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The receptor-binding and membrane-fusion properties of influenza virus variants selected using anti-haemagglutinin monoclonal antibodies

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A monoclonal antibody raised against X-31 influenza virus reacted with the majority of natural H_3N_2 viruses isolated between 1968 and 1982. A number of variants of X-31 and of a receptor-binding mutant of X-31 were selected by the antibody during virus replication in eggs and MDCK cells. Antibody-binding assays indicated that the viruses selected were not antigenic variants and analyses using derivatized erythrocytes showed that their receptor-binding properties differed from those of the parent viruses. The amino acid substitutions in the variants were all located in the vicinity of the receptor-binding site and the structural consequences are discussed in relation to the three-dimensional structure of X-31 HA. In addition all of the variants fused membranes at higher pH than wild-type virus indicating that structural modifications in the distal globular region of HA influence the low pH-induced conformational change required for membrane fusion.

Key words: X-31 influenza virus/receptor binding/amino acid substitutions

Introduction

The haemagglutinin glycoproteins of influenza virus membranes bind to cell surface glycoconjugates containing sialic acid (reviewed in Gottschalk, 1959). Haemagglutinins of different viruses have different binding specificities (e.g. Carroll *et al.*, 1981) and in previous studies differences in the amino acid sequences of the haemagglutinins of selected receptor-binding variants have been used to locate the binding site in a pocket at the distal tip of each haemagglutinin subunit (Rogers *et al.*, 1983). The periphery of this pocket contains amino acid residues of known antigenic importance (Wiley *et al.*, 1981) and interrelationships between antigenic and receptor-binding properties have been directly observed in analyses of the reactions of receptor variants with monoclonal antibodies (Daniels *et al.*, 1984) and in studies of the different receptor-binding properties of naturally occurring and monoclonal antibody-selected variants (Underwood, 1984; Underwood *et al.*, 1986; Yewdell *et al.*, 1986).

We report here the selection and properties of a number of X-31 influenza virus variants which have altered receptor-binding properties and discuss the significance of these properties in relation to the structure of the receptor-binding site. In addition we present evidence that as a consequence of the amino acid sequence changes which influence the receptor-binding properties of the mutants, the pH at which their haemagglutinins mediate membrane fusion is elevated. The basis of the decrease in haemagglutinin stability which this implies (Daniels *et al.*, 1985) is also discussed in relation to the structure of X-31 haemagglutinin at pH 7 and at the lower pH of fusion.

Results

Antigenic analysis of mutant haemagglutinins

The majority of monoclonal antibodies produced against the haemagglutinin of a particular influenza virus react only with viruses isolated within a short period of time, often no more than 2 years before or after the date of isolation of the immunizing virus. However, a number of antibodies are more widely cross-reactive and this is the case for HC63, the main antibody used in this study. The results presented in Table I indicate that HC63 reacts with the majority of haemagglutinins of H_3N_2 viruses isolated between 1968 and 1982. To determine the specificity of HC63, variants of X-31 were selected by growing the virus in the presence of the monoclonal antibody and their properties were analysed in haemagglutination inhibition (HI) tests using antibodies of known specificity (Table II). Three variants selected in eggs 63-3, 63-D and 63-E were readily distinguished from X-31 by the inability of antibodies HC110X and HC68X to inhibit their haemagglutination activity. In addition, mutant 63-3 was further distinguished by not reacting with antibodies HC31, HC125X and HC3M. Two variants were selected by growing X-31 in MDCK cells in the presence of HC63. One of these behaved similarly in the HI test to mutant 63-3 above; the other, V9A, appeared to be like wild-type X-31 virus except for a decreased reaction with HC63. The properties of the receptor-binding variant X-31/HS selected by growing X-31 virus in the presence of non-immune horse serum are also shown in Table II. Like variants 63-3, 63-D and 63-E haemagglutination by X-31/HS was not inhibited by antibodies HC110X and HC68X and a mutant of X-31/HS selected by growing virus in the presence of antibody HC63 was distinctively unaffected in the HI test with the majority of monoclonal antibodies. The results indicate that the failure of certain mutants to react with specific monoclonal antibodies as judged by HI is not necessarily a reflection of changes in their antigenicity; it can be a consequence of changes in receptor-binding properties.

The antigenic properties of the variants selected with HC63 and HC68X were therefore analysed directly in ELISA antibody-binding assays. Like all of the other antigenic variants listed in Table II which do not bind the antibodies used in their selection, V68X showed no reaction with HC68X. The variants selected with HC63, on the other hand, bound HC63 to similar titres as wild-type X-31 in these tests. They therefore appear not to be antigenic variants, and their behaviour in HI tests suggests that they may be adsorption mutants.

