Premature Stop Codons in the G Glycoprotein of Human Respiratory Syncytial Viruses Resistant to Neutralization by Monoclonal Antibodies

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Mutants of human respiratory syncytial (RS) virus which escaped neutralization by monoclonal antibodies directed against the G glycoprotein were selected from the Long strain. Most mutants showed drastic antigenic changes, reflected in the lack of reactivity with several anti-G antibodies, including the one used for selection. Sequence analysis revealed the presence of in-frame premature stop codons in the mutated G genes which shortened the G polypeptide by between 11 and 42 amino acids. In contrast, two mutants selected with monoclonal antibody 25G contained two amino acid substitutions (Phe-265→Leu and Leu-274→Pro) and had lost only the capacity to bind the antibody used in their selection. These results demonstrate that the carboxy-terminal end of the G molecule is dispensable for infectivity in tissue culture and indicate the importance of this part of the G protein in determining its antigenicity.

The G glycoprotein of human respiratory syncytial (RS) virus is responsible for attachment of the virus to the cell membrane receptor during the initial stages of an infectious cycle (15). The nucleotide sequence of the G protein predicts polypeptides of 298 and 292 amino acids for virus of the A and B antigenic subgroups, respectively (12, 22, 25, 30). The G molecule belongs to the type II glycoproteins, having a single hydrophobic domain between residues 38 and 66 which serves as both signal and transmembrane anchor. In addition, the G protein contains several sequence and structural features unusual among the attachment proteins of other paramyxoviruses. The protein precursor is synthesized as a 32-kDa polypeptide which is cotranslationally modified by the addition of high-mannose N-linked sugars to form an intermediate of 45 kDa (31). This step is followed by conversion of the N-linked sugars to the complex type and addition of O-linked sugar side chains to achieve the mature form with an apparent molecular mass in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of 80 to 90 kDa. This preferential contribution of O-linked sugars to the molecular mass of the mature protein reflects the presence of more than 70 potential sites (Ser or Thr) for this type of modification in the G protein ectodomain.

Monoclonal antibodies (MAbs) directed against the G protein are able to neutralize infectivity in vitro (1, 8) and protect in vivo against a virus challenge in experimental animals (26, 29). Protection is also achieved after immunization with purified G protein (28) or inoculation of vaccinia virus recombinants expressing the G protein (18, 23). Although these results point to the G protein as an important immunogen for the development of effective and safe vaccines, the high degree in G antigenic variation among RS virus isolates (2, 8, 17) poses some problems. For instance, the protective immune response induced by the G glycoprotein in animals is subgroup specific, whereas the protection mediated by the other external RS virus glycoprotein (F) is cross-reactive. It remains to be tested whether individual G proteins protect against a challenge with antigenically distinct viruses of the same subgroup.

To explore the structural basis of G protein antigenicity, we have initiated the isolation and characterization of mutants which escape neutralization to individual MAbs. Five such mutants, selected with MAb 63G, have been reported recently (9). The 63G escape mutants contained frameshift mutations in the carboxy-terminal third of the G protein gene which originated from single adenosine deletions. Four of the mutants had lost reactivity with most anti-G MAbs and encoded G polypeptides 25 amino acids shorter than the wild type, with the last 69 or 81 amino acids changed. These results indicated that the carboxy-terminal third of the G protein can accommodate many sequence changes and plays an essential role in determining the antigenic structure of the G molecule. This concept is reinforced by the results presented here on the characterization of other escape mutants selected with five different anti-G antibodies.

Isolation and antigenic characterization of escape mutant viruses. The Long strain of RS virus was plaque purified in monolayers of HEp-2 cells as described previously (7). Escape mutants were then independently selected with MAb 25G, 27G, 64G, 62G, or 74G. Each selection cycle involved incubation of the virus with each MAb in the presence of complement and rabbit anti-mouse serum prior to inoculation of cell monolayers as described in detail for the selection of mutants resistant to MAb 63G (8, 9). After viral adsorption, growth medium was added with a 1/5 dilution of the corresponding MAb-containing supernatant. Complete inhibition of cytopathic effect was observed after 3 days at 37°C, but resistant virus emerged, and cytopathic effect was detected, after five to seven selective cycles. The resistant mutants were plaque purified, and the virus of a single plaque from each culture supernatant was recovered for further analysis.

Seven escape mutants were tested in an enzyme-linked immunosorbent assay (ELISA) for reactivity with a panel of 19 anti-G MAbs (Fig. 1). For comparison, two previously

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The mutants had lost reactivity with antibodies indicated at the top were used to coat microtiter plates. The reactivity of each virus with the panel of anti-G Mabs was tested by ELISA (8). Data represent less than 25% (■), 25 to 50% (■), and more than 50% (■) of the absorbance values obtained with the Long strain.

Reported mutants selected with MAb 63G (R63/2/4/8 and R63/2/4/1) (9) were included in the assay. The epitopes recognized by the anti-G antibodies have been clustered in 10 antigenic areas by competitive virus-binding assays (8).

