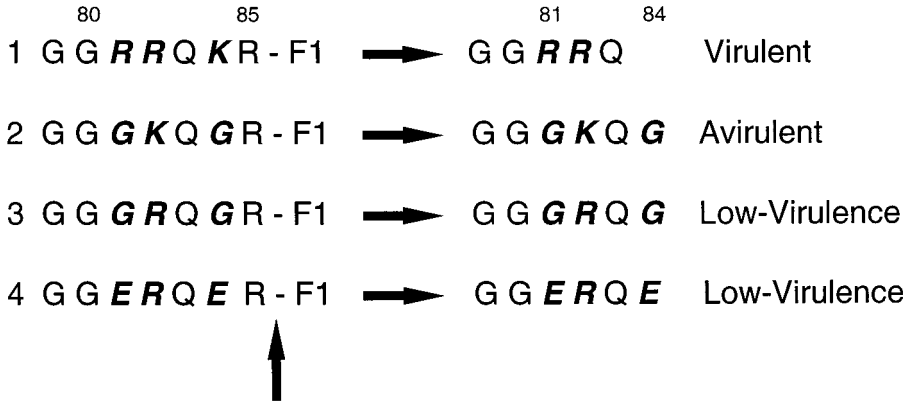

Figure 1. Variation of amino acids at the F2 polypeptide *C*-termini of four major pathotypes of NDV before and after proteolytic activation. Variable amino acids in the *C*-termini of the F2 polypeptide domains of the various F0s and the active F proteins are indicated in bold italics. The vertical arrow indicates the site of endoproteolytic cleavage between the *C*-termini of the F2 polypeptide domains (residue 85) and the *N*-terminus of the F1 polypeptides. Subsequent trimming of the basic amino acids exposed by the endoproteolytic cleavage results in the *C*-termini of the mature F2 polypeptides indicated by the horizontal arrows.

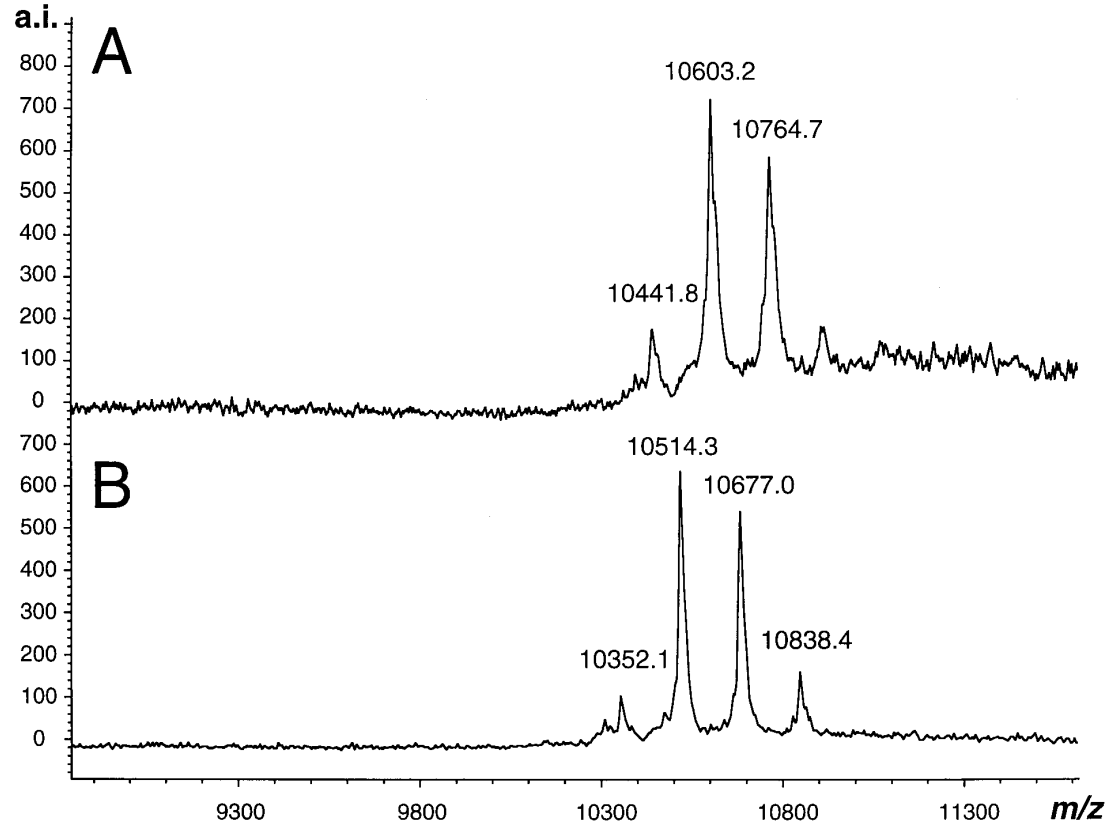

Figure 2. MALDI/TOF mass spectra of intact (A) V4 and (B) VRI 82-6409 F2 polypeptides. Both spectra were acquired in reflector mode operation using external calibration but without delayed extraction. DHAP–DAHC was used as the matrix.

the F1 and F2 polypeptides of their F proteins.[3,13] The resultant cysteine residues were subsequently alkylated by reaction for 2 h in the dark at 22 °C with 50 mM 5-*N*-[(iodoacetamidoethyl)amino]naphthalene-1-sulphonic acid.[19,20,31–33] Adjustment of the pH to ~8 with NaOH was required upon addition of the alkylating agent. Proteins rendered fluorescent as a consequence of alkylation were precipitated with 90% (v/v) methanol at −20 °C and washed twice with methanol to remove excess alkylating agent. Individual proteins were separated by electrophoresis on polyacrylamide slab gels (14.2 cm wide × 11 cm high × 1.5 mm thick) in the presence of SDS and recovered from gel slices by electroelution.[31] Digestion of intact F2 polypeptides was performed for 4 h at 37 °C using two additions of 1% (w/w) of sequencing grade AspN-protease (Boehringer Mannheim) with the second addition made at 2 h.