Nucleotide sequence analysis of the mutant genes for haemagglutinin

To characterize these variants further the amino acid sequences of their haemagglutinins were deduced from the complete se-

Table I. Haemagglutination-inhibition reactions of H_3N_2 subtype reference viruses with monoclonal antibodies against X-31 haemagglutinin

	Monoclonal antibodies										
	HC63	HC45	HC159X	HC3	HC113X	HC19	HC263X	HC110X	HC83X	HC21	HC125
A/Hong Kong/1/68	6400	800	6400	6400	6400	6400	6400	6400	6400	6400	3200
A/England/878/69	3200	<	<	<	<	6400	6400	6400	6400	6400	3200
A/England/42/72	3200	400	<	<	<	<	6400	6400	6400	6400	3200
A/Pt.Chalmers/1/73	3200	<	<	<	<	<	6400	<	6400	<	<
A/Hannover/61/73	3200	400	<	<	<	<	6400	<	6400	<	3200
A/Victoria/3/75	<	<	<	<	<	<	6400	<	<	<	<
A/Victoria/112/76	<	<	<	<	<	<	6400	<	<	<	<
A/Texas/1/77	400	<	<	<	<	<	<	<	<	<	<
A/Roumania/293/78	3200	<	<	<	<	<	<	<	<	<	<
A/Bangkok/1/79	3200	<	<	<	<	<	<	<	<	<	<
A/Belgium/2/81	400	<	<	<	<	<	<	<	<	<	<
A/Phillipines/2/82	200	<	<	<	<	<	<	<	<	<	<
Amino acid substitution in selected variants of X-31		63 D → N	143 P → T	144 G → D	145 S → N	157 S → L	158 G → E	189 Q → K	193 S → I	198 A → E	199 S → P

The titres are the reciprocals of the highest dilutions of the antibodies at which haemagglutination is inhibited.
< = <100.

Table II. Reactions of X-31 and X-31 variants in haemagglutination inhibition tests

	Monoclonal antibodies												
	HC159X	HC3	HC113X	HC19	HC263X	HC110X	HC126X	HC68X	HC83X	HC31	HC125X	HC3M	HC63
X-31	6400	6400	3200	3200	6400	1600	6400	3200	3200	3200	6400	1600	12 800
63-3	6400	6400	3200	3200	6400	<	6400	<	3200	<	<	<	40
63-D	6400	6400	6400	6400	6400	<	6400	<	6400	3200	6400	1600	160
63-E	6400	6400	3200	6400	6400	200	6400	<	3200	3200	6400	1600	40
V9A	6400	6400	6400	6400	6400	6400	6400	6400	6400	6400	6400	6400	1280
X-31/HS	6400	6400	6400	6400	6400	<	6400	<	3200	<	3200	<	640
H-31/HS-V63	800	6400	<	<	<	<	6400	<	<	3200	3200	1600	<20
Amino acid substitution in selected variant	143 P → T	144 G → D	145 S → N	157 S → L	158 G → E	189 Q → K	189 Q → H	193 S → R	193 S → I	198 A → E	199 S → P	219 S → F	Table III

< = <100.

quences of the appropriate RNAs. The only sequence differences, compared with the sequence of X-31 haemagglutinin, were detected in the HA_1 polypeptide (Table III). All amino acid substitutions resulted from single base changes and no silent base changes were detected. Mutants 63-3 and V9A contained single amino acid substitutions at residue HA_1 218: glycine to glutamic acid and glycine to arginine respectively. 63-E contained two substitutions: HA_1 193, serine to asparagine and HA_1 226, leucine to proline. Mutant 63-D contained a seven amino acid deletion between residues HA_1 224 and 230 and the mutant of X-31/HS selected using antibody HC63 had this deletion and amino acid substitutions serine to isoleucine at HA_1 145 and glycine to glutamic acid at HA_1 158. Subsequent references to these mutants will indicate these sequence changes and their location, e.g. mutant 63-E containing two substitutions at residues 193 and 226 is abbreviated 63-E (197, S → N; 226 L → P).

Receptor-binding properties of the mutant haemagglutinins

As noted above, variants selected by antibody HC63 except V9A (218, G → R) behaved similarly to the receptor-binding variant X-31/HS (225, L → Q) in HI tests with antibodies HC110X and HC68X which recognize HA_1 residues 189 and 193 respectively. Since in addition the antibody-binding assays indicated that they are not antigenic variants we compared their receptor-binding specificities by their reactions with derivatized erythrocytes and horse serum inhibitors. As reported before (Rogers *et al.*, 1983) X-31 specifically agglutinates erythrocytes enzymically modified to contain oligosaccharides terminating in the sequence SAα2,6 Gal and is sensitive in HI tests to a glycoprotein α_2-macroglobulin present in non-immune horse serum. The receptor-binding variant X-31/HS (226, L → Q), on the other hand, agglutinates erythrocytes modified to contain terminal SAα2,3 Gal sequences and by virtue of the procedure used in its selection is insensitive to the horse serum inhibitor. The variants selected by antibody HC63 showed differences from X-31 in their receptor-binding properties. As shown in Table III they either acquired the ability to agglutinate erythrocytes containing the SAα2,3 Gal linkage [63-3 (218, G → E)], lost their sensitivity to inhibition by equine α_2-macroglobulin [63-D (224−230 deleted) and 63-E (193, S → N; 226, L→P)] or exhibited transient haemagglutination of all derivatized cells [63-E (193, S → N; 226, L → P), 63-D (224−230 deleted) and V9A (218, G → R)]. Variant X-31/HS-V63 (224−230 deleted, 145, S → I; 158, G → E) was able to agglutinate cells containing the SAα2,6 Gal linkage in contrast

