PROTEINS: Structure, Function, and Genetics 15:121–132 (1993)

Identification of Critical Contact Residues in the NC41 Epitope of a Subtype N9 Influenza Virus Neuraminidase

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ABSTRACT **We have examined amino acids on influenza virus neuraminidase (NA) subtype N9 (A/tern/Australia/G70c/75) which are in contact with monoclonal antibody NC41 to analyze individual interactions important for antibody recognition. The crystal structure of NA complexed with NC41 Fab[1] shows antibody contacts at 19 amino acid residues on the NA surface which are localized on five polypeptide loops surrounding the enzyme active site. Fifteen mutant NA genes were constructed to encode a protein which contained a single amino acid substitution and these were tested for effects of the replacement on NC41 binding. Our data revealed that NAs with changes at 368, 400, and 434 completely lost NC41 recognition. NAs with side chains replaced at residues 346 and 373 exhibited binding reduced to less than 50% of wild-type binding. Changes in seven other contacting residues, including substituted side chains which differed considerably from wild-type NA in size and charge, had no significant effect on NC41 binding. These results indicate that only a few of the many residues which make up an epitope are crucial for interaction and provide the critical contacts required for antibody recognition. This implies that antibody escape mutants are selected only if they contain changes at these crucial sites, or changes which introduce bulky side chains that sterically prevent antibody attachment.**

Key words: epitope, neuraminidase, critical contacts, antigen–antibody complex, monoclonal antibody, site-directed mutagenesis, influenza virus

INTRODUCTION

The two major antigenic determinants found on the surface of influenza virus types A and B are the hemagglutinin (HA) and neuraminidase (NA) glycoproteins. HA permits attachment of the virus to sialic acid moieties on the surface molecules of host cells and, upon endocytosis, triggers fusion between virus and host cell membranes (reviewed in ref. 2). While the role of NA in the viral life cycle is not clear, it is considered a receptor-destroying enzyme. This activity allows it to remove sialic acid residues from the surface of infected cells and from newly synthesized HA and NA which permits release of progeny virions and prevents self-aggregation of virus particles.[3] Most neutralizing antibodies directed against HA inhibit infection by blocking its interaction with sialic acid on the surface of host cells.[4] Antibodies directed against NA, while not inhibiting virus entry and therefore not neutralizing in the usual sense, still protect against spread of the virus and against challenge with the same or similar virus.[5] Antigenic variation in either of these proteins results in loss of antibody recognition and consequently vaccines must be redesigned each year to combat new influenza strains.

The NA is found as a tetramer on the virus, anchored in the lipid bilayer by the hydrophobic amino terminus (for a review, see ref. 6). A stalk region consisting of approximately 40 amino acids supports the enzymatically active, box-shaped head. Each monomer comprising the head consists of six four-stranded antiparallel β-sheets arranged somewhat resembling a propeller.[7] The enzyme active site lies within a depression located on top of each monomer. In some strains, fully active NA heads can be released from the stalk by digestion with pronase[8] and subsequently crystallized alone or in complex with antibody. Crystal structures of influenza A subtype N2 and N9 NAs have been determined[7,9] and refined[10–12] and more recently the structure of influenza B NA, isolated from B/Beijing/1/87[13,14] has been determined. Despite only 30% sequence identity between B/Beijing NA and N2 or N9 NA,[13] the protein fold is essentially the same, although the B NA is more compact and each subunit is tilted 25°

Received April 9, 1992; revision accepted July 10, 1992.
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such that the enzyme active site is directed towards the side rather than on top as in the influenza type A NAs.[14] The tilting results in the formation of a central hole around the 4-fold axis on the underside of the tetramer.

The crystal structure of a complex of N9 NA from A/tern/Australia/G70c/75 with NC41 Fab has been determined[15,16] and refined[1] and shows that the antibody contacts the NA over a surface area of 900 Å^2. Although earlier estimates varied, the refined structure shows 19 amino acids on the NA in contact with 17 residues on the NC41. To investigate the antigenic structure of N9 NA, Webster et al.[17] generated 35 monoclonal antibodies to the NA. Eighteen escape mutants were selected with these antibodies and the sequence changes determined. Each of the escape mutants contained a single amino acid change located on the top surface of the NA near the rim of the active site depression. Those escape mutants that did not react with NC41 had single sequence changes at positions subsequently found to be contacts with Fab in the complex structure. To more fully characterize the NC41 epitope, multiple selections of escape mutants by NC41 were made to see if changes in all of the NC41 contact residues could be selected. Surprisingly, of the 19 escape mutants selected by NC41, ten were at residue 373, six at 368 and two at 400. The other escape mutant had two changes, at residues 434 and 437.[18] Some escape mutants selected with other monoclonal antibodies had changes at NC41 contact amino acids 370 and 371, and these mutants did not bind NC41. However, while obviously present in the population, these non-binding mutants were not selected by NC41. Due to the limited sites of sequence changes in escape mutants, it was hypothesized that the antibody selection process may be extremely rigorous, and only in contact residues most crucial for interaction will a single change be sufficient to abolish binding and allow escape from antibody inhibition.[18]

The present study was designed to understand how NA is able to escape antibody recognition by determining which NC41 contact residues are most crucial for antibody binding. Fifteen N9 NA mutants were constructed by oligonucleotide-directed, site-specific mutagenesis. Fourteen contained single amino acid changes at NC41 contact residues which were not changed in the escape mutants. The other N9 NA mutant contained a change at a residue which is adjacent to the NC41 epitope, but is not in contact with the Fab. The mutations spanned each of the five polypeptide loops that make up the NC41 epitope. We have identified amino acids Ser-368, Asn-400, and Lys-434 within the NC41 epitope as critical contact residues for the recognition of Fab. The data presented here support the hypothesis that only a few residues are absolutely necessary for interaction with antibody such that any single change in those residues totally abolishes antibody recognition. Antibody escape mutants are selected at other contact residues only if the side chain substitution results in substantial steric interference.

MATERIALS AND METHODS

Viruses and Cells

A cloned, full-length cDNA copy of influenza virus neuraminidase gene isolated from the reassortant strain A/NWS/33(H1)-A/tern/Australia/G70c/75(N9) has been described.[19] For transient expression of the proteins in tissue culture, CV-1 cells (an African green monkey kidney cell line) and baby hamster kidney (BHK) cells were used. CV-1 cells were grown in DMEM containing 10% supplemented calf serum while BHK cells were grown in Eagle's modified medium (GIBCO) containing 10% supplemented calf serum (Hyclone).

DNA Clones and Transfection

All DNA manipulations and mutagenesis experiments were performed by standard procedures.[20–24] Mutagenic oligonucleotides were synthesized using an Applied Biosystems DNA Synthesizer. Each was designed to create a single amino acid change at positions identified by crystallographic data as NC41 contact residues. The following oligonucleotides were named according to the position of the 5′ nucleotide, with positions of the mismatches underlined, and the corresponding amino acid sequence change in parentheses: N9-997M, 5′-AACCCCCGAAAGAATGACCCA-3′ (P330K); N9-1000M, 5′-CCCCGACCTA AGGACCCAACT-3′ (N331K); N9-1039M, 5′-CCTTATCCTGGCATTAACAAC-3′ (N346I); N9-1045M, 5′-CCAGGCAATGGCAACAATGGG-3′ (N347G); N9-1111MT, 5′-AGGACAATAACCATAGCT-3′ (S368T); N9-1114M, 5′-ACAATAAGCTATGCTTCAAGA-3′ (I369Y); N9-1116M, 5′-AATAAGCATAGGCTCAAGAT-3′ (A370G); N9-1120M, 5′-AGCATAGCAGCAAGATCC-3′ (S371A); N9-1126MG, 5′-GCTTCAAGAGGCGGATATG AG-3′ (S373G); N9-1126MT, 5′-GCTTCAAGAACCGGATAT-3′ (S373T); N9-1207M, 5′-ATCGTCTTACAGACTGACTGG-3′ (N400Q); N9-1210M, 5′-GTCTTAAACCTTGACTGG AGT-3′ (T401L); N9-1216M, 5′-AACACTGACGGTAGTGGTTAC-3′ (W403G); N9-1309MR, 5′-GGGAGACCTCGGGAGGATAA-3′ (K434R); N9-1309MD, 5′-GGGAGACC TGACGAGGATAAA-3′ (K434D). Three of the mutagenic oligonucleotides (N9-1000M, N9-1039M, and N9-1120M) contained an additional nucleotide substitution, which while having no effect on the amino acid sequence, increased the change in the T_m for denaturation between wild-type and mutant sequences. This assisted in identification of candidate mutant clones, since they are distinguished from wild-type sequences by increasing wash temperatures after hybridization with ^{32}P-labeled muta-