Proteolytic fragments were isolated by reversed-phase high-performance liquid chromatography (HPLC) using slight variations of a previously described protocol.[33] These involved use of a 25 cm × 2.1 mm i.d. column of octadecasilica (Vydac), a flow-rate of 150 μl min^{-1} and a linear gradient from 0.1% (v/v) aqueous trifluoroacetic acid to 80% (v/v) aqueous acetonitrile containing 0.09% (v/v) trifluoroacetic acid, developed over 90 min. Gradients were generated using a Hewlett–Packard chromatography system comprised of a Model 1090M solvent-delivery system under the control of an HP 2170AA Chemstation and elution of peptides was monitored at 214 nm using a diode-array detector.

Mass spectrometry

Mass spectrometry was performed using a Bruker Reflex mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) operated exclusively in the reflectron mode. Samples were deposited on target surfaces after mixing with an equal volume of the supernatant fraction of a saturated mixture of α-cyano-4-hydroxycinnamic acid (CHCA) in 33% (v/v) aqueous CH_3CN containing 0.1% (v/v) trifluoroacetic acid[34] or a matrix solution comprising 10 mg ml^{-1} 2,6-dihydroxyacetophenone (DHAP) in CH_3CN, ethanol and water (45:45:10 by volume) containing 0.1 M diammonium hydrogencitrate (DAHC).[35,36] Ionization was achieved using a nitrogen laser pulsed at a repetition rate of 3 Hz. The laser irradiance was adjusted to threshold levels in order to observe intact molecular ions which were accelerated to a potential of 28.5 kV and subsequently reflected with a reflectron potential of 30 kV on to a dual microchannel plate detector. Most measurements were performed with a 100 ns delay between the laser pulse and extraction of ions from the source. A digitization rate of 1 GHz was employed for delayed extraction mode and masses were assigned using an external calibration file created using the monoisotopic *m/z* values for angiotensin II ($MH^+ = 1046.56$) and adrenocorticotropic hormone 18–39 ($MH^+ = 2465.2$) or by internal calibration with the same calibrants. Alternatively, continuous extraction of ions was employed and masses were assigned to intact peptide ions by reference

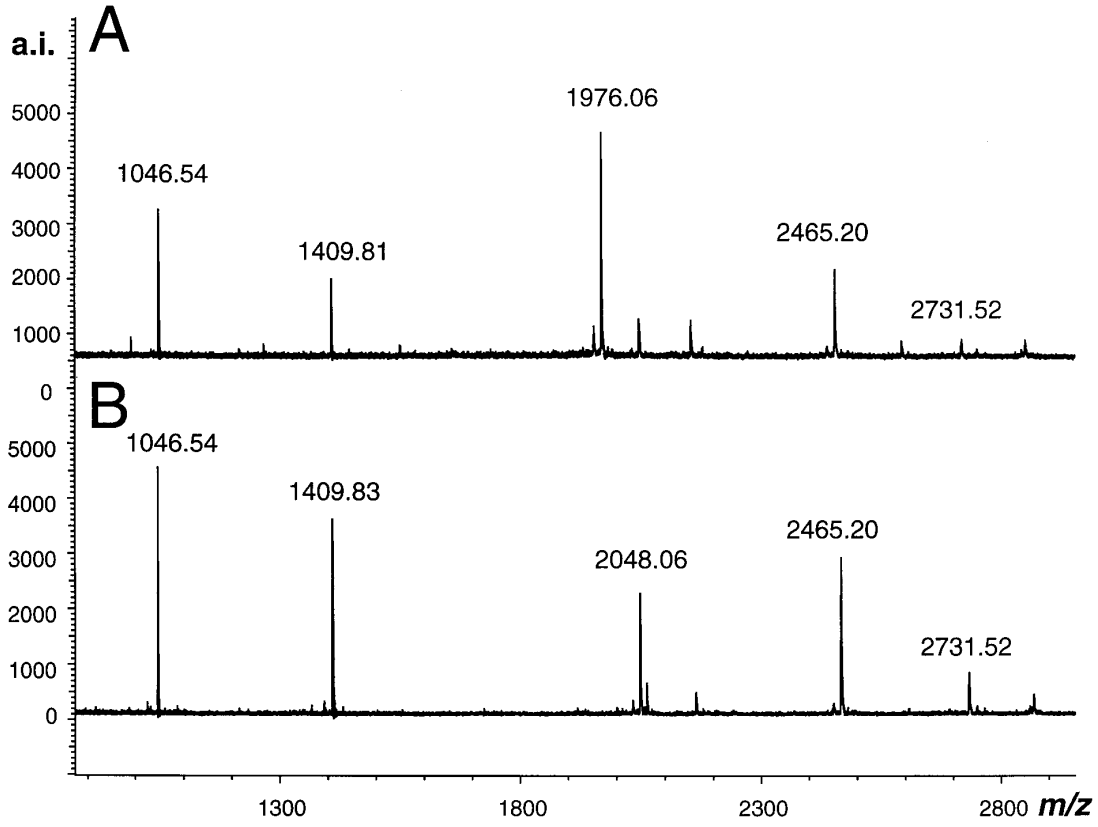

Figure 3. MALDI/TOF mass spectra of unfractionated AspN protease digests of (A) the V4 and (B) VRI 82-6409 F2 polypeptides. Both spectra were acquired in the reflector mode of operation using delayed extraction and CHCA as the matrix. Ions at *m/z* 1046.54 and 2465.20 are the monoisotopic ions of angiotensin II and ACTH 18–39, respectively, used as internal calibrants.

to an external calibration file created using the flight times of the components of a mixture of 200 fmol of angiotensin II ($MH^+ = 1046.56$; monoisotopic), 400 fmol of adrenocoticotropic hormone residues 18–39, ($MH^+ = 2466.73$; average mass peak centroid) and 2 pmol of bovine insulin ($MH^+ = 5734.54$; average mass peak centroid). Measurements were made at a digitization rate of 250 MHz in the continuous extraction mode.