Table III. Receptor binding, fusion pH and amino acid substitutions in X-31 and X-31/HS antibody-selected variants

Variant	Amino acid at position					Receptor specificity				HI[c] Eq α_2M	Change in fusion pH[d]
	145	158	193	218	226	Haemagglutination[a] (HA titre)		Adsorption[b] (nmol/ml packed cells)			
						SAα2,6 Gal	SAα2,3 Gal	SAα2,6 Gal	SAα2,3 Gal		
X-31	S	G	S	G	L	1024	0	13	>142	4096	–
V83X			I			2048	0	14	>142	2048	−0.05
63-E			N		P	512[t]	2[t]	30	93	0	+0.53
63-D					*	256[t]	256[t]	30	68	0	+0.20
63-3				E		1024	1024	10	54	1024	+0.43
V9A				R		256[t]	0	32	>142	1024	+0.37
V68X			R			1024	512	24	42	0	0
X-31/HS					Q	0	1024	53	46	0	0
X-31/HS-V63	I	E			*	128	256	23	45	0	+0.20

[a]Haemagglutination assays were performed using 1.5% (v/v) human erythrocytes enzymatically modified to contain 37 nmol NeuAc/ml packed cells for the SAα2,6 Galβ, 4GlcNAc (SAα2,6 Gal) sequences and 107 nmol NeuAc/ml packed cells for SAα2,3 Galβ1, 3GalNAc (SAα2,3 Gal) sequences. All of the viruses agglutinated native (untreated) erythrocytes but did not agglutinate sialidase treated cells.
[b]These values represent the amount of sialic acid (in nmol/ml packed erythrocytes) required to bind 50% of the applied virus. The actual values are an average of the 50% binding point determined by assaying the amount of virus bound to derivatized cells and the amount of virus remaining in the supernatant.
[c]Haemagglutination inhibition (HI) by equine α_2-macroglobulin was determined at initial Eq α_2M concentrations of 3.5 mg/ml.
[d]A resonance energy transfer assay was used to measure haemagglutinin-mediated fusion. By this assay X-31 exhibited 50% fusion at pH 5.6.
[t]Indicates 'transient' agglutination where agglutination of derivatized erythrocytes was apparent after 30 min at room temperature, but no longer evident after 60 min.
*These variants have deleted amino acids 224−230 inclusive (RGLSSRI).

to its parent X-31/ HS (226, L → Q). Of five variants isolated with other monoclonal antibodies used in the HI test, only one, V68X (193, S → R) showed altered binding properties in acquiring the ability to agglutinate erythrocytes containing the SAα2,3 Gal linkage (Table III).

For such haemagglutination tests erythrocytes were derivatized to contain amounts of sialic acid optimal for detecting differences in receptor-binding properties. Information on the relative affinities of the viruses for the SAα2,6 Gal and SAα2,3 Gal linkages was obtained by determining the adsorption of viruses to preparations of derivatized erythrocytes containing different amounts of sialic acid (Paulson and Rogers, 1986). Figure 1 shows results obtained with X-31 and X-31/HS (226, L → Q). As indicated by haemagglutination, X-31 is specific for adsorption to sialic acid in the SAα2,6 Gal linkage and binds weakly to SAα2,3 Gal-derivatized cells even at the highest level of incorporation examined. In contrast X-31/HS binds to almost the same extent to cells containing either the SAα2,3 Gal linkage or the SAα2,6 Gal linkage but haemagglutinates only SAα2,3 Gal-derivatized cells simply because the amount of sialic acid incorporated into the SAα2,6 Gal-derivatized cells used in the haemagglutination tests was adjusted <40 nmol/ml packed cells, the minimum threshold required for virus adsorption.

Results of adsorption assays with HC63 and HC68X antibody-selected variants are compared with those obtained with X-31 in Figure 2 and a summary of the results expressed as the amount of sialic acid required to support adsorption of 50% of the virus is presented in Table III. Viruses are grouped in three panels according to the location of the amino acid substitution. Panel A compares variants with substitutions in residue HA_1 218. The most striking differences are in the apparent affinities of the variants for the SAα2,3 Gal linkage which was highest for variant 63-3 containing glutamic acid at position HA_1 218, intermediate for wild-type X-31 with glycine, and lowest for variant V9A with arginine. In panel B the highest apparent affinity for the SAα2,3 Gal linkage of mutants with substitutions at HA_1 193 was seen in variant V-68X with arginine, followed by decreasing affinity