TABLE I. Rationale for the Construction of Mutant Neuraminidase Clones*

Mutant		Background	Rationale
P 330 K	(328)		Peptide angle altered as P removed- long, charged side chain added
N 331 K	(329)	N331D escaped inhibition by other mAbs (not NC41) and this change had a limited reduction in binding NC41	Contacts 2 residues in Fab light chain. Replace with bulky charged group
N 346 I	(344)	Asn frequently occurs in this and other antibody epitopes. May be important for antibody recognition	Change to a bulky, hydrophobic group
N 347 G	(345)	Not an NC41 contact residue; in other subtypes the sidechain at this position acts as a Ca^{2+} coordinate	Test for effect of removal of side chain on Ca^{2+} binding and NC41 recognition
S 368 T	(367)	In escape mutants selected by NC41, changes to R, G, and N abolished binding	Creates a mutant NA with similar H-bonding potential
I 369 Y	(368)	Change to R in escape mutant had little effect on binding	Replace with bulky, aromatic group
A 370 G	(369)	Other mAbs (not NC41) selected mutants with a change to I, which were not inhibited by NC41	Removal of side chain
S 371 A	(370)	Escape mutant with change to L was selected by other mAbs, but did not bind NC41	Reduce side chain size
S 373 G	(372)	S373F was selected in 10/19 NC41 escape mutants	Removal of side chain
S 373 T			Restore H-bonding potential
N 400 Q	(400)	Escape mutant N400K selected by NC41	Conservative change maintains neutrality
T 401 L	(401)	Contacts 3 Asn on antibody	Replacing with apolar side chain
W 403 G	(403)	Only aromatic in epitope	Remove side chain
K 434 R	(432)	Forms a salt link with Fab H chain. Escape mutant selected by NC41 contained E at this position	Retains charge
K 434 D			Similar to change observed in escape mutant

*Mutant NAs were named using the N9 numbering system, preceded by the 1 letter codes for the wild-type amino acid and followed by the amino acid substituted at that position. Corresponding positions in N2 NA are indicated in parentheses.

genic oligonucleotides. All mutant sequences were confirmed by dideoxy sequencing[25] and expressed from the T7 promoter in the vector Bluescript (Stratagene) using the vaccinia virus-T7 polymerase transient expression system[26] with the modifications as previously described.[24] The mutant NAs were named using the single letter code of the wild-type amino acid, amino acid position (N9 numbering), and the single letter code of the substituted amino acid.

Protein Analysis

NA activity assays, inhibition assays, and immunoprecipitation procedures were performed in CV-1 cells as described earlier.[24] For some experiments, BHK cells were used. These are not as sensitive to vaccinia infection and thus are able to maintain macromolecular synthesis for longer periods of time and produce more of the mutant protein. The neuraminidase inhibition (NI) assays are identical to NA activity assays except that prior to addition of fetuin substrate, antibody is added at a dilution which inhibited wildtype NA activity by 80%. For the NA and NI assays, infected and transfected BHK cells were harvested at 40 hr posttransfection, and for immunoprecipitation, cells were labeled at 25 hr posttransfection. For quantitation of immunoprecipitated proteins, dried gels were exposed overnight to a storage phosphor screen, and the screens analyzed on a phosphor imager (Molecular Dynamics).

RESULTS

Expression of Mutant NAs

A series of NA mutants were designed based on information available about the N9 NA-NC41 Fab complex,[16] each containing a single amino acid change at positions identified by the crystal structure as being in contact with light and/or heavy chain Fab residues. Shown in Table I is a summary of all the mutants constructed and the rationale behind each of the changes made in 12 out of 19 NC41 contact residues. Changes were not made in the other 7 contacts since their interactions with NC41 either involve main chain atoms (Arg-329, Ile-367 and Asp-402) or are van der Waals contacts (Pro-