Analysis of metastable ions arising from post-source decay (PSD) was performed using 20 stepwise decrements in the reflectron potential and increasing the laser irradiance to optimize the production of fragment ions in each voltage window.[37–40] Masses were assigned to metastable ions by reference to a calibration table created by determining the behaviour of metastable ions of known mass, produced from adrenocorticotropic hormone residues 18–39, at various reflectron potentials.[41] Metastable ion analysis data were acquired in the continuous extraction mode at a digitization rate of 250 MHz. Assembly of the individual spectra for each reflectron voltage on to a continuous mass scale was performed using Bruker FAST software routines within the Bruker XTOF software package.

Theoretical masses of proteolytically derived fragments of the V4 F2 polypeptide and of fragment ions resulting from PSD were calculated using a commercially available program (MacBiospec 1.0.1 from Perkin–Elmer SCIEX, Foster City, CA, USA) that computes fragment ion *m/z* values according to defined fragmentation pathways.[42,43]

Edman degradation

Stepwise amino terminal sequence analysis was performed by automated Edman degradation[44] using a Hewlett–Packard G1000A solid-phase protein sequencer.

RESULTS

As a first step in the process of determining the reason for the previously observed failure of the VRI 82-6409 isolate of NDV to react with antipeptide antibodies targeted at the *C*-termini of the F2 polypeptides of the common pathotypes of NDV,[21,22] intact F2 polypeptides of both the V4 and VRI 82-6409 isolates were purified by a combination of SDS-PAGE and electroelution[19,20,31] and subjected to mass analysis by MALDI/TOF-MS. The intact V4 F2 polypeptide revealed a series of peaks separated by 162 Da [Fig. 2(A)] which is indicative of high mannose oligosaccharides with varying numbers of mannose units at the single consensus sequence for N-linked glycosylation involving Asn54. Subtraction of the amino acid contribution to the mass (9061.4 Da) of the most prominent ion (*m/z* 10603.2) left a mass (1541.8 Da) corresponding to an oligosaccharide with a composition of (GlcNAc)2(Man)7 (1541.4 Da). The amino acid contribution was based on the previous observation[19,20] that the *C*-terminus of this F2 polypeptide was formed by

Table 1. Characterization of AspN protease fragments of V4 and VRI 82-6409 F2 polypeptides

Source	Found	*m/z*[a] Theory	Difference	Match
V4 F2				
Digest	1409.81	1409.81	0	1–15
Digest	1976.06	1976.02	0.04	66–84
Digest	2731.52	2731.51	0.01	16–40
HPLC, 46 min	1975.99	1976.02	0.07	66–84
HPLC, 56 min	1409.73	1409.81	0.08	1–15
HPLC, 66.5 min	4538.78	2997.46[b]	1541.32	41–65
HPLC, 68 min	2731.58	2731.51	0.07	16–40
VRI 82-6409 F2				
Digest	1409.83	1409.81	0.02	1–15
Digest	2048.06	1976.02	72.04	66–84
Digest	2731.52	2731.51	0.01	16–40
HPLC, 45 min	2047.94	1976.06	71.88	66–84
HPLC, 56 min	1409.78	1409.81	0.03	1–15
HPLC, 66 min	4376.07	2997.46[b]	1378.61	41–65
HPLC, 68 min	2731.46	2731.51	0.05	16–40

[a] Isotopic resolution was achieved for all peptides except glycosylated residues 41–65. Hence monoisotopic values are listed for all but the glycosylated peptides.
[b] Theoretical average value for residues 41–65 which accounts for modification of Cys45 with 5-*N*-[(acetamidoethyl)amino]naphthalene-1-sulphonic acid but not glycosylation of Asn54.

Gly84. Similar analysis of the VRI 82-6409 F2 polypeptide also revealed a series of ions separated by 162 Da [Fig. 2(B)], with the most prominent ion (*m/z* 10514.3) being smaller than the most prominent ion of the V4 F2 polypeptide by 89 Da. However, it was not possible to determine the reason for this difference by subtraction of the amino acid composition contribution to the mass of the VRI 82-6409 F2 polypeptide since its gene sequence has not been determined. The difference in the masses of the two F2 polypeptides could not be reconciled in terms of a single amino acid or monosaccharide difference.

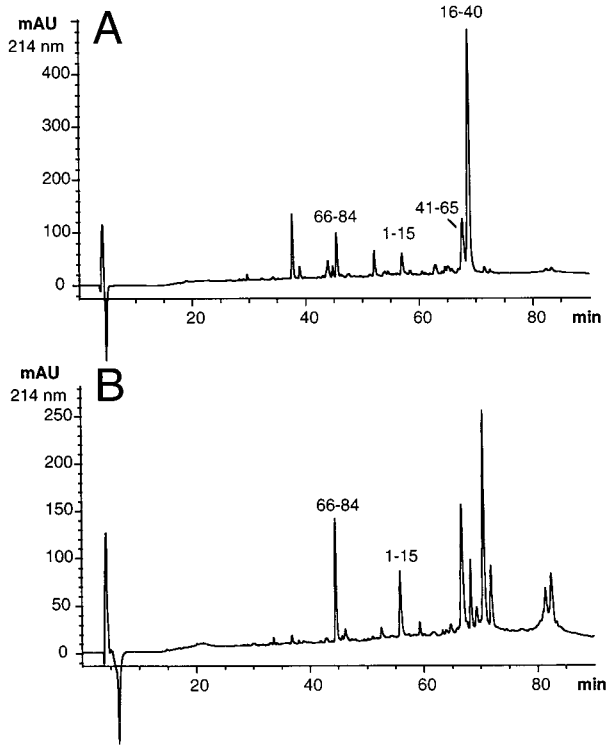

Figure 4. Reversed-phase HPLC separation of peptides derived by AspN protease digestion of the (A) V4 and (B) VRI 82-6409 F2 polypeptides. Numbers above peaks represent residue numbers spanned by peptides contained in the respective peaks.