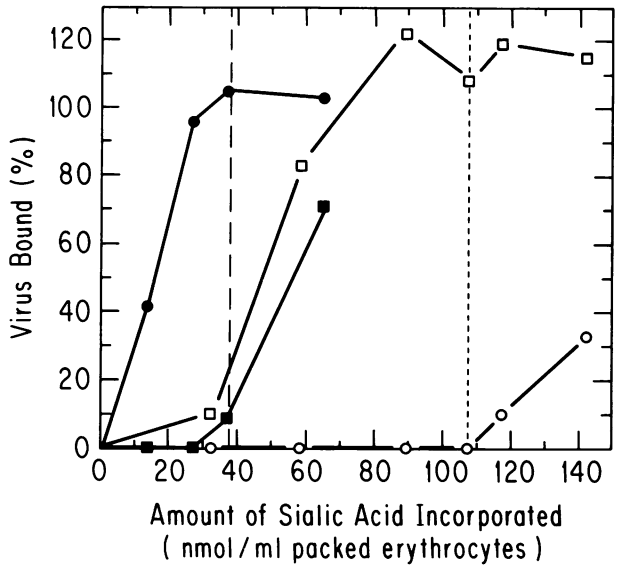

Fig. 1. The relative affinities of X-31 and X-31/HS for their receptors. The results shown are the neuraminidase activities of erythrocyte-bound virus; estimates of unbound virus were their reciprocals. When 100% virus binding occurred no unbound virus was detected indicating that there were sufficient receptors on 5% derivatized erythrocytes to bind all virus added. Experimental details of the neuraminidase assay are as described by Paulson and Rogers (1986). Vertical lines at 37 nmol/ml and 107 nmol/ml indicate the amounts of SA in α2,6 and α2,3 linkage respectively, used in the assignation of receptor specificity in Table III. ○,● X-31; □,■ X-31/HS (226, L → Q); closed symbols SAα2,6; open symbols SAα2,3.

for 63-E with asparagine, X-31 containing serine and V-83X with isoleucine. Finally in panel C the deletion of residues HA_1 224−230 in variants 63-D and X-31/HS-V63 is seen to have resulted in increasing affinity for the SAα2,3 Gal linkage: small differences between these variants may be due to the two ad-

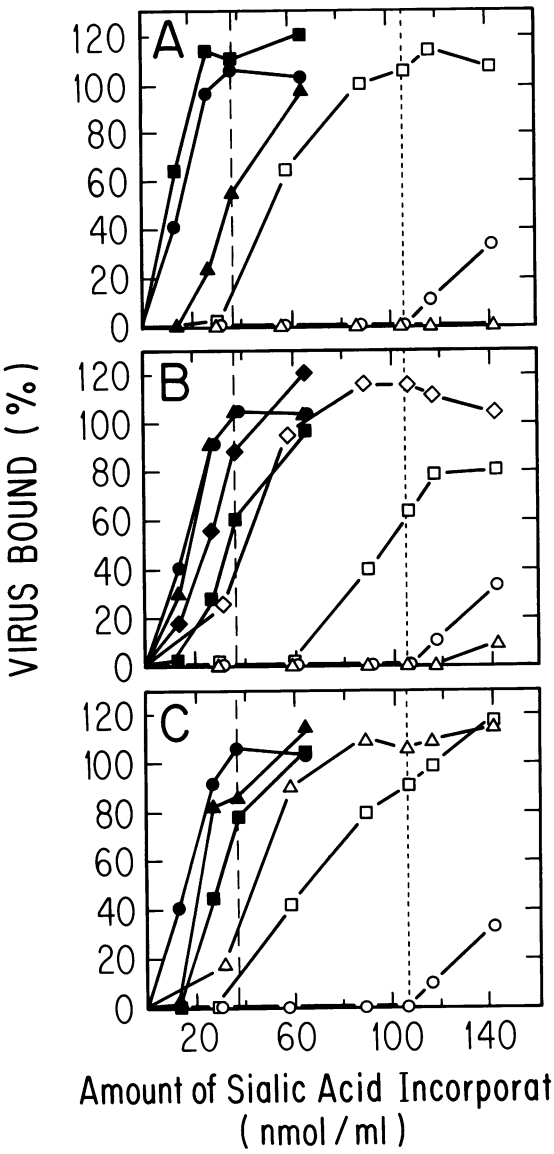

Fig. 2. The relative affinities of monoclonal antibody-selected variants of X-31 for their receptors. Affinities were determined as described in the legend to Figure 1. Closed symbols, SAα2,6; open symbols SA2,3. Viruses studied are **(a)** ● X-31, ■ 63-3 (218, G → E), ▲ V9A (218, G → R); **(b)** ● X-31, ■ 63-E (193, S → N; 226, L → P), ◆ V68X (193, S → R), ▲ V83X (193, S → I); **(c)** ● X-31, ■ 63-D (224–230 deleted), ▲ X-31/HS-V63 (224–230 deleted; 145, S → I; 158, G → E).