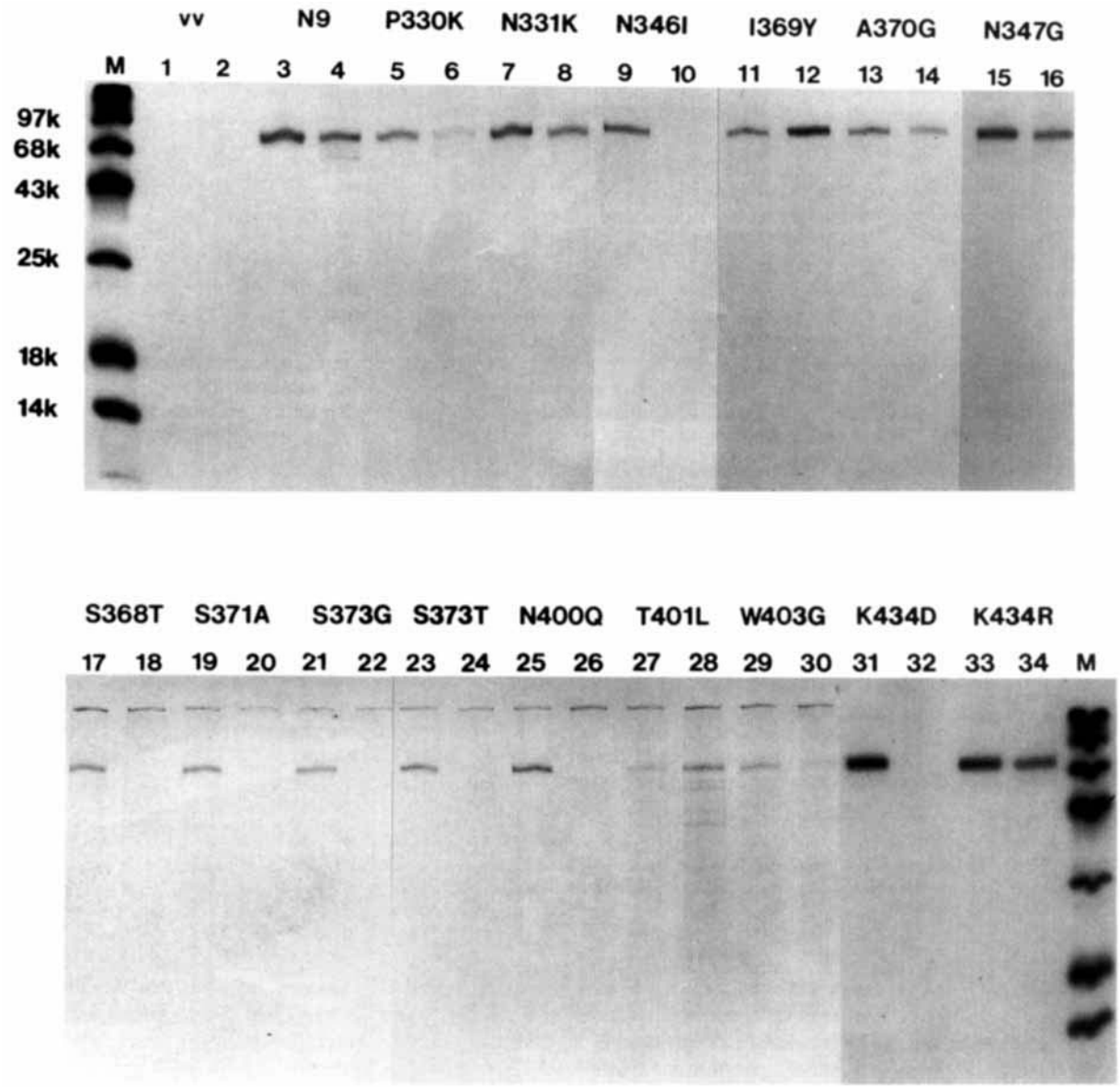

Fig. 1. Immunoprecipitation of wild-type and mutant N9 NAs. Confluent BHK cells on 35 mm plates were infected with the recombinant vaccinia virus vvTF7-3 containing the T7 polymerase gene, and then transfected with Bluescript containing the mutant NA gene of interest. Cells were labeled with EXPRE^{35}S^{35}S-Protein Labeling mix (NEN) at 25 hr posttransfection, and one-half of samples immunoprecipitated with 10 μl of a 1/1,000 dilution of antibody, either polyclonal antisera to A/tern/Australia/G70c/75 (odd-numbered lanes) or N9 monoclonal antibody NC41 (even-numbered lanes). Immune complexes were recovered by binding to Protein A Sepharose (Pharmacia) and one-eighth of total sample was electrophoresed in a 12% SDS-polyacrylamide gel.

328, Asn-349, Leu-399, and Pro-433), which are not as specific since they lack directionality.

Mutant NA genes were subcloned from M13mp11[27] into the vector Bluescript downstream of the T7 promoter and transfected into CV-1 or BHK cells, which had been infected with the recombinant vaccinia virus, vvTF7-3, which expresses the T7 polymerase gene.[26] To determine how much of the mutant NA polypeptides were made, cell extracts were immunoprecipitated with an anti-G70c NA polyclonal antibody, which contains antibodies against folded and unfolded NA. All of the mutant proteins were expressed (Fig. 1, odd-numbered lanes), though mutants P330K, N400Q, and W403G were at lower levels than wildtype N9 NA and the other mutant NAs.

To test if the expressed mutant NAs were folded correctly such that they retained their enzymatic activity, NA assays[28] were performed and results are depicted in Figure 2. Enzyme activity measurements were standardized so that they took into account levels of total protein measured as ^{35}S-labeled

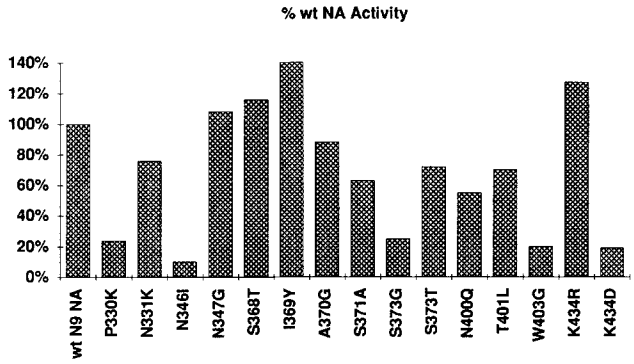

Fig. 2. Neuraminidase activity in wild-type and mutant NAs. Enzyme activity of each of the mutant NAs was measured using the standard thiobarbituate assay method[28] and fetuin as substrate (SIGMA). Standard deviation in the assay for inhibition of NA activity by NC41 is ± 10%. Assays were normalized for total protein expressed by quantitation of the immunoprecipitated proteins (Fig. 1) using a Molecular Dynamics Phospor Imager. The *y* axis depicts the percentage of wild-type enzyme activity exhibited by the mutant NAs.

immunoprecipitated NA protein quantitated using the Molecular Dynamics phosphor imager. While all of the NA mutants were enzymatically active, mutants P330K, N346I, S373G, N400Q, W403G, and K434D exhibited 50% or less of wild-type N9 NA activity, after correcting for total NA protein produced.

Effect of Mutation on Inhibition by NC41

Escape mutants selected in the presence of monoclonal antibody NC41 each contained a single amino acid change at positions known to be Fab contacts. Our mutant NAs contained contact residue changes which had not been observed in escape mutants. The effect of these single site mutations on the ability of NC41 to inhibit the mutant NA enzyme activity was measured, and results are shown in Figure 3. The mutants were titrated to determine the maximum extent of inhibition. NA mutants S368T, N400Q, and K434D retained all of their enzyme activity even at the highest concentrations of antibody (Fig. 4). NA mutants N346I, S373G, and S373T exhibited reduced inhibition by NC41. Mutants S371A and W403G had reduced inhibition by NC41 at lower concentrations of NC41, but reached wild-type inhibition of NA activity at high antibody concentrations. The extent of inhibition of NA activity at the varying antibody dilutions was the same for the rest of the mutant NAs as for wild-type NA.

It was possible that the loss of inhibition, or reduced inhibition of mutant NA activity by NC41 was not coupled to loss of, or reduced binding. To test if these mutants were still binding antibody, but in a different manner such that they were not inhibiting enzyme activity, mutant NAs were immunoprecipitated using monoclonal antibody NC41. Figure 1 (even-numbered lanes) shows that mutants S368T, N400Q, and K434D are no longer bound by NC41, while mutants N346I, S371A, S373G, S373T, and W403G have significantly reduced binding to NC41 in that only a small fraction can bind to the monoclonal antibody compared with the amount immunoprecipitated with the polyclonal antibody to G70c NA. The percent inhibition of enzyme activity in the NA mutants calculated by NI assays correlated very well with binding by NC41 (Figs. 1 and 4), as determined by quantitation of radiolabeled proteins immunoprecipitated. In every case, the loss of or severely reduced inhibition of enzyme activity by NC41 was due to loss of or reduced binding by NC41.

Summary of Mutant Neuraminidases

Seven out of 15 single-site mutations at Fab contact residues had little or no effect on the ability of NC41 to bind NA and inhibit enzyme activity, suggesting that many changes within the epitope can be tolerated without loss of antibody binding. Conservative substitutions made at 346 (Asn to Ile) and 373 (Ser to Gly and Thr) resulted in less than 50% binding by NC41, but nevertheless still maintained some interactions with Fab. Escape mutants have been selected containing bulky sidechain replacements at 373, implicating a steric inhibitory effect rather than loss of crucial contacts. However, we have identified Ser-368 and Asn-400 as critical contact residues within the epitope in that no change can be tolerated at these side chains without completely abolishing antibody recognition. Lys-434 is also critical for NC41 recognition in that a side chain substitution which prevents its salt link interaction with the Fab results in total loss of antibody binding. Table II summarizes the data obtained from the previous escape mutant analyses, and the site-directed mutants discussed here.