Digestion of the V4 F2 polypeptide was performed with AspN protease and an aliquot of the unfractionated digest was analysed by MALDI/TOF-MS. Ions corresponding to residues 1–15 (*m/z* 1409.81) and residues 66–84 (*m/z* 1976.02) of the V4 F2 polypeptide were clearly evident in the spectrum of the digest in addition to ions produced by the internal calibrants and a less abundant ion representing residues 16–40 (*m/z* 2731.52) [Fig. 3(A)]. Use of internal calibration resulted in experimental masses within 20 ppm of the theoretical values for the AspN protease fragments (Table 1). Additional ions were evident in the AspN mass map; however, none of these matched sequences between aspartic acid and glutamic acid residues. One of these at *m/z* 2164.09 was close to the value for a non-specific cleavage to produce a peptide spanning residues 16–35 of the V4 F2 polypeptide (*m/z* 2164.22). Another ion at *m/z* 2054.94 appeared to reflect non-specific cleavage at residues 55 and 72 (*m/z* 2054.21). However, in- or post-source decay cannot be ruled out as the genesis of these ions. The AspN protease fragments were also isolated by HPLC [Fig. 4(A)] and analysed by MALDI/TOF-MS (Table 1). The masses of these peptides corresponded to those expected from the known sequence of the V4 F2 polypeptide (Table 1) and covered the complete sequence (Fig. 5). The peptide fraction at 46 min produced an ion at *m/z* 1975.99 which corresponded to residues 66–84 of the V4 F2 polypeptide sequence as observed in the unfractionated digest. The presence of high-mannose glycans on Asn54 of the V4 F2 polypeptide was corroborated by the spectrum of a peptide fraction at 66.5 min. This was evident from peaks spaced by 162 Da and a mass that corresponded to residues 41–65

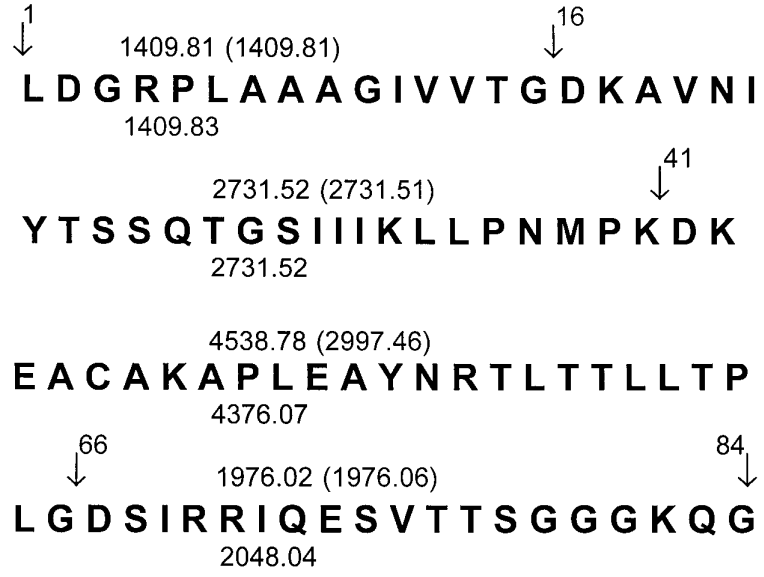

Figure 5. Alignment of masses expected from AspN protease cleavage of the V4 F2 polypeptide with the amino acid sequence. Boundaries between each peptide are indicated with vertical arrows and numbered amino acid residues form the *N*-termini of the peptides or the *C*-terminus of the ultimate peptide. Labels above each AspN protease peptide correspond to the experimental *m/z* values for the V4 peptides followed by the theoretical *m/z* values in parentheses. Labels below the sequence represent the experimental *m/z* values for the VRI 82-6409 isolate. Monoisotopic values are presented for all but the glycosylated sequence spanning residues 41–65 for which average values are used. Except for the glycosylated peptides, the data are from the unfractionated digest.

of the V4 F2 polypeptide sequence upon subtraction of the mass of (GlcNAc)2(Man)7 from the most prominent ion in the spectrum [Fig. 6(A)].

The VRI 82-6409 F2 polypeptide was also subjected to AspN protease cleavage and an aliquot of the digest subjected to MALDI/TOF-MS without fractionation [Fig. 3(B)]. Intense ion signals were observed in the unfractionated digest of the VRI 82-6409 F2 polypeptide at *m/z* values that corresponded to within 20 ppm of the theoretical values for residues 1–15 (*m/z* 1409.83)

