

# Antigenic and Structural Properties of the Hemagglutinin-Neuraminidase Glycoprotein of Human Parainfluenza Virus Type 3: Sequence Analysis of Variants Selected with Monoclonal Antibodies Which Inhibit Infectivity, Hemagglutination, and Neuraminidase Activities

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The hemagglutinin-neuraminidase (HN) gene sequence was determined for 16 antigenic variants of human parainfluenza virus type 3 (PIV3). The variants were selected by using monoclonal antibodies (MAbs) to the HN protein which inhibit neuraminidase, hemagglutination, or both activities. Each variant had a single-point mutation in the HN gene, coding for a single amino acid substitution in the HN protein. Operational and topographic maps of the HN protein correlated well with the relative positions of the substitutions. There was little correlation between the cross-reactivity of a MAb with the bovine PIV3 HN and the amount of amino acid homology between the human and bovine PIV3 HN proteins in the regions of the epitopes, suggesting that many of the epitopes are conformational in nature. Computer-assisted analysis of the HN protein predicted a secondary structure composed primarily of hydrophobic  $\beta$  sheets interconnected by random hydrophilic coil structures. The HN epitopes were located in predicted coil regions. Epitopes recognized by MAbs which inhibit neuraminidase activity of the virus were located in a region which appears to be structurally conserved among several paramyxovirus HN proteins and which may represent the sialic acid-binding site of the HN molecule.

Human parainfluenza virus type 3 (PIV3) appears to be endemic in all areas of the world and is a significant cause of acute bronchiolitis and pneumonia in infants (5). Because of this important role in childhood illness, we are interested in developing an effective vaccine for PIV3. Since antibodies directed to the surface glycoproteins of other paramyxoviruses play a major role in prevention of infection in vitro and in vivo (14, 15), we studied the hemagglutinin-neuraminidase (HN) glycoprotein of PIV3 by constructing operational and topological antigenic maps by using monoclonal antibodies (MAbs) (6, 7). Competitive-binding radioimmunoassays identified six topographically distinct antigenic sites on the PIV3 HN glycoprotein (A to F). MAbs which neutralize virus infectivity and inhibit hemagglutination defined eleven unique epitopes which are located in antigenic sites A, B, and C. MAbs to several site A epitopes also inhibited neuraminidase activity when the small substrate *N*-acetyl neuraminylactose was used, suggesting that site A is located on the HN molecule in a region topographically close to the sialidase active site. Three additional antigenic sites (D, E, and F) were detected by anti-HN MAbs which do not neutralize infectivity or inhibit either hemagglutination or neuraminidase activities.

Several MAbs to site A epitopes cross-neutralize the bovine PIV3 strain. To identify the amino acids important for the integrity of those shared epitopes, we recently sequenced the HN genes of antigenic variants selected in the presence of those cross-reactive MAbs and compared them with the sequences of the HN genes of the parental human PIV3 and of the bovine virus (7). As expected, the deduced

amino acid substitutions in these variants occurred in regions of high amino acid homology between the human and bovine strains. We have now characterized the mutations in variants selected with MAbs directed to epitopes in sites A, B, and C which do not neutralize the bovine strain. The present report describes amino acid substitutions in the HN molecule of antigenic variants selected with MAbs which inhibit virus infectivity, neuraminidase, or hemagglutination activities of human PIV3, or a combination thereof. The positions of the substitutions correlate well with previously published operational and topographic maps. Computer-assisted structural analysis predicts that the HN epitopes are located in coil structures which interconnect regions of  $\beta$ -sheet topology.

## MATERIALS AND METHODS

**Cells and viruses.** LLC-MK<sub>2</sub> cells were grown in Eagle modified minimum essential medium supplemented with 10% fetal bovine serum. Semiconfluent monolayers were infected with PIV3 at a multiplicity of 0.1 in serum-free Eagle minimum essential maintenance medium. After 72 h, the medium was harvested, and virus was concentrated by centrifugation. Virus was purified by sedimentation through 30% sucrose onto a cushion of 60% sucrose.