DISCUSSION

Recently, much emphasis has been placed on determining the types of interactions involved for specific antibody recognition of protein antigens. The structures of 3 lysozyme–antibody complexes,[29–32] and 2 neuraminidase–antibody complexes[1,16] have been solved. Structure determinations of these antibody-antigen complexes have allowed some generalizations to be made concerning the types of interactions involved, such as shape complementarity, hydrogen bonding potential, and salt links (for reviews, see refs. 33 and 34). Thus far, there appears to be a high degree of shape complementarity such that water molecules tend to be excluded from the antigen-antibody interface. A notable exception is the FabD1.3-lysozyme complex,[32] in which several water molecules remain in the interface. There is also an array of hydrogen bonds formed between the proteins in the complex which are necessary and deter-

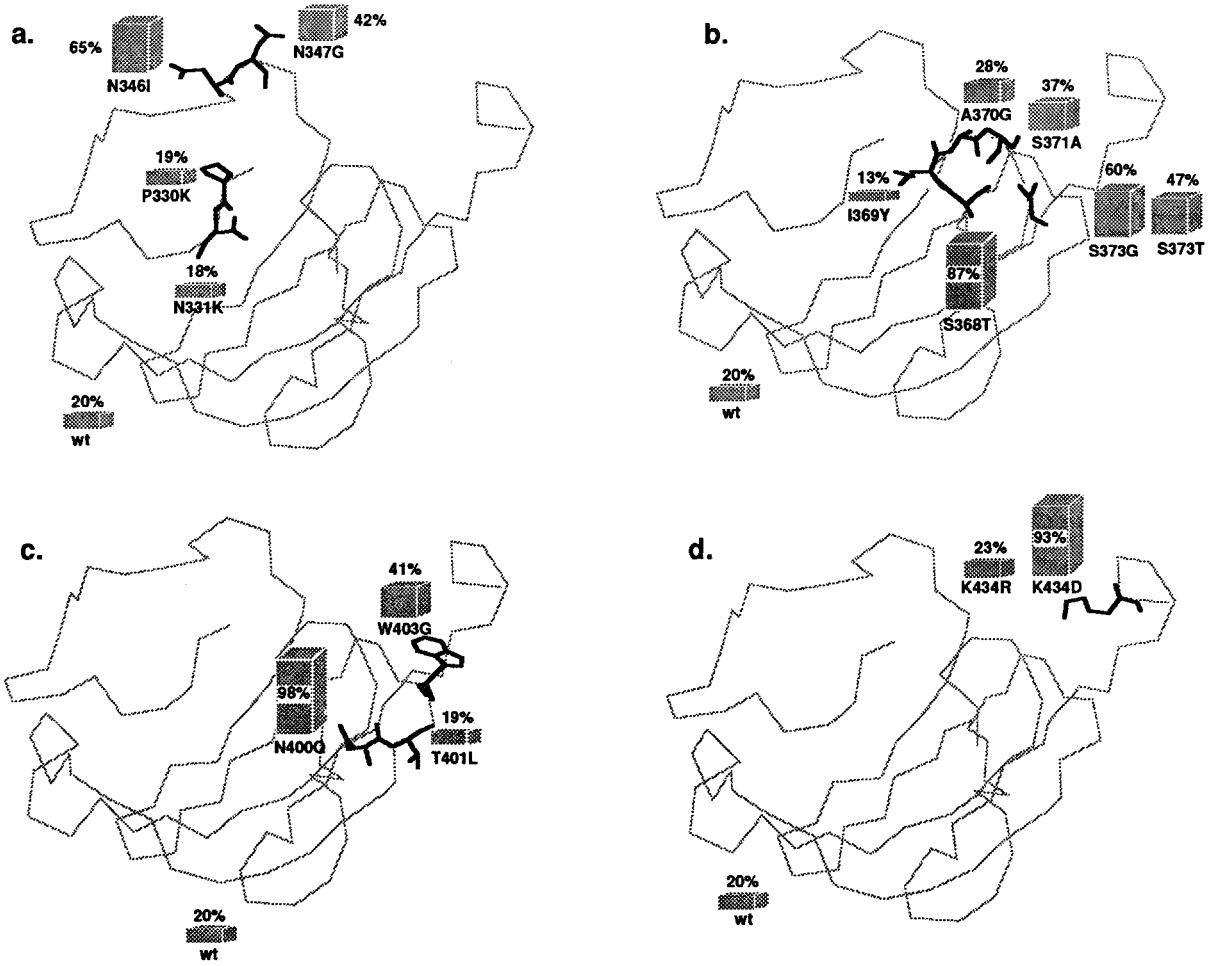

Fig. 3. Inhibition of enzyme activity of mutant NAs by NC41. A schematic representation of sections of the N9 NA monomer[12] shows the positions of mutated side chains in each of the N9 NA surface loops in contact with NC41. Percent NA activity as measured in the presence of NC41 is indicated by the bars. The mutant NA clones were transiently expressed as described in Figure 1, and 4% of each sample was measured for NA activity in the presence of a 1/1,000 dilution of monoclonal antibody NC41 (concentration of undiluted antibody is 15 mg/ml) which inhibits 50 ng of wild-type N9 NA activity by 80%. (**a**) Loop 315–350, (**b**) loop 367–373, (**c**) loop 399–403, and (**d**) loop 431–435. The diagrams were created using the MacImdad graphics program (Molecular Applications Group). Since the coordinates of N9 NA[11] were not available to us, the structure of N9 NA was independently solved by Whitaker et al. and refined to 2.5 Å.[12]

mine specificity. Although not always observed, electrostatic interactions are highly energetically favorable and are likely to be crucial to those complexes in which they are found. The complementarity determining regions (CDRs) which comprise most of the paratope contain an unusually high number of aromatic residues that greatly contribute to the overall binding energy.[35]

In influenza virus, we have a more biologically significant system in which escape from antibody binding can be measured and distinguished from loss of binding as determined by ELISA. In order to obtain a better understanding of how influenza NA is able to escape antibody inhibition, we examined the importance of residues in contact with monoclonal antibody NC41. Each of the mutant NAs constructed contained a single amino acid substitution at a contacting residue on one of five polypeptide loops which make up the NC41 epitope. A summary of these mutant NAs is discussed below.

Pro-330 to Lys. Pro 330 is located on a surface loop in close proximity to the Ca^{2+} binding site,[11,14] and has two contacts between its main chain N and the NC41 Fab light chain.[1] The replacement of the rigid proline side chain with a charged, flexible lysine side chain resulted in a 75% decrease in enzyme activity, possibly due to disruption of tertiary structure stability. However, the ability of NC41 to bind and inhibit the enzyme activity of this mutant NA was unaffected by this substitution, and we conclude that this residue is not playing a significant role in NC41 recognition or binding.

Asn-331 to Lys. Escape mutants selected by other antibodies contained a change at 331 to Asp,[17] and