Among the new escape mutants, only those selected with MAb 25G had lost the binding site for the antibody used in their selection and retained reactivity with all other anti-G Mabs. Their behavior resembled that of mutant R63/2/4/1, which had lost only reactivity with MAb 63G. In contrast, the mutants selected with Mabs 27G, 64G, 62G, and 74G showed drastic antigenic changes, reflected in the loss of reactivity with a large number of anti-G Mabs, which recognized epitopes of the overlapping antigenic areas VI to IX. In some cases the epitopes recognized by unique antibodies, such as 25G and 78G, were also lost in the mutant viruses. The ELISA results were confirmed by Western immunoblot (not shown), indicating that the loss of reactivity of the mutant viruses with Mabs was not a consequence of major conformational changes introduced in the mature G molecule. All of the escape mutants shown in Fig. 1 except R63/2/4/8 retained reactivity with polyclonal antisera raised against either the soluble form of the Long G protein or purified Long virus (data not shown).

Sequence analysis of the escape mutant mRNAs. To identify the genetic alterations selected in the escape mutants, direct sequencing of the G mRNAs was carried out by the dideoxy method (21), using 32P-labeled primers synthesized according to the G sequence of the Long strain (9, 12). Total RNA was extracted from infected cells by the isothiocyanate-CsCl method (4), and poly(A) + RNA was purified by oligo(dT)-cellulose chromatography. The reverse transcriptase reactions were followed by a chase with terminal deoxynucleotidyld transferase to eliminate sequence ambiguities (6). Table 1 summarizes the changes detected in comparison with Long G mRNA. Each of the two mutants selected with MAb 25G contained two U→C transitions at nucleotides 808 and 836 which predicted the amino acid changes Phe-265→Leu and Leu-274→Pro. The other mutants contained single base transitions orversions which introduced premature in-frame stop codons in the G protein gene. Mutant R64/8/2 contained, in addition, a silent A→G transition at nucleotide 426. No special features were found in the G gene sequence which could explain the selection of the premature stop codons at the sites indicated in Table 1. The transition C→U was selected more frequently and with three Mabs, whereas the transversion A→U was selected only once.

The sequence changes observed in the mutant viruses predicted structural alterations of the G protein (Fig. 2). Figure 2 also includes changes previously reported for the mutants selected with MAb 63G (9). Only the mutant viruses R63/2/4/1, R25/7/12, and R25/8/3 encoded proteins of wild-type length. The former mutant, however, contained seven amino acid changes (residues 205 to 211) resulting from two frameshifts, whereas the last two mutants contained two amino acid substitutions, at residues 265 and 274. The mutants which contained premature stop codons have between 11 and 42 amino acids truncated from the carboxyterminal end. These truncations implied the loss of 4 to 17 potential sites for O-linked oligosaccharides and 1 to 2 sites for N-linked sugar chains. Mutants R63/1/2/3, R63/2/4/8, R63/4/10/5, and R63/8/7/2 encoded proteins that were 25 amino acids shorter than the wild type and with the last 81 (first mutant) or 69 (last three) amino acids changed. These changes also implied a considerable reduction in the number of potential O- and N-linked oligosaccharide side chains.

Determination of the G protein size from escape mutants. To confirm the G protein size reduction predicted from the nucleotide changes detected in the escape mutants, proteins from purified virions were separated by SDS-PAGE (24) and

![Table 1. Summary of sequence changes detected in escape mutant viruses](image)

**Table 1. Summary of sequence changes detected in escape mutant viruses**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Nucleotides changed</th>
<th>Amino acids changed</th>
<th>No. of amino acids lost (residues)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R25/7/2</td>
<td>f 808 (U→C)</td>
<td>265 (Phe→Leu)</td>
<td>42 (257-298)</td>
</tr>
<tr>
<td>R25/8/3</td>
<td>836 (U→C)</td>
<td>274 (Leu→Pro)</td>
<td></td>
</tr>
<tr>
<td>R27/8/3</td>
<td>784 (A→U)</td>
<td>257 (Lys→stop)</td>
<td>42 (257-298)</td>
</tr>
<tr>
<td>R64/8/2</td>
<td>426 (A→G)</td>
<td>No change</td>
<td></td>
</tr>
<tr>
<td>R62/6/1</td>
<td>796 (C→U)</td>
<td>261 (Gln→stop)</td>
<td>38 (261-298)</td>
</tr>
<tr>
<td>R62/C2/10</td>
<td>847 (C→U)</td>
<td>278 (Gln→stop)</td>
<td>21 (278-298)</td>
</tr>
<tr>
<td>R74/1/1</td>
<td>876 (C→U)</td>
<td>278 (Gln→stop)</td>
<td>21 (278-298)</td>
</tr>
</tbody>
</table>

* a Shown are the nucleotide differences of each mutant mRNA compared with Long, indicating the amino acid changes predicted for each virus.
FIG. 2. Diagram of the G protein primary structure and changes in the antibody escape mutants. The Long G protein amino acid sequence (9) is represented. Shown are the transmembrane domain ( ), the potential acceptor sites for O ( ) and N ( ) glycosylations, and the cysteine residues ( ). The structures of the mutant G proteins indicate the sites of frameshift changes ( ) and the amino acid substitutions ( ). The