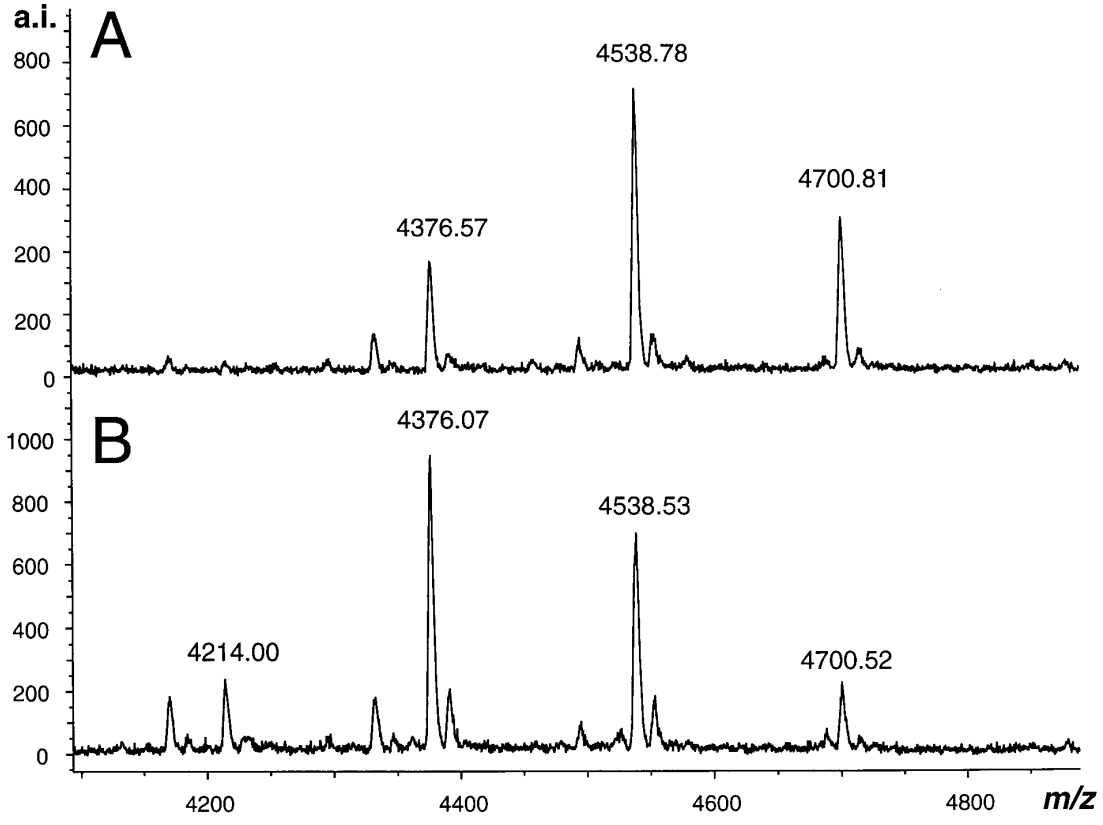

Figure 6. MALDI/TOF mass spectra of the glycopeptides corresponding to residues 41–65 of the (A) V4 and (B) VRI 82-6409 F2 polypeptides. Both spectra were acquired in the reflectron mode of operation with delayed extraction, external calibration and DHAP–DAHC as the matrix.