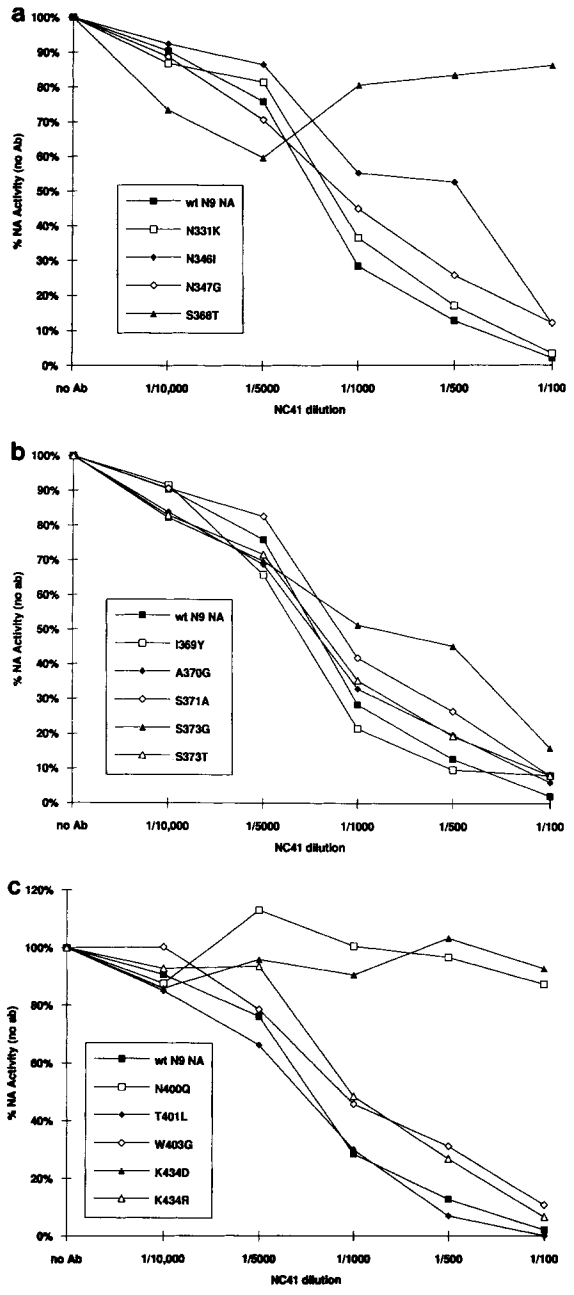

Fig. 4. NA Inhibition by NC41. To determine the maximum extent of inhibition by NC41, varying dilutions of antibody ranging from 1/100 to 1/10,000 were added to the wild-type and mutant NAs prior to addition of fetuin substrate. On the *y* axis, 100% represents the amount of NA activity measured in the absence of antibody.

these mutants exhibited reduced binding to NC41. The structures of both uncomplexed N331D[11] and N331D complexed with NC41[36] have been determined. They reveal that while both the Asn and Asp side chains at 331 in wildtype or mutant NAs occupy the same spatial positions in the uncomplexed structures, a conformational change is observed in the mutant NA when complexed with NC41 in that the Asp carboxylate group is rotated 150° about the Cα–Cβ bond.[36] In our current study, we inserted a bulkier, positively charged side chain (Lys), and this had no significant effect on either the NA activity of the mutant or on the ability of NC41 to recognize the protein. It is possible that the flexibility of the lysine side chain allows the antibody to accommodate this substitution. We hope to determine the structure of the N331K–NC41 complex.

Asn-346 to Ile. This residue makes two contacts with the NC41 light chain in the CDR2 domain. Escape mutants with changes at this position were never selected by the panel of monoclonal antibodies to N9 NA, including NC41.[17,18] When we replaced the Asn side chain with Ile, we observed an 85% decrease in enzyme activity. However, this NA activity was only inhibited by 35% in the presence of NC41, whereas wild-type NA activity is inhibited by 80% with the same dilution of antibody. Immunoprecipitation with NC41 compared with a polyclonal antiserum to G70c NA revealed a significant loss of binding to NC41. Model building suggested that the Ile could be accommodated in the complex. One possible explanation for loss of binding by NC41 to N346I is that replacement of Asn with Ile may have caused this hydrophobic side chain to bury into the protein and disrupt the conformation enough to destabilize the mutant NA's interaction with NC41.

Asn-347 to Gly. Although not an NC41 contact, Asn-347 is located adjacent to the epitope and Ca^{2+} binding site.[11] Many other NAs have a Gly residue at this position, and coordinate the Ca^{2+} ion through the main chain carbonyl oxygen, as is observed with B/Beijing NA.[14] Although Asn-347 does not coordinate the Ca^{2+} ion in N9 NA,[11] we were curious to see if replacement of Asn-347 with the Gly found in many other NAs would have any affect on Ca^{2+} binding or NC41 recognition. As depicted in Figure 4, this change had no effect on the NA activity or NC41 binding of N347G. Enzyme activity of this NA versus wild-type was also measured in the presence of 10 mM EGTA, which chelates Ca^{2+} ions. Under these conditions, no differences were observed between wild-type NA and N347G (data not shown). In both wild-type and mutant, the Ca^{2+} ion is buried and not accessible to EGTA.

Ser-368 to Thr. Six of the 19 escape mutants selected by NC41 contained a single change at 368 to either Asn, Gly or Arg,[18] targeting this residue as a critical contact with NC41 Fab. Upon complex formation, the Ser Oγ atom is hydrogen bonded with Glu-96 Oε1 on the heavy chain (H96). We replaced the Ser with Thr to see if preservation of the hydrogen bonding potential would allow NC41 to bind. This change completely abolished the ability of NC41 to bind to the NA, as revealed by immunoprecipitation with NC41 (Fig. 3). The methyl group of Thr could be disrupting the antibody–antigen inter-

TABLE II. Summary of Mutant Neuraminidases*

Site-directed mutants	Escape mutants	Binding to NC41	Selected by NC41
P 330 K		+	
N 331 K		+	
N 346 I		(+)	
N 347 G		+	
	S 368 N	No	Yes
	S 368 G	No	Yes
	S 368 R	No	Yes
S 368 T		No	
I 369 Y		+	
A 370 G		+	
	A 370 D	No	No
S 371 A		+	
	S 371 L	No	No
S 373 G		(+)	
S 373 T		(+)	
	S 373Y	No	No
	S 373 F	No	Yes
N 400 Q		No	
	N 400 K	No	Yes
T 401 L		+	
W 403 G		+	
K 434 R		+	
K 434 D		No	
	K 434 N	No	No
	K 434 E + K 437 G	No	Yes

*Effects of the site-directed mutations in NC41 contact residues on antibody binding are compared with escape mutants selected in the presence of NC41.[17] Mutants set in italics have reduced binding by NC41 as indicated by (+), whereas mutants set in bold face have completely lost binding by NC41 and have changes in residues which are critical contacts for NC41.

action. We conclude that Ser-368 is absolutely critical for NC41 binding, and it is located at the center of the epitope.

Ile-369 to Tyr. Webster et al.[17] selected an escape mutant containing Arg at 369 with monoclonal antibody NC24. This mutant NA retained partial binding to NC41 and the structure of the I369R–NC41 Fab complex has recently been solved.[36] According to the structure, I369R is able to maintain partial binding to NC41 due to the formation of a solvated salt link between NH1 and Asp H101 (Oδ1) on the NC41 heavy chain. Insertion of the bulky, hydrophobic Tyr side chain at this position had no effect on either the NA activity, or inhibition by NC41. Contact residue 369 can be ruled out as essential to the interaction, since drastic side chain replacements can be tolerated at this position without affecting the interaction. This is not surprising since it is the main chain N atom which is hydrogen-bonded to the carboxylate oxygen Oε2 of Glu H96.[1]

Ala-370 to Gly. While an escape mutant with a change at 370 was never selected by NC41, six other monoclonal antibodies selected escape mutants with an Ala to Asp substitution at 370 which also did not bind NC41. It is unknown why the A370D mutants were unable to grow in the presence of NC41, yet did not bind this antibody as determined by ELISA[17] and inhibition assays. One explanation is that the in vitro assays are not sensitive enough to detect a low level of binding, while assays involving virus replication (escape mutant selection) can.

According to the structure,[1,16] this amino acid has one heavy chain and two light chain contacts, although only A370 main chain atoms are involved in these contacts. Removal of the side chain in this experiment by substitution of Ala for a Gly maintains enzymatic activity and NC41 binding equivalent to wild-type NA.