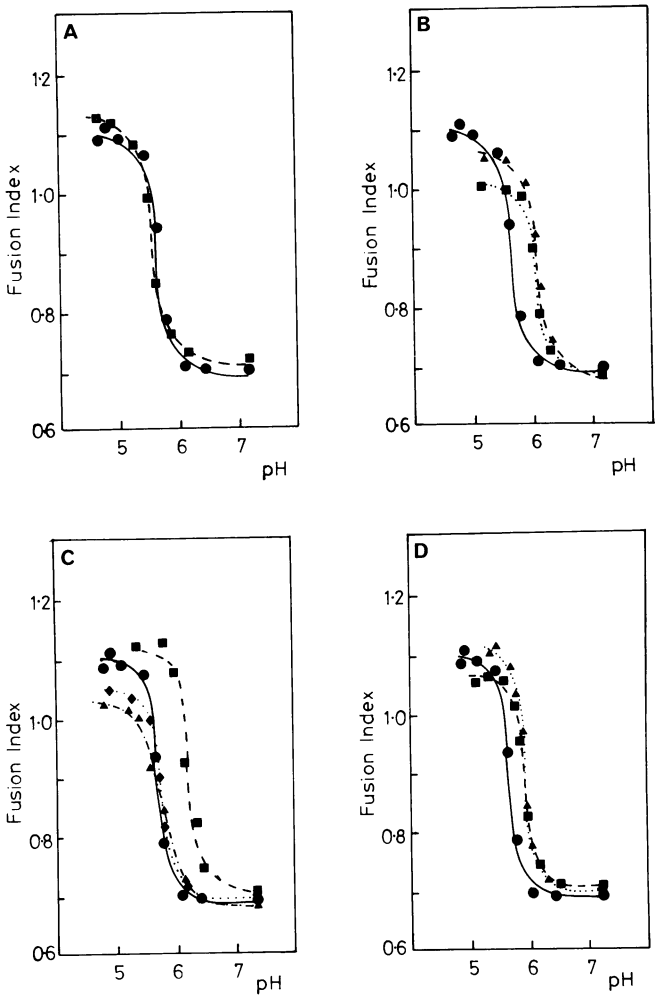

Fig. 3. The pH dependence of the membrane fusion by X-31 and selected mutant viruses. The fusion index is the ratio of the fluorescence of cholesteryl anthracene-9-carboxylate at 460 nm (donor fluorophore) to the fluorescence of *N*-4-nitrobenzo-2-oxa-1,3-diazole phosphatidylethanolamine at 530 nm (acceptor fluorophore). The values indicate the extent of lipid dilution and hence of membrane fusion. Virus suspensions were adjusted to equal haemagglutination titres. Viruses studied are: **(a)** —●— X-31, — — ■ — — X-31/HS; **(b)** —○— X-31, ●···■··· 63-3 (218, G → E), — —▲— — V9A (218, G → R); **(c)** —●— X-31, — — ■ — —63-E (193, S → N; 226, L → P), ···◆··· V68X (193, S → R), · —▲— · V83X (193, S → I); **(d)** —○— X31, — — ■ — — 63-D (224–230 deleted), ···▲··· X-31/HS-V63 (224–230 deleted 145, S → I; 158, G → E).

ditional amino acid substitutions in X-31/HS-V63 at HA_1 145 and 158 or to inaccuracies in the procedure.

Changes in the relative affinities of the variants for the SAα2,6 Gal linkage were also observed. In most cases a decrease in the affinity for the SAα2,6 Gal linkage accompanied an increase in the affinity for the SAα2,3 Gal linkage. However in mutant 63-3 (218, G → E) a slightly increased affinity for the SAα2,6 Gal linkage accompanied the increased affinity for SAα2,3 Gal and in mutant V9A (218, G → R) decreased affinity for both linkages was observed. Although several of the variants had an affinity for the SAα2,3 Gal linkage equivalent to that of the receptor variant X-31/HS (226, L → Q), their affinity for the SAα2,6 Gal linkage was always higher (Table III).

The transient haemagglutination by variants V9A (218, G → R), 63-E (193, S → N; 226, L → P) and 63-D (224–230 deleted) (Table III) does not result from neuraminidase action since resuspension of the erythrocytes allowed their repeated agglutination, but is due to their low affinity for sparsely derivatized erythrocytes. In general, stable haemagglutination appeared to require twice the level of incorporation of sialic acid at which 50% of virus was adsorbed in assays such as those in Figures 1 and 2.

Finally the sensitivity of haemagglutination by the variants to inhibition by equine α_2-macroglobulin correlated with high affinity for the SAα2,6 Gal linkage (Table III). The single exception was V9A (218, G → R) which unlike the other variants with low affinity for the SAα2,6 Gal linkage did not bind SAα2,3 Gal-derivatized cells and was therefore inhibited in HI tests using native erythrocytes by the α-2,6 linkage-rich α_2-macroglobulin.