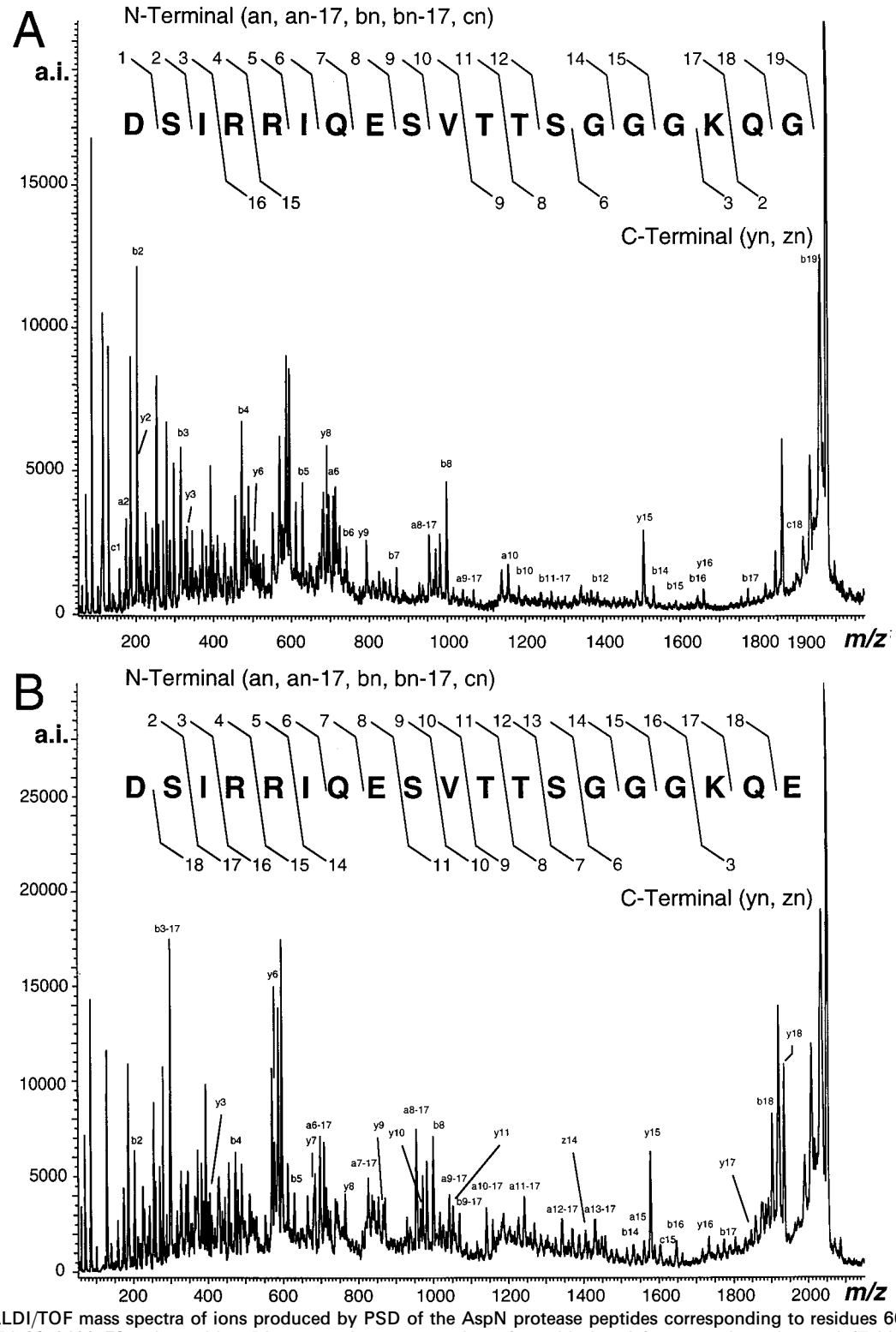

Figure 7. MALDI/TOF mass spectra of ions produced by PSD of the AspN protease peptides corresponding to residues 66–84 of the (A) V4 and (B) VRI 82-6409 F2 polypeptides. Diagrammatic representation of peptide bond fragmentations detected (Table 2) are shown above the respective spectra. Labels above ions in the spectra are representative of fragmentation at the indicated peptide bonds and are not necessarily the only ions corresponding to fragmentations at particular bonds.

and 16–40 (m/z 2731.52) of the V4 F2 polypeptide sequence (Table 1). No ion was observed corresponding to residues 66–84 of the V4 sequence even allowing for a decrease in mass of 89 Da to account for a variation in this region of the VRI 82-6409 F2 polypeptide. However, an abundant ion was observed that did not match any of the predicted AspN fragments of the V4 F2 polypeptide sequence. This ion (m/z 2048.06) was 72.04 Da heavier than residues 66–84 of the V4 sequence. As was seen for the V4 sequence, other ions were observed that appeared to represent non-specific enzymatic cleavage or fragmentation in the gas phase.

Peptides were isolated from the AspN protease digest of the VRI 82-6409 F2 polypeptide [Fig. 4(B)] that had

Table 2. Analysis of fragments formed by PSD of metastable ions of residues 66–84 of V4 F2 and VRI 82-6409 polypeptides

			m/z		
Fragment	Predicted V4 66–84	Observed V4 66–84	Difference	Observed VRI 82-6409 66–84	Difference
N-Terminal					
c1	131.1	131.2	0.1	—	—
a2	175.2	174.9	−0.3	175.4	0.2
b2	203.2	203.4	0.2	203.3	0.1
a3	288.3	288.2	−0.1	288.5	0.2
b3	316.3	316.2	−0.1	316.4	0.1
b3–17	299.3	—	—	298.5	−0.8
a4	444.5	—	—	444.6	0.1
b4	472.5	472.0	−0.5	472.6	0.1
b4–17	455.5	455.5	0	455.6	0.1
b5	628.7	628.7	0	629.0	0.3
b5–17	611.7	611.7	0	611.8	0.1
a6	713.9	713.5	−0.4	713.7	−0.2
a6–17	696.9	696.6	−0.3	696.9	0
b6	741.9	741.4	−0.5	742.2	0.3
b6–17	724.9	—	—	724.7	−0.2
a7	842.0	841.7	−0.3	842.0	0
a7–17	825.0	824.8	−0.2	825.4	0.4
b7	870.0	869.7	−0.3	870.3	0.3
b7–17	853.0	852.5	−0.5	853.0	0
a8	971.1	970.6	−0.4	971.3	0.2
a8–17	954.1	953.7	−0.4	954.3	0.2
b8	999.1	998.5	−0.6	999.3	0.2
b8–17	982.1	981.4	−0.7	982.2	0.1
a9–17	1041.2	1040.8	−0.4	1041.6	0.4
b9	1086.2	1085.8	−0.4	—	—
b9–17	1069.2	—	—	1068.6	−0.6
a10	1157.3	1157.0	−0.3	1157.6	0.3
a10–17	1140.3	1140.5	0.2	1140.8	0.5
b10	1185.3	1184.5	−0.8	1186.1	0.8
a11	1258.4	—	—	1258.1	−0.3
a11–17	1241.4	1240.4	−1	1242.0	0.6
b11	1286.4	—	—	1286.0	−0.4
b11–17	1269.4	—	—	1268.4	−1
a12	1359.5	1359.6	0.1	—	—
a12–17	1342.5	1342.9	0.4	1342.8	0.3
b12	1387.5	1386.9	−0.6	1387.9	0.4
b12–17	1370.5	1369.3	−1.2	1370.2	−0.3
a13–17	1429.6	—	—	1430.3	0.7
b13–17	1457.6	—	—	1457.2	−0.4
b14	1531.7	1531.0	−0.7	1532.2	0.5
a15	1560.7	—	—	1560.4	−0.3
b15	1588.7	1588.4	−0.3	1588.5	−0.2
c15	1603.7	—	—	1603.9	0.2
b16	1645.8	—	—	1646.8	1
b17	1773.9	1772.8	−1.1	1773.1	−0.8
b18	1902.1	—	—	1902.2[a]	0.1
c18	1717.1	1916.2[a]	−0.9	—	—
b19	1959.1	1960.0	0.9	—	—
C-Terminal					
y2	204.2	204.4	0.2	—	—
y3[b]	332.4	332.4	0	404.7	72.3
y6	503.6	503.6	0	576.5	72.9
y7	590.6	—	—	663.0	72.4
y8	691.7	691.4	−0.3	764.0	72.3
y9	792.8	792.3	−0.5	865.3	72.5
z9	775.8	775.1	−0.7	—	—
y10	892.0	—	—	965.0	73
y11	979.1	—	—	1051.4	72.3
z14	1332.4	—	—	1405.4	73
y15	1505.6	1505.2	−0.4	1577.6	72
y16	1661.8	1660.1	−1.7	1732.7	70.9
y17	1775.0	—	—	1846.4	71.4
y18	1862.1	—	—	1934.4	72.3