Ser-371 to Ala. The structure of a mutant NA S371L selected with monoclonal antibody 32/3 has been reported.[11] This mutant NA is not inhibited by NC41 and analysis of the N9-NC41 complex[1] suggests that the larger Leu side chain at this position is preventing the antigen–antibody complex from forming. In our present study, we have replaced Ser with Ala, which reduces the size of the substituted side chain but also removes the hydrogen bond between the Ser Oγ and Oδ1 of Asp H97. The specific enzyme activity in this mutant is somewhat reduced (62%) compared with wildtype NA activity. At higher concentrations of antibody, S371A maintained its interaction with NC41, although at a slightly reduced efficiency compared to wt N9 with the antibody (63% inhibition of NA activity in the mutant versus 80%). Our results indicate that the hydrogen bond formed by the Ser Oγ is not an important contact with NC41.

Ser-373 to Gly and Thr. Ten out of 19 escape mutants selected by NC41 contained a Ser to Phe substitution at 373.[18] In addition, an escape mutant selected by antibody NC44 containing Tyr at this position also did not bind NC41. Ser-373 has 2 heavy chain contacts in the CDR3 of NC41 Fab, one being the formation of a hydrogen bond between the Oγ of Ser-373 and Asn H98 N.[1] One explanation for the failure to select S373Y as an escape mutant by NC41 is that the Tyr hydroxyl may still be maintaining some hydrogen bonding with Asn H98 of NC41 (albeit very weak, since no binding is observed by ELISA[17]) or another antibody side chain sufficiently such that it could not grow in the presence of NC41. Insertion of Phe at this position prevents any type of hydrogen bond from occurring with NC41 and mutant NAs containing this substitution can grow in the presence of this antibody. NC41 inhibition is reduced to 40% by removal of the side chain at 373 (Ser to Gly). This is a significant reduction in binding when compared to wild-type binding, and is possibly due to the loss of the hydrogen bond. When a mutant NA containing a Thr side chain which has similar hydrogen-bonding potential was tested, NC41 inhibition of NA activity increased to 53%; this was still less than wild-type N9

NA. As with the S368T mutant, the Thr methyl group may have inhibitory effects on the interaction. S373G lost enzyme activity (32% of wild-type) whereas the enzyme activity of S373T was unaffected by this change.

Asn-400 to Gln. Asn-400 makes a total of 5 contacts with NC41 heavy chain residues, 3 of which are hydrogen bonds. Escape mutants which contained a Lys at this position were selected by NC41.[17,18] To determine if loss of binding by NC41 was a consequence of the long, bulky, positively charged side chain, we conservatively constructed a mutant NA containing a Gln at this position, which we anticipated would regain NC41 binding. This mutant completely lost NC41 recognition, as indicated in both the inhibition experiments (Fig. 3), and failure to immunoprecipitate with NC41 (Fig. 1). As with Ser-368, Asn-400 appears to be absolutely crucial to the interaction, such that any substitutions would completely abolish NC41 binding. Both of these critical NC41 contact residues are located at the center of the epitope.

Thr-401 to Leu. An escape mutant with a change at Thr-401 has never been selected by NC41 or any other N9 NA monoclonal antibodies. However, since this residue makes 3 heavy chain contacts, one of which is a hydrogen bond, the effects of insertion of a Leu side chain were examined. This mutant NA retained most of its enzyme activity (57%), and its recognition by NC41 as measured by inhibition assays and immunoprecipitation is equivalent to wild-type N9 NA. Despite several heavy chain interactions with NC41 as identified by the structure, this contact can tolerate changes without any effect on binding.

Trp-403 to Gly. Trp-403 is the only aromatic side chain on the NA interacting with NC41 and makes hydrophobic interactions with heavy chain residues in the CDR3 region.[1] This residue is located on the end of a β-sheet and is solvent exposed.[12] Removal of the side chain by the construction of mutant W403G inserts more flexibility at this position. This NA maintained 59% binding to NC41, but lost 68% of its NA activity. We conclude that this residue, while important in maintaining the structural integrity of the protein due to its location, has only a complementary role in NC41 recognition. A change to an Ala or Val side chain probably would not have as drastic an effect on the structure.

Lys-434 to Arg and Asp. As the only buried salt link in the complex, the Nζ atom of Lys-434 interacts with Oδ1 of Asp-H97. The structure of an escape mutant K434N selected by monoclonal antibody NC34 which also did not bind NC41[11] revealed a shift in the location of the side chain with this substitution. The salt link contact abolished with this change may be necessary for a stable antibody–antigen interaction. The K434R mutant was constructed in this study in an attempt to preserve the salt link. As indicated by the results, Arg was able to successfully replace Lys and this mutant NA maintained 77% binding to NC41, and had wild-type enzyme activity. In the previous study of the NC41 epitope,[18] one of the escape mutants selected with NC41 contained changes at 434 from Lys to Glu and at 437 from Lys to Gly. To show that a single change at 434 from a positively charged to a negatively charged residue was sufficient to abolish binding by NC41, we constructed a mutant NA replacing the Lys at 434 with Asp. NC41 did not interact with this NA and binding was completely lost (see immunoprecipitation, Fig. 1). This mutant also exhibited an 83% loss of NA activity. We conclude by these results that the formation of the salt link between the NA and antibody is absolutely crucial for NC41 recognition.

As far as we are aware, this is the first extensive study of effects of altering side chains in a protein epitope. However, there are reports of similar studies on the antibody paratope. It has been shown that antibody affinity can be greatly increased by replacement of a few amino acids in the variable region.[37] Lavoie et al.[38] demonstrated that changing somatically mutated residues within CDR 3 of the heavy chain of monoclonal antibody HyHEL-10 back to germline sequences resulted in loss of affinity for lysozyme, even though none of the amino acids changed contact lysozyme in the crystal structure of the HyHEL-10 Fab–lysozyme complex. In the lysozyme–antibody complexes, all six of the antibody CDRs are in contact with the protein, whereas four and five of the CDRs are involved, respectively, when monoclonal antibodies NC10 and NC41 bind to neuraminidase. It will be interesting to investigate residues in the NC41 combining site and their relative importance in antigen recognition.

Shown in Figure 5 is a representation of the N9 NA molecule oriented as NC41 sees it. The critical contact residues Ser-368, Asn-400, and Lys-434 within the epitope are indicated and are centrally located within the entire contact region. Novotny et al.[39,40] have used the program CONGEN[41,42] and atomic coordinates from X-ray analyses of antibody–antigen complexes to calculate the Gibbs free energy of complex formation, and define which amino acids are most energetically favorable to the interaction, or "energetic" epitope. Using this program, Tulip (personal communication, unpublished results) included residues Lys-434, Asn-400, and Ser-368 as part of the "energetic" epitope of NC41. Our results are in agreement with Novotny's hypothesis in that there are only a few amino acid residues which are most crucial for favorable interactions with the antibody. The other contact residues are providing "passive surface complementarity" and their size and shape is more important than any specific interactions they might form. Also this complementarity allows for the exclusion of water molecules, as