The stability and membrane fusion properties of the mutant haemagglutinins

In attempts to isolate antibody HC63-selected mutant haemagglu-

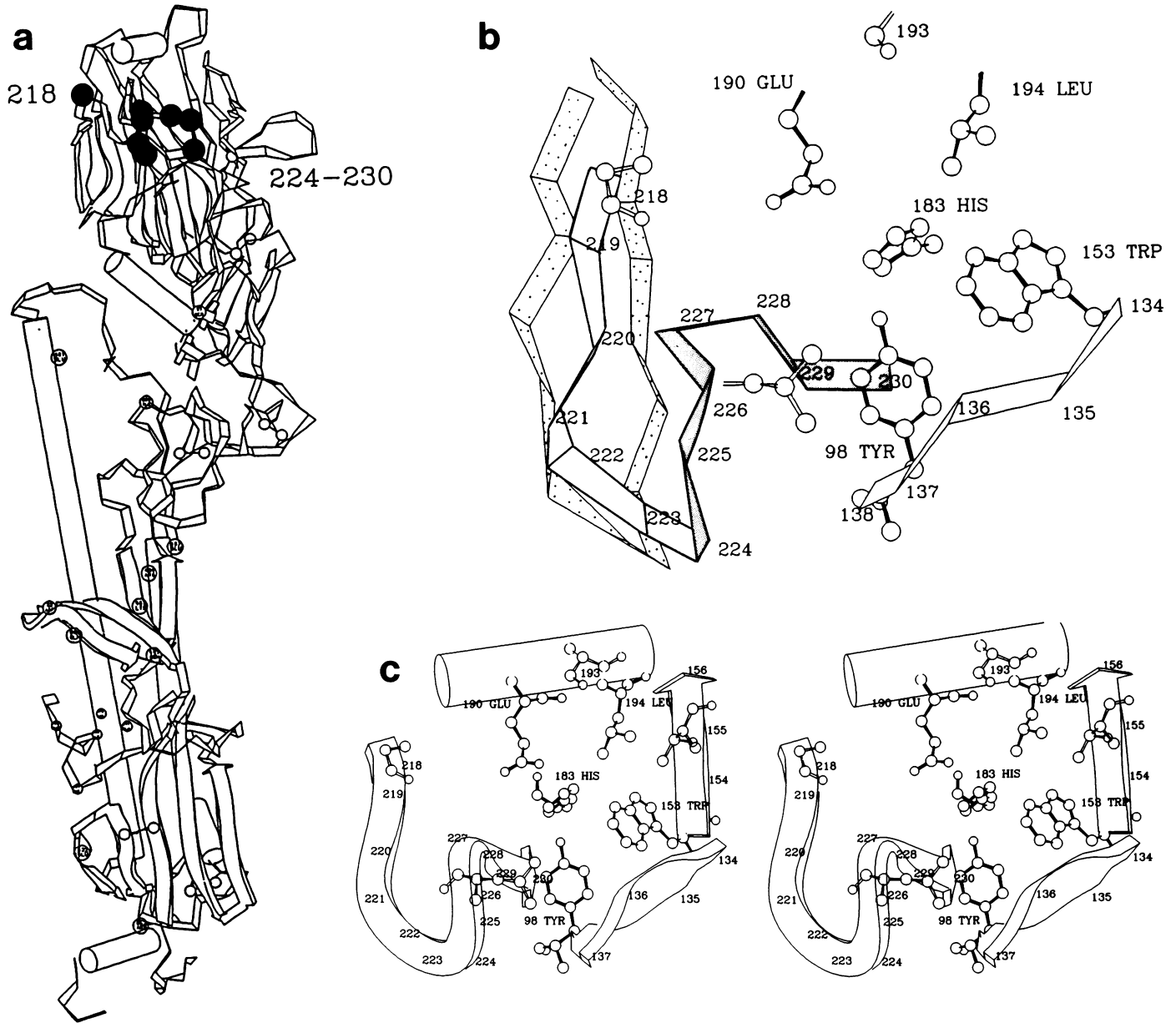

Fig. 4. The haemagglutinin receptor-binding site. **(a)** A schematic diagram of an X-31 haemagglutinin subunit. The locations of the amino acid substitutions close to the receptor-binding site in variants 63-D, 63-3 and V9A are indicated. **(b)** A schematic diagram showing the conserved residues in the binding site in dark bonds. The amino acids substituted in adsorptive mutants 193, 218, 226 are shown in open bonds. The deletion 224–230 is shaded. A stippled ribbon shows the position of residues on an adjacent subunit in the HA trimer. **(c)** A stereo-diagram of the same region as in **(b)**. Flat ribbons represent extended polypeptide chains and the cylinder segments of α-helix.

tinins by bromelain digestion (Brand and Skehel, 1972) it was observed that the haemagglutinins were more readily degraded than that of wild-type X-31 virus and that once isolated the mutant haemagglutinins were less stable. These properties were reminiscent of the unstable haemagglutinins of fusion mutants of X-31 which undergo the structural transition required for membrane fusion, at higher pH than wild-type virus. We therefore compared the pH at which membrane fusion was mediated by HC63-selected mutants with the pH of fusion by X-31. Figure 3 shows the pH dependence of membrane fusion by X-31 and mutant viruses. The pH at which 50% fusion occurred is shown in Table III. X-31/HS, V83X and V68X had the same pH dependence as wild-type virus whereas all mutants selected with HC63 fused membranes at a higher pH. The more important amino acid substitutions occurred at residues HA_1 226 and 218. Both of the substitutions at HA_1 218, glycine to arginine (V9A) and glycine to glutamic acid (63-3), resulted in fusion at about pH 6.0, an elevation of 0.4 pH units and the leucine to proline change at HA_1 226 had the biggest effect on fusion pH with an elevation of 0.53 pH units. These elevations of fusion pH are in the same range as those observed previously for mutants selected specifically for their ability to fuse membranes at higher pH (Wharton *et al.*, 1986).