[a] These ions are diagnostic of a *C*-terminal glutamic acid and *C*-terminal glycine for the VRI 82-6409 and V4 sequences, respectively.
[b] These data were diagnostic of a difference of a glycine and glutamic acid within the last three residues at the *C*-termini of the V4 and VRI 82-6409 sequences.

masses covering residues 1–65 of the V4 F2 polypeptide sequence (Fig. 5; Table 1). The fraction that eluted at 66 min revealed a series of ions separated by 162 Da [Fig. 6(B)] that indicated correspondence with residues 41–65 of the V4 F2 polypeptide. The predominant glycoform in the spectrum of this peptide was 162.7 Da smaller than the corresponding V4 peptide. One peptide from the VRI 82-6409 F2 polypeptide digest eluted in approximately the same position as residues 66–84 of the V4 F2 polypeptide but it had a mass 71.92 Da greater (m/z 2047.94) than this region of the V4 sequence.

The mass difference between residues 66–84 of the two F2 polypeptides, apparent from analysis of the unfractionated AspN protease digests and analysis of HPLC-isolated fractions, was consistent with variation of one of the four glycine residues of the last six residues of the V4 F2 polypeptide to a glutamic acid in the VRI 82-6409 F2 polypeptide (Δ72.06). The only other potential amino acid differences that come close to accounting for the mass differences between ions attributed to residues 66–84 of the two sequences are glycine to lysine (Δ71.07) or glutamine (Δ71.04). However, this would have required a mass error of 450 ppm, which is much greater than was observed for the other peptides in the unfractionated digests using internal calibration (<20 ppm) or isolated HPLC fractions with external calibration (<60 ppm). The combination of theoretical mass differences due to the difference between a glycine and a glutamic acid and one less mannose in the VRI 82-6409 F2 polypeptide (Δ90) is close to the experimentally found difference of 89 Da between the major glycoforms of the two intact F2 polypeptides.

Additional peaks were observed in the chromatograms of digests of both F2 polypeptides. In the case of the V4 F2 polypeptide these were probably due to nonspecific cleavages by AspN protease. However, the late elution times of the majority of the additional peaks in the VRI 82-6409 F2 polypeptide digest is suggestive that there was also incomplete and/or partial enzymatic cleavage [Fig. 4(B)] of this F2 polypeptide.

Residues 66–84 of the V4 F2 polypeptide produced a PSD spectrum containing abundant fragment ions [Fig. 7(A)]. This included an extensive series of *N*-terminal (a, b and c) ions expected for this peptide [Fig. 7(A); Table 2]. A limited series of y ions was also observed with the V4 peptide that was consistent with a *C*-terminal glycine [Fig. 7(A); Table 2]. Analysis of fragment ions arising from PSD of the peptide corresponding to residues 66–84 of the VRI 82-6409 F2 polypeptide also revealed extensive fragmentation [Fig. 7(A); Table 2]. Ions could be discerned as corresponding to the nearly complete b and/or b − 17 series expected for residues 66–84 of the V4 F2 polypeptide [Fig. 7(B), Table 2]. Many of these b series ions were accompanied by corresponding a and a − 17 ions and c series ions were sometimes observed. Importantly, fragmentation of the *C*-terminal amino acid from the parent ion to conclude this ion series (b18) was consistent with the loss of glutamic acid [Fig. 7(B), Table 2]. An incomplete set of y ions of the sequence was also observed for residues 66–84 of the VRI 82-6409 F2 polypeptide. These y fragments were all 72–73 Da larger than the fragment ions theoretically feasible for the corresponding V4 peptide. This included the y3 ion which was also consistent with a variation of glycine to glutamic acid at the *C*-terminus of the VRI 82-6409 sequence.

Edman degradation analysis verified that the peptide containing residues 66–84 of the VRI 82-6409 F2 polypeptide varied from the V4 F2 polypeptide sequence only at the *C*-terminus. The PTH-amino acid signal for the *C*-terminal glutamic acid was evident for the VRI 82-6409 peptide, albeit at a considerably reduced level compared with preceding residues in the sequence; however, the *C*-terminal glycine of the V4 peptide was not detected at all. Diminished yields or failure to observe *C*-terminal residues is not unusual during Edman sequencing. This is due to the high solubility properties of PTC-amino acids, particularly small apolar amino acids, relative to PTC-peptides. Enhanced solubility of the PTC-amino acids results in their premature extraction from the reaction cartridge to waste during wash steps between coupling of phenyl isothiocyanate and transfer of the PTC-amino acid to the conversion flask for production of PTH derivatives.

DISCUSSION

The present study provided an explanation for the previously observed failure of the VRI 82-6409 isolate[22] to react with a panel of antipeptide antisera against the F2 polypeptides of various strains of NDV. This panel of antisera was produced to react specifically with sequences at the *C*-termini of the F2 polypeptides of fusion proteins of the common pathotypes of NDV (Fig. 1).[21] These sequences are generated as a consequence of endoproteinase cleavage of the various F0s and 'carboxypeptidase-like' trimming of the exposed basic amino acids.[19,20] Individual antisera were raised against synthetic peptides representing the sequence of each of the common pathotypes.[21] The only variations between the four sequences exists within the last three residues of sequence 1 and the last four residues of sequences 2–4 (Fig. 1). Previous studies have demonstrated that cross-reactivities were only observed between antisera raised using peptides 2 and 3 and their counterpart F2 polypeptides and that this cross-reactivity was only minor.[21,22] These two F2 polypeptides vary only by the presence of lysine or arginine at position 3 from their *C*-termini. Consequently, it is apparent that the specificities of the antisera are for the last four residues of the various F2 polypeptides. Hence it is not surprising to find that the VRI 82-6409 isolate, which did not react with the currently available panel of antipeptide antisera,[22] has a variation within the last four residues of its F2 polypeptide.