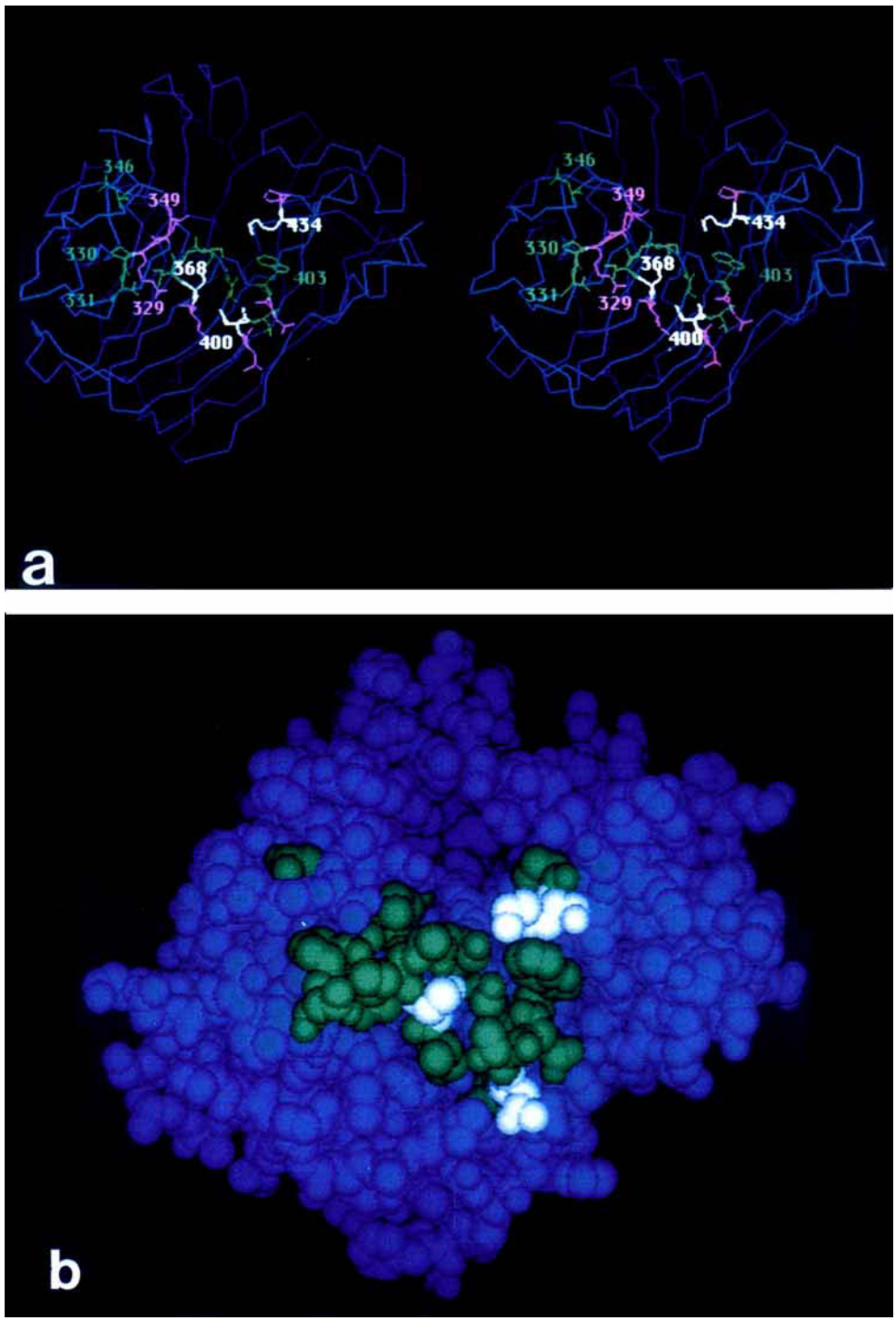

Fig. 5. The NC41 epitope on N9 NA. (**a**) The orientation of N9 NA as NC41 recognizes it reveals that antibody contacts surface loops of the NA without steric interference of the enzyme active site. These stereo diagrams were created as in Figure 3. NC41 contact residues which were changed in our experiments are shown in green, with critical contact residues 368, 400, and 434 highlighted in white. NC41 residues not altered in these experiments are indicated in magenta. (**b**) Space-filling model of a monomer of subtype N9 influenza virus neuraminidase. Residues in contact with monoclonal antibody NC41 are indicated in green and critical contact residues are shown in white. The enzyme active site is seen as a depression on top.

observed in most antigen–antibody complexes studied at this time.

We have shown through site-directed mutagenesis of many of the NC41 contact residues that a wide range of substitutions can be tolerated in residues identified by structural data as antibody contacts, and that only a few of the interactions seen in the crystal structure play a major role in the stability of the complex. In this report, and in agreement with the escape mutant data, Ser-368 and Asn-400 are absolutely essential for this interaction to take place. At these critical contact sites, antibody binding is lost when very conservative changes are made. More drastic side chain replacements were not made at these key positions since they may have structural consequences in addition to loss of NC41 binding. In addition, replacement of Lys 434 with Asp also abolished binding, indicating the importance of the salt link formed between antigen and antibody. Mutant K434R was able to maintain this salt bridge with NC41, and antibody binding was not affected. This result differs from the previous observation of Smith-Gill et al.[43] in which an Arg to Lys substitution at position 68 in bobwhite quail lysozyme reduced the binding affinity of FabHyHEL-5 by two orders of magnitude. It is noteworthy that all of the escape mutants selected by NC41 had amino acid substitutions at positions identical to those site-directed mutants which are not inhibited by NC41. Current efforts are focused on the development of an in vivo system whereby these and other laboratory-generated mutant NA genes can be incorporated into virus. We will determine if these viruses can be grown in the presence of NC41, or other monoclonal antibodies.

The exact mechanism of neuraminidase inhibition by NC41 is not immediately clear since the antibody does not appear to interfere sterically with substrate binding, although CDR L1 does sit close to the edge of the active site[1] and could feasibly prohibit substrate entry or release of the product. In addition, Tulip et al.[1] have suggested that upon complex formation, the immobilization of the 366–372 loop could affect the ability of Arg-371 to carry out its role in substrate binding or enzyme catalysis. It has also been proposed[14] that some antibodies which inhibit enzyme activity but don't block the active site may act by disrupting the Ca^{2+} binding site and destabilizing the NA structure. NC41 recognizes NA surface loops which include Ca^{2+} atom coordinating residues and could feasibly be inhibiting NA activity in this manner. Experiments to test these possibilities are underway.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Robert Webster, St. Jude Children's Hospital, Memphis, TN, for his monoclonal antibody NC41. We also thank Dr. Jeffrey Engler, University of Alabama at Birmingham, for synthesis of mutagenic oligonucleotides on an Applied Biosystems DNA Synthesizer. We thank Dr. Ming Luo (University of Alabama at Birmingham) and Dr. William Tulip (University of Oregon) for helpful comments and suggestions and access to unpublished data. We gratefully acknowledge Pamela Pruett for purification of NC41 and all the members of the laboratory for critical reading of the manuscript. This work was aided by NIH Grants AI 19084 and AI 26718. JMN and PBW were supported in part by NIH training Grants T32 AI 07150 and T32 CA 09467.