**Production of MAbs and selection of antigenic variants.** Neutralizing MAbs specific for the HN protein of the human PIV3/WASH/47885/57 strain were used to select antigenic variants. The biological activities of the MAbs and the antigenic properties of the variants have been previously described (6, 7), with the exception of variant 454/11V3f1b. Variant 454/113f1b does not react with epitope I MAbs

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TABLE 1. MAb and variant characterization

MAb characterization				Variant characterization		
Mab	Epitope	Antigenic site	Activities inhibited <sup>a</sup>	Variant	Codon change	Amino acid change
170/7	I		H, N	170/7V4a <sup>b</sup>	CCT → ACT	370 Pro → Thr
	I	A		170/7SV1a <sup>b</sup>	CCT → CAT	370 Pro → His
271/7	I		H, N	271/7V2a <sup>b</sup>	GCA → GTA	281 Ala → Val
423/4	I		H, N	423/4V2a <sup>b</sup>	CCT → ACT	370 Pro → Thr
451/4	VI	A	H (N)	451/4V31a <sup>b</sup>	TCA → TTA	278 Ser → Leu
				451/4SV1a <sup>b</sup>	TCA → TTA	278 Ser → Leu
166/11 <sup>c</sup>	VII	A	H (N)			
149/3 <sup>c</sup>	IIIB		H, N			
128/9	IIIA	A	H, N	128/9V1c	AAA → GAA	500 Lys → Glu
454/11	IIIA		H (N)	454/11V3f1b	AGT → AAT	369 Ser → Asn
101/1	IIIA		H	101/1V6a	AAT → GAT	461 Asn → Asp
403/7	IIA	C	H	403/7V1b	AAT → GAT	364 Asn → Asp
61/5	IIA	C	H	61/5V8a	AAT → GAT	345 Asn → Asp
447/12	IIC	A	H	447/12V2a	GAG → GGG	346 Glu → Gly
77/5	IIB	C	H	77/5V4a	AAT → AGT	347 Asn → Ser
429/5	IV		H	429/5V4a3a	AAA → AAC	171 Lys → Asn
66/4	VA	B	H	66/4V2a	AAG → AAT	395 Lys → Asn
68/2	VB		H	68/2V4a	TGG → TTG	397 Trp → Leu

<sup>a</sup> MAbs were assayed in standard HI assays and neuraminidase inhibition assays as previously described (7). Parentheses denote that MAbs 451/4, 166/11, and 454/11 inhibit neuraminidase activity only when fetuin is used as a substrate, whereas all other MAbs which inhibit neuraminidase activity to do so with both fetuin and *N*-acetyl neuraminylactose substrates. N, Neuraminidase; H, hemagglutination.

<sup>b</sup> The sequence changes of these variants have been previously described (7).

<sup>c</sup> We were unable to select a stable mutant for sequence analysis with these antibodies.

(170/7, 272/7, and 423/4), and in this respect it differs from variants previously selected with MAb 454/11.

**Nucleic acid sequence analysis.** Nucleic acid sequence analysis of the variants was done by using virion RNA obtained by proteinase K-sodium dodecyl sulfate treatment of purified virions followed by phenol-chloroform extraction (3). Propagation of variants in 12 roller bottle cultures of LLC-MK<sub>2</sub> cells yielded 30 to 100 µg of virion RNA. Synthetic oligonucleotide primers complementary to PIV3/47885/57 virion RNA at intervals of about 300 nucleotides were used to sequence virion RNA by reverse transcription, as previously described (7).

## RESULTS

**Sequence changes in variants selected with MAbs which inhibit hemagglutination and neuraminidase activities.** The coding sequences of the HN genes of the antigenic variants were determined, and a single-point mutation was found in each case. The nucleotide changes and deduced amino acid substitutions in these variants, as well as those described previously (7), are shown in Table 1. Also shown are the biological activities, hemagglutination and neuraminidase, inhibited by the selecting antibodies. The amino acid substitutions in the variants are generally of a nonconservative nature, resulting in a change in charge or hydrophobicity. In one case (variant 77/5V4a), the substitution at position 347 introduces a new N-glycosylation site, which is located near an existing glycosylation site (position 351 to 353). Whether this new site is used is not known.

There is no strong correlation between the biological activity inhibited by the selecting antibody and the position of the amino acid substitution in the variant. However, MAbs which inhibit neuraminidase activity select variants with substitutions which appear to cluster in three regions of the HN sequence (positions 278 and 281, 369 and 370, and 500). The remaining MAbs, which inhibit only hemagglutination, select variants with mutations at widely separated

positions in the linear sequence, with the only obvious clustering occurring at residues 345, 346, 347, and 395 and 397.