Antisera for fusion protein cleavage motifs are potentially very valuable for pathotyping NDV isolates.[21–23] However, it seems reasonable to anticipate that other isolates of NDV may be detected through failure to react with the currently available panel of antisera. As with the VRI 82-6409 isolate, these negative reactions would have to be investigated further. Subsequent investigation could be at the viral genome level, such as by using the polymerase chain reaction (PCR)[17,18,25] or the protein structural level, or both. However, protein

characterization would be required to reveal details of post-translational modifications that occur during propagation of these viruses.[5,6,9,19,20] In the instance of the F2 polypeptide these include glycosylation and post-cleavage trimming of basic amino acids.[19,20] Determination of the actual *C*-termini of variant F2 polypeptides provides an option of producing specific antipeptide antisera to detect the variant sequences.

Although reasonably accurate determination of the intact molecular masses of the F2 polypeptides of the V4 and VRI 82-6409 isolates was possible by MALDI/TOF-MS and did indicate structural differences, this was not sufficient to characterize the differences. This was due in part to differences in the glycoforms of the two isolates. Even if this difference had been taken into account it would have allowed only an approximation of the actual difference in mass due to amino acid composition differences. The mass difference due to one less mannose ($\Delta - 162.14$) plus the experimental mass difference ($\Delta + 88.9$) is indicative of an actual mass difference due to amino acid compositions of 73.24 Da which is over 1 Da more than the theoretical difference due to the sequence variation of glycine to glutamic acid. Furthermore, determination of the compositional difference within the intact F2 polypeptide would not have identified the position of the variation. Definitive determination of the compositional variations between the two F2 polypeptides was achieved by analysis of the masses of peptides derived by AspN protease cleavage, including the glycosylated peptides. This revealed that the compositional differences involved a combination of an amino acid change and a difference in glycosylation, localized these differences to particular AspN peptides and provided peptides to perform PSD analysis to identify the variant amino acids. In addition, alignment of the data set for the VRI 82-6409 F2 polypeptide with the known V4 sequence provided a provisional determination of the sequence of the VRI 82-6409 F2 polypeptide which had not previously been performed at the protein or gene level.

Determination of the masses of the AspN protease peptides corresponding to residues 66–84 of the VRI 82-6409 and V4 F2 polypeptides demonstrated that a difference in mass of 72 Da arose from a difference in amino acid composition within this region. The most likely variation to account for a mass difference of 72 Da is a glycine to a glutamic acid. Four glycine residues are present within the last six residues of the V4 F2 polypeptide, two of which (Gly81 and Gly84) occur within the last four residues (Fig. 1). Failure of the VRI 82-6409 isolate to react with the panel of antisera may have been due to a variation for any one of these glycine residues but, as discussed above, the antisera specificity suggests positions 81 and 84 as the most likely to be affected by the variation. Isolates had previously been identified with a glycine to glutamic acid variation at position 81[24] or at both positions 81 and 84.[19–24] However, PSD analysis established that the variation of the VRI 82-6409 isolate involved the *C*-terminal position 84. This finding was confirmed by Edman degadation. These findings also have implications concerning the specificities of the antisera used to identify the VRI 82-6409 isolate. It is apparent that the amino acids at positions 81 and 84 of the F2 polypeptides influence recognition by the various antisera. For instance, the VRI 82-6409 isolate was not detected by antisera to sequences 2, 3 and 4 (Fig. 1), despite the fact that the VRI 82-6409 isolate only has the additional side-chain of glutamic acid at position 84 relative to sequence 2 and only lacks the side-chain of glutamic acid at position 81 relative to sequence 4. Mass analysis of the AspN protease peptides of the VRI 82-6409 F2 polypeptide also indicated a difference from the V4 F2 polypeptide in the nature of glycosylation at Asn54. It is not clear whether this represents a systematic difference between the two isolates of NDV or if it is simply a random finding associated with particular preparations of the viruses.

The original characterization of the *C*-termini of the F2 polypeptides of the common pathotypes of NDV and variant isolates involved the use of FAB ionization on a sector instrument.[19,20,22,23] That approach required the use of considerably more material than was needed for the present study and did not allow the determination of the mass of the intact F2 polypeptides or glycosylated peptides or the sequence of the *C*-termini of the F2 polypeptides. The present study demonstrated the comparative advantage gained through the use of MALDI/TOF-MS in terms of both accuracy, sensitivity and structural analysis capability. AspN peptides were detected with accuracies of within 20 ppm using internal calibration, even with unfractionated digests. The impact of this accuracy was to allow exclusion of potential variations in composition in favour of identification of the actual variation. It may have been valid to have identified invariant proteolytically generated fragments, for example residues 1–15 and residues 16–40, using external calibration and to have used their theoretical masses as internal calibrants for accurate determination of the mass of the variant part of the F2 polypeptide, residues 66–84. This would have avoided the complication of admixture of exogenous internal calibrants and potential losses in sensitivity. Although the limiting amount of F2 polypeptide required for characterization by MALDI/TOF-MS was not determined in the present study, it may be feasible to use MALDI/TOF-MS for the rapid and sensitive characterization of unusual isolates of NDV on a routine basis without the need to extract the intact F2 polypeptide from gels and isolate specific proteolytic fragments by HPLC. This approach would utilize in-gel digestion of F2 polypeptides separated by SDS-polyacrylamide gel electrophoresis, parent ion selection to isolate specific ions of interest in the unfractionated digests and PSD analysis to establish their sequences. Avoidance of electroelution and HPLC would be more economical in terms of both time and quantities of samples required.

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