Discussion

Analyses of monoclonal antibody-selected variants of influenza HA (Gerhard, 1977; Laver *et al.*, 1979, 1980; Webster and Laver, 1980; Caton *et al.*, 1982; Skehel *et al.*, 1984) and of other virus proteins (for review, see Yewdell and Gerhard, 1981) have been important in studies designed to enumerate and locate anti-

genically important regions of the molecule and in assessments of the antigenic significance of amino acid sequence differences between the haemagglutinins of natural isolates. In this study selections with monoclonal antibody HC63 prepared against X-31 haemagglutinin are shown to result in the isolation of mutants with haemagglutinins which differ from that of X-31 in their receptor-binding and membrane-fusion properties but which are not antigenically different since they continue to bind the selecting antibody in ELISA tests. Similar observations with regard to changes in the receptor-binding properties of variants isolated under antibody pressure have been made before (Fazekas de St. Groth, 1977; Yewdell *et al.*, 1986) by using in these cases antibody mixtures and recording increases in the binding affinities of the selected mutants, and by Underwood (1984) and Underwood *et al.* (1986) in analyses of the receptor-binding properties of natural and monoclonal antibody-selected antigenic variants. In the analyses described here comparative estimates of affinity for erythrocytes were also made and distinct changes in receptor-binding specificity including acquisition of the ability to recognize sialic acid in α2,3 linkage to galactose were observed which may influence the overall affinity for native erythrocyte surface receptors.

The amino acid sequence differences in HC63-selected mutants principally involved substitutions in positions HA_1 218, HA_1 226 and deletions of HA_1 224–230. No equivalent changes have been detected in natural isolates of the H_3 subtype made between 1968 and 1982; Gly-218 is conserved in all isolates, residue 226 is almost invariably leucine, and deletions have not been detected in the haemagglutinins of any H_3 isolates from humans. This degree of conservation is consistent with the ability of HC63 to react in HI tests with isolates made over a long period of time (Table I). However, since the variants selected are adsorptive mutants the results of HI tests even with reagents of known specificity cannot be used to define the residues recognized by the antibody. The mutations detected do not prevent binding of the antibody to the variant haemagglutinins in ELISA tests and they are therefore presumably not located in the epitope recognized by HC63. The location of these changes in the X-31 haemagglutinin structure (Wilson *et al.*, 1981) is, however, consistent with their effects on receptor binding. Residue HA_1 226 is located on the left side of the receptor-binding site projecting towards its centre and in the case of X-31/HS, HA_1 226 glutamine appears to interact with bound sialic acid (Weiss *et al.*, in preparation). Substitutions at this position are known to influence receptor specificity and the small change resulting from the proline substitution in 63-E is consistent with the size and probable orientation of this residue in the variant haemagglutinin. The deletion of residues HA_1 224–230 in mutants 63-D and X-31/HS-V63 would obviously change the structure of this side of the binding pocket (Figure 4) and the observation that discrimination between the SAα2,3 Gal linkage and the SAα2,6 Gal linkage is lost also supports the importance of this region in dictating linkage specificity. Residue HA_1 218 is less directly involved in the receptor-binding site. It is located in the interface between subunits of the haemagglutinin trimer in close proximity to HA_1 201 arginine of the neighbouring subunit and HA_1 220 arginine of the same subunit. The substitution HA_1 218 glycine to arginine in variant V9A would disturb the interface by introducing a large, positively charged side-chain and possibly as a result change the structure of the left-hand side of the site (Figure 4). The substitution HA_1 218 glycine to glutamic acid in variant 63-3 may have a similar effect but the consequences of introducing a negatively charged amino acid in this position are difficult to predict.

The variant V68X (193, S → R), in recognizing both SAα2,6 Gal and SAα2,3 Gal linkages has similar receptor-binding specificity to the mutants selected with HC63. In this case, however, no change in the pH of fusion from that of X-31 virus was detected (Table III) and in ELISA antibody binding assays the variant did not react with the selecting antibody. HC68X has previously been shown to distinguish between X-31 and the receptor binding variant X-31/HS in HI tests and to recognize HA_1 193 (Daniels *et al.*, 1984). This residue is located on the top of the back wall of the binding site in a short α-helix between conserved residues HA_1 190 glutamic acid and HA_1 194 leucine (Figure 4). The substituted amino acid in this case, HA_1 193 serine to arginine, may interact with HA_1 glutamic acid 190 which appears to be directly involved in receptor binding (Weiss *et al.*, in preparation) or bind directly to the receptor and as a result modify binding specificity. This observation indicates, however, that components of the left side of the pocket, including HA_1 226, are not the exclusive determinants of receptor-binding specificity.