**Correlation between cross-reactive and specific MAbs and amino acid homology between human and bovine HN proteins.** We have previously examined the antigenic relationships between the human PIV3 and the bovine PIV3 by using MAbs. MAbs 170/7, 271/7, 423/4 (epitope I), and 451/4 (epitope VI) cross-react with the bovine strain in the enzyme-linked immunosorbent assay and in hemagglutination inhibition and neuraminidase inhibition tests. We found that these cross-reactive MAbs select variants with amino acid substitutions in stretches of hydrophilic residues which are highly conserved between human and bovine viruses (7). These results are compatible with the idea that the cross-reactive MAbs recognize linear sequences of amino acids which are highly homologous between the two HN proteins. This hypothesis predicts that non-cross-reactive MAbs will select variants with amino acid substitutions in regions of relatively low homology. To test this hypothesis, we compared the homology between the human and bovine PIV3 HN proteins in the regions of substitutions selected by MAbs which recognize either common or human PIV3-specific epitopes (Fig. 1). Although MAb 128/9 does not inhibit hemagglutination activity of the bovine virus, it does cross-react in the enzyme-linked immunosorbent assay and the neuraminidase inhibition test, and therefore it is grouped with antibodies recognizing common epitopes. In some cases, the homology in the flanking sequences of the human-specific epitopes is lower than that of the common epitopes. For example, residues 345, 346, and 347 are flanked by nonhomologous amino acids extending from positions 343 to 348. Similarly, residue 171 and residues 395 and 397 are found in regions of relatively low homology (amino acids 167 to 175 and 388 to 402). However, in several other cases the amino acids flanking the human-specific epitopes are highly conserved. The most striking example of this is residue 364 which, in addition to being flanked by conserved amino

Common Epitopes		Specific Epitopes	
MAb		MAb	MAb
451/4	271 ∇ ∇ 291 TPKVDERSDYASSGIEDIVLD	61/5	336 ∇ ∇ ∇ 356 GYGGLLEHPINENAICNTTGCP
271/7	TPKVAERSDYASTGIEDIVLD	447/12	GYGGLLEHEDNGDVICNTTGCP
170/7	360 ∇ 380 QRDCNQASHSPWFSDRRMVNS	429/5	161 ∇ 181 SGLPGLMKTPIRLMPGPGLL
423/4	QRDCNGASYSWFSNRRMVNS	101/1	451 ∇ 471 WHNLSRPGNNECPWGHSCFD
128/9	490 ∇ 510 IVSSVILDSQKSRVNPVITYS	66/4	386 ∇ ∇ 406 KGLNSIPKLVWTISMQRNYW
	VVSSVILDSQKSRNPVITYS	68/2	KGIDATFSLRVWTIPMSQNYW
		403/7	354 ∇ 374 GCPGKTQRDCNQASHSPWFSN

FIG. 1. Amino acid homologies between the human (top) and bovine (bottom) PIV3 HN proteins in regions flanking common and human-specific epitopes. Residue numbers of the human PIV3 HN are shown above the sequence. Dots between the sequences indicate homology. Alignments were done by using the Protaln program (19) with a K-tuple size of 2 and a gap penalty of 4. Arrowheads mark the residues substituted in variants selected with each MAb.

acids, is itself conserved in the bovine HN. Clearly, the specificity of the MAbs for the human PIV3 HN cannot be explained solely by the differences in the linear sequences of the two HN proteins. A likely explanation for the data is that the MAbs recognize conformational epitopes composed of nonlinear amino acids. Indeed, variant 403/7V1b, which has the above-described substitution at position 364, is no longer recognized by MAbs which select variants with substitutions at residues 345, 346, and 347, suggesting that these four amino acids are juxtaposed on the folded molecule, bringing residue 364 from an area of high homology into an area of low homology (residues 345, 346, and 347). The conformational nature of these epitopes is supported by their loss of binding under denaturing Western blotting conditions (V. Hemming, unpublished observations). Thus, it appears that at least some of our MAbs recognize conformational

epitopes composed of nonlinear amino acids contributed by conserved and nonconserved regions of the HN molecule.

**Secondary structure predictions for the HN protein immunogenic regions.** Computer-assisted analysis of the HN amino acid sequence was done to predict the structural context in which the HN epitopes are located. The algorithms of Garnier et al. and Hopp and Woods were used to generate secondary structure probabilities and hydropathicity values (10, 11). These algorithms, when applied to the influenza A virus neuraminidase (NA), predict regions of  $\beta$ -sheet and loop structures with 54 and 66% accuracy, respectively. When applied to the PIV3 HN, these algorithms predict that the N-terminal 30 amino acids and residues 55 to 89 of the HN are regions of  $\alpha$ -helix. Between these two helical regions is a stretch of  $\beta$ -sheet-forming residues which is very hydrophobic and represents the

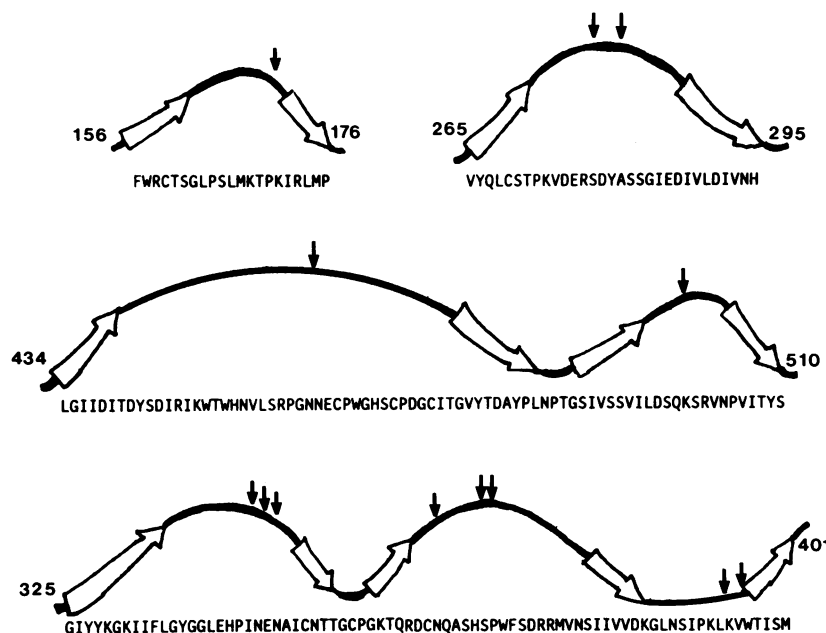


FIG. 2. Schematic diagram of secondary structure analysis of HN epitopes and flanking regions (residues 156 to 176, 265 to 295, 434 to 510, and 325 to 401). Below each diagram, the amino acid sequence of the parental wild-type HN protein is shown. Large arrows depict regions predicted to be  $\beta$ -sheet structures, arched solid lines represent hydrophilic coils, and cupped solid lines represent hydrophobic coils. Vertical small arrows mark the positions of the epitopes as determined by sequence analysis of antigenic variants.

putative N-terminal membrane-anchorage sequence (9). The two aforementioned regions at the N terminus and a third stretch of residues at the C terminus (residues 516 to 572) are virtually the only areas of the HN molecule yielding a high probability of  $\alpha$ -helix structure.

The algorithm of Garnier et al. predicts that the remainder of the molecule is predominantly hydrophobic  $\beta$  sheets connected by hydrophilic regions with high random coil and turn probabilities. The results of secondary structure analysis of areas where amino acid substitutions occur in PIV3 HN antigenic variants are diagrammed in Fig. 2. Nearly all of the epitopes are found in hydrophilic random coil or turn regions flanked by areas of  $\beta$ -sheet topology. The two exceptions to this pattern are residues 395 and 397, which are located in a hydrophobic coil region.

### DISCUSSION

We sequenced the HN genes of antigenic variants of PIV3 selected with MAbs to identify immunogenic amino acid residues on the HN protein. These experiments assume that the site of amino acid substitution corresponds to the actual epitope. This assumption has recently been supported by X-ray diffraction analysis of an influenza A variant hemagglutinin (HA) (12). The work presented here has identified single amino acids which are important for the integrity of HN epitopes. Most of these epitopes are located in the C-terminal half of the molecule, as expected for a protein anchored at its N terminus (9). Previously published operational and topographic maps of the PIV3 HN indicated that our MAbs recognize six distinct groups of epitopes (I to VI) organized into two topographically separate sites (A and B), which are partially bridged by site C. Epitope groups (I to VII) were based on reactivity patterns of the MAbs with laboratory-selected antigenic variants. Epitope group subdivisions (e.g., IIA, IIB, IIC) were based on fine specificities of the antibodies for a large panel of clinical isolates (6). It is reasonable to expect that MAbs belonging to the same epitope group should select variants with amino acid substitutions which cluster in the linear sequence of the HN molecule, and in some cases this was indeed found. For example, variants selected with MAbs recognizing epitopes IIA, IIB, and IIC have substitutions at positions 345, 346, and 347. Similarly, variants selected with MAbs to epitopes VA and VB have substitutions at positions 395 and 397. On the other hand, three MAbs to epitope IIIA selected variants with substitutions at scattered positions (369, 461, and 500), suggesting that these residues are juxtaposed on the folded molecule. It is evident from the antigenic analysis of variant 454/11V3flb that a substitution at residue 369 affects the binding of epitope I MAbs, two of which select variants with substitutions at neighboring residue 370. Thus, the antigenic maps of the HN molecule correlate well with the sequence changes in the variants.

To test whether the MAbs recognize linear amino acid sequences, we compared the homology between human and bovine PIV3 HN proteins in the regions of the epitopes recognized by MAbs which are human PIV3 specific and by MAbs which cross-react with the bovine strain. We previously noted that all of the substitutions in variants selected by cross-reactive MAbs involved amino acids which were conserved between the two strains (7). We have now shown that several of the substitutions selected by non-cross-reactive MAbs involve residues located in regions of low homology. These results support the hypothesis that the amino acid substitutions in the variants correspond to the

actual sites of antibody binding. However, in several cases, although a MAb did not cross-react with the bovine PIV3, the amino acids in question were located in regions of high homology with the bovine strain. This indicates either that the substitution produces allosteric effects or that the conserved amino acid in question is part of a conformational epitope composed of conserved and nonconserved residues contributed by different regions of the HN molecule. We are currently sequencing the HN genes of several clinical PIV3 isolates with differences in their HN epitopes to identify additional residues which may participate in these epitopes.

There are two strong parallels between the influenza virus NA and the paramyxovirus HN proteins: the N-terminal anchorage sequence and similar enzymatic functions. The HN possesses the additional function of hemagglutination, which has also recently been described for the NA of the N9 subtype of influenza virus (13). These similarities have prompted speculation that the HN and NA genes may have evolved from a common ancestral gene (4). The three-dimensional structures of both influenza A surface glycoproteins, HA and NA, have been determined, and the HA receptor-binding site and NA catalytic site have been identified (8, 17, 20, 21). Sequence analyses of influenza NA antigenic variants have shown that MAbs which inhibit enzyme activity with the low-molecular-weight substrate select variants with mutations bordering the catalytic site (for a review, see reference 2). By analogy with the influenza system, our sequence analysis of HN antigenic variants, which contain both hemagglutination and neuraminidase activities on a single glycoprotein, should identify not only the HN epitopes but also residues proximal to the receptor-binding site, the enzyme active site, or both. Our MAbs could not completely dissociate the two biological activities of the HN protein: some of the MAbs inhibited only hemagglutination, but all of the MAbs which inhibited neuraminidase activity also inhibited hemagglutination. Whether this means one active site performs both functions is not known. However, MAbs which inhibit exclusively either hemagglutination or neuraminidase activity of the Sendai virus HN have been produced, supporting the two-site hypothesis (16). The present study indicates that PIV3 HN MAbs which inhibit hemagglutination and neuraminidase activities or only hemagglutination select mutations which appear to cluster in different segments of the HN molecule, but their relative locations on the three-dimensional molecule remain to be determined.

Computer-assisted analysis of the HN protein predicts that the molecule will have a secondary structure of alternating hydrophobic  $\beta$  sheets and hydrophilic random coil-turn topology, but very little  $\alpha$ -helix content. This relatively unusual structure is also characteristic of the influenza NA (17). A recent comparison of predicted secondary structures of the HN proteins of four paramyxoviruses revealed that one region in particular was highly conserved structurally and that it strongly resembled the sialic acid-binding site of the influenza NA (E. Jorgensen and P. T. Lomedico, submitted for publication). This region corresponds to residues 234 to 323 of the PIV3 HN, and to residues 208 to 287 of the N2 NA. MAbs which inhibit influenza virus neuraminidase activity with *N*-acetyl neuraminylactose select NA variants with substitutions in this area (positions 221 and 253) (1, 18). The present study shows that two MAbs which inhibit PIV3 neuraminidase activity with *N*-acetyl neuraminylactose or fetuin select variants with mutations in the corresponding PIV3 HN sequence (positions 278 and 281). Furthermore, MAbs to the Sendai virus HN protein which inhibit

neuraminidase activity also select variants with mutations in this region (S. Thompson and A. Portner, personal communication). These results reinforce the idea that the functional domains of the HN and NA proteins are structurally similar, but confirmation awaits the determination of the three-dimensional structure of the HN molecule by crystallography.

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