REFERENCES

1. Tulip, W.R., Varghese, J.N., Laver, W.G., Webster, R.G., Colman, P.M. Refined structure of the influenza virus neuraminidase-NC41 Fab complex. J. Mol. Biol., 227:122–148, 1992.
2. Wiley, D.C., Skehel, J.J. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu. Rev. Biochem. 56:365–394, 1987.
3. Palese, P., Tobita, K., Ueda, M., Compans, R.W. Characterization of temperature-sensitive influenza virus mutants defective in neuraminidase. Virology 61:397–410, 1974.
4. Wiley, D.C., Wilson, I.A., Skehel, J.J. Structural identification of the antibody-binding sites of Hong Kong influenza hemagglutinin and their involvement in antigenic variation. Nature (London) 289:373–378, 1981.
5. Webster, R.G., Reay, P.A., Laver, W.G. Protection against lethal influenza with neuraminidase. Virology 164:230–237, 1988.
6. Air, G.M., Laver, W.G. The neuraminidase of influenza virus. Proteins. 6:341–356, 1989.
7. Varghese, J.N., Laver, W.G., Colman, P.M. Structure of the influenza virus glycoprotein antigen neuraminidase at 2.9Å resolution. Nature (London) 303:35–40, 1983.
8. Laver, W.G. Crystallization and peptide maps of neuraminidase "heads" from H2N2 and H3N2 influenza virus strains. Virology 86:78–87, 1978.
9. Baker, A.T., Varghese, J.N., Laver, W.G., Air, G.M., Colman, P.M. Three-dimensional structure of neuraminidase of subtype N9 from an avian influenza virus. Proteins 2: 111–117, 1987.
10. Varghese, J.N., Colman, P.M. Three-dimensional structure of the neuraminidase of influenza virus A/Tokyo/3/67 at 2.2Å resolution. J. Mol. Biol. 221:473–486, 1991.
11. Tulip, W.R., Varghese, J.N., Baker, A.T., van Donkelaar, A., Laver, W.G., Webster, R.G., Colman, P.M. Refined atomic structures of N9 subtype influenza virus neuraminidase and escape mutants. J. Mol. Biol. 221:487–497, 1991.
12. Whitaker, P.B., Carson, M., Babu, Y.S., Laver, W.G., Smith, C.D., Air, G.M. Three-dimensional structure of influenza A N9 neuraminidase and its complex with the inhibitor 2-deoxy 2,3-dehydro-N-acetyl neuraminic acid. Submitted, 1992.
13. Burmeister, W.P., Daniels, R.S., Dayan, S., Gagnon, J., Cusack, S., Ruigrok, R.W.H. Sequence and crystallization of influenza virus B/Beijing/1/87 neuraminidase. Virology 180:266–272, 1991.
14. Burmeister, W.P., Ruigrok, R.W.H., Cusack, S. The 2.2Å resolution crystal structure of influenza B neuraminidase and its complex with sialic acid. EMBO J. 11:49–56, 1992.
15. Colman, P.M., Laver, W.G., Varghese, J.N., Baker, A.T., Tulloch, P.A., Air, G.M., Webster, R.G. Three-dimensional structure of a complex of antibody with influenza virus neuraminidase. Nature (London) 326:358–363, 1987.
16. Tulip, W.R., Varghese, J.N., Webster, R.G., Air, G.M., Laver, W.G., Colman, P.M. Crystal structures of neuraminidase-antibody complexes. Cold Spring Harbor Symp. Quant. Biol. 54:257–263, 1989.
17. Webster, R.G., Air, G.M., Metzger, D.W., Colman, P.M., Varghese, J.N., Baker, A.T., Laver, W.G. Antigenic structure and variation in an influenza N9 neuraminidase. J. Virol. 61:2910–2916, 1987.

18. Air, G.M., Laver, W.G., Webster, R.G. Mechanism of antigenic variation in an individual epitope on influenza virus N9 neuraminidase. J. Virol. 64:5797–5803, 1990.
19. Air, G.M., Ritchie, L.R., Laver, W.G., Colman, P.M. Gene and protein sequence of an influenza neuraminidase with hemagglutinin activity. Virology 145:117–122, 1985.
20. Maniatis, T., Fritsch, E.F., Sambrook, J. "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1982.
21. Zoller, M., Smith, M. Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol. 100:468–500, 1983.
22. Norris, K., Norris, F., Christiansen, L., Fiil, N. Efficient site-directed mutagenesis by simultaneous use of two primers. Nucl. Acids Res. 11:5103–5112, 1983.
23. Lentz, M.R., Air, G.M. Loss of enzyme activity in a site-directed mutant of influenza neuraminidase compared to expressed wild-type protein. Virology 148:74–83, 1986.
24. Nuss, J.M., Air, G.M. Transfer of the hemagglutinin activity of influenza virus neuraminidase subtype N9 into an N2 background. Virology 183:496–504, 1991.
25. Sanger, F., Nicklen, S., Coulson, A.R. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. 74:5463–5467, 1977.
26. Fuerst, T.R., Niles, E.G., Studier, F.W., Moss, B. Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 83:8122–8126, 1986.
27. Norrander, J., Kempe, T., Messing, J. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. Gene 26:101–106, 1983.
28. Aymard-Henry, M., Coleman, M.T., Dowdle, W.R., Laver, W.G., Schild, G.C., Webster, R.G. Influenza virus neuraminidase and neuraminidase inhibition test procedures. Bull. WHO 48:199–202, 1973.
29. Amit, A.G., Mariuzza, R.A., Phillips, S.E.V., Poljak, R.J. Three dimensional structure of an antigen-antibody complex at 2.8Å resolution. Science 233:747–753, 1986.
30. Sheriff, S., Silverton, E.W., Padlan, E.A., Cohen, G.H., Smith-Gill, S.J., Finzel, B.C., Davies, D.R. Three-dimensional structure of an antibody-antigen complex. Proc. Nat'l. Acad. Sci. U.S.A. 84:8075–8079, 1987.
31. Padlan, E.A., Silverton, E.W., Sheriff, S., Cohen, G.H., Smith-Gill, S.J., D.R., D. Structure of an antigen-antibody complex: Crystal structure of the HyHEL-10 Fab-lysozyme complex. Proc. Natl. Acad. Sci. U.S.A. 86:5938–5942, 1989.
32. Fischmann, T.O., Bentley, G.A., Bhat, T.N., Boulet, G., Mariuzza, R.A., Phillips, S.E.V., Tello, D., Poljak, R.J. Crystallographic refinement of the three-dimensional structure of the FabD1.3-lysozyme complex at 2.5Å resolution. J. Biol. Chem. 266:12915–12920, 1991.
33. Davies, D.R., Padlan, E.A., Sheriff, S. Antibody-antigen complexes. Annu. Rev. Biochem. 59:439–473, 1990.
34. Mian, I.S., Bradwell, A.R., Olson, A.J. Structure, function and properties of antibody binding sites. J. Mol. Biol. 217: 133–151, 1991.
35. Padlan, E.A. On the nature of antibody combining sites unusual structural features that may confer on these sites an enhanced capacity for binding ligands. Proteins 7:112–124, 1990.
36. Tulip, W.R., Varghese, J.N., Webster, R.G., Laver, W.G., Colman, P.M. Crystal structures of two mutant neuraminidase-antibody complexes with amino acid substitutions in the interface. J. Mol. Biol., 227:149–159, 1992.
37. Sharon, J. Structural correlates of high antibody affinity: Three engineered amino acid substitutions can increase the affinity of an anti-*p*-azophenylarsonate antibody 200-fold. Proc. Natl. Acad. Sci. U.S.A. 87:4814–4817, 1990.
38. Lavoie, T.B., Drohan, W.N., Smith-Gill, S.J. Experimental analysis by site-directed mutagenesis of somatic mutation effects on affinity and fine specificity in antibodies specific for lysozyme. J. Immunol. 148:503–513, 1992.
39. Novotny, J., Bruccoleri, R.E., Saul, F.A. On the attribution of binding energy in antigen-antibody complexes McPC603, D1.3 and HyHEL-5. Biochemistry 28:4735–4749, 1989.
40. Novotny, J. Protein antigenicity: a thermodynamic approach. Mol. Immunol. 28:201–207, 1991.
41. Brooks, B.R., Bruccoleri, R.E., Olafson, B.D., States, D.J., Swaminathan, S., Karplus, M. CHARMM: A program for macromolecular energy, minimization and dynamics calculations. J. Comput. Chem. 4:187–217, 1983.
42. Bruccoleri, R.E., Karplus, M. Prediction of the folding of short polypeptide segments by uniform conformational sampling. Biopolymers 26:137–168, 1987.
43. Smith-Gill, S.J., Wilson, A.C., Potter, M., Prager, E.M., Feldmann, R.J., Mainhart, C.R. Mapping the antigenic epitope for a monoclonal antibody against lysozyme. J. Immunol. 128:314–322, 1982.