X-31 haemagglutinin mediates membrane fusion and haemolysis at pH 5.6 (Wharton *et al.*, 1986). In previous analyses of mutants which fuse membranes at higher pH the amino acid sequence changes detected in mutant haemagglutinins were grouped into two categories. In one of these the amino acid substitutions destabilized the location of the hydrophobic amino-terminal region of HA_2 which may be directly involved in membrane fusion (Daniels *et al.*, 1985). Substitutions in the second category either destroyed or distorted contacts in the interfaces between the chains and subunits of the trimer and as a consequence destabilized its quaternary structure. Altered contacts were located in regions remote from the amino terminus of HA_2 ranging 77 Å along the length of the HA_2–HA_2 subunit interface and the observations reported here that changes in the HA_1–HA_1 interface of the trimer also elevate the pH of fusion extend this range to over 100 Å. Such changes may decrease the stability of the trimer sufficiently to facilitate extrusion of the amino terminus of HA_2 from its buried location or may predominantly influence the local stability of the distal region of the molecule. In the latter case structural changes in this region may precede extrusion of the amino terminus of HA_2 but distinction between this possibility and, for example, the occurrence of simultaneous structural changes in both regions is not possible from the information available. The substitution of proline for leucine at HA_1 226 and the HA_1 224–230 deletion may also influence the stability of the HA_1–HA_1 interface to elevate the pH of fusion but without more direct information their structural consequences cannot be assessed.

We have discussed before (Daniels *et al.*, 1985) the various selective pressures which influence the synthesis and structure of a functionally complex oligomer such as haemagglutinin and the multiple effects which single amino acid substitutions can have. The inclusion of the membrane distal HA_1–HA_1 interface as a structurally important region for both receptor-binding and membrane-fusion activities emphasizes the importance of these constraints and raises the possibility of a role for receptor binding in triggering fusion activity.

Materials and methods

Monoclonal antibody production and characterization

Antibodies were produced in BALB/c mice as described before (Daniels *et al.*, 1983) using SP2/0-Ag14 myeloma cells except for HC63, HC31 and HC3M which resulted from fusions using NS1/Ag4 cells. HC63, which is extensively used in the experiments reported here, was classified in double-immunodiffusion tests as a member of the IgG_1 subclass. Isoelectric focusing and polyacrylamide gel electrophoresis in SDS indicated the presence of a single antibody in the medium of [^{35}S]methionine-labelled HC63-producing cell cultures.

Variant selection and virus growth

Variants of X-31 were obtained by mixing equal volumes of allantoic fluid containing virus and undiluted ascitic fluid containing monoclonal antibodies, and using the mixture as egg inoculum as before (Daniels *et al.*, 1983).

Variant selections with HC63 were also done in MDCK cells. Allantoic fluid containing X-31 was mixed with antibodies at different dilutions for 1 h and 200-μl aliquots used to infect MDCK cells maintained in Linbro Fb6 plates. After 30 min cultures were washed and overlayed with 3 ml MEM/trypsin (Worthington; TPCK-treated 0.6 U/ml) containing HC63 at a 5-fold greater dilution than used in the preincubation. After 3 days at 37°C, supernatant fluids were collected and the 'variant enriched' tissue culture fluid was subjected to a further antibody selection followed by plaque purification (Appleyard and Maber, 1974).

For all antigenic, receptor-binding, fusion and nucleotide sequence analyses X-31 and the variants were grown in the allantoic cavity of 10-day-old embryonated hens' eggs and purified as described by Skehel and Schild (1971). No changes in the sequences of genes for HA occurred when variants selected in MDCK cells were subsequently passaged in eggs.

Antigenic analyses

Haemagglutination inhibition tests were done using standard methods (WHO, 1953). ELISA assays for antibody binding were done as described by Bos *et al.* (1981) using horseradish peroxidase with 3,3′,5,5′-tetramethylbenzidine as substrate.

Nucleotide sequence analyses

Sequences were determined using the dideoxynucleotide chain terminating procedure of Sanger *et al.* (1977) as described before (Daniels *et al.*, 1983).

Receptor-binding analyses

Receptor specificities of the variants were defined by their ability to agglutinate native, asialo and enzymically resialated human erythrocytes. Sialyltransferase derivatized erythrocytes were prepared as described previously (Rogers and Paulson, 1983). On the basis of earlier receptor specificity studies of X-31 (Rogers *et al.*, 1983) derivatized erythrocytes were made to contain either SAα2,3 Galβ1,3 Gal NaAc or SAα2,6 Galβ1,4 Glc NAc sequences in amounts of 107 and 37 nmol SA/ml packed cells, respectively. Haemagglutination titres were determined at room temperature using serial 2-fold dilutions of virus (25 μl) in phosphate-buffered saline (pH 7.0) mixed with 25 μl of a 1.5% suspension of native, asialo or derivatized human erythrocytes. Titres were determined after 1 h and expressed as the reciprocal of the maximum dilution of virus that caused complete agglutination.

Estimates of virus adsorption were made using a neuraminidase assay to quantitate the amount of virus bound to erythrocytes derivatized to contain different amounts of SA (Paulson and Rogers, 1986).

Fusion analyses

Haemolysis and resonance energy transfer methods described before were used (Daniels *et al.*, 1985; Wharton *et al.*, 1986). Assays were done over the pH range 5.0–7.4 at 37°C and the results are expressed as the differences (ΔpH) between the pHs at which either haemolysis or resonance energy transfer were 50% of the maximum for the variants and X-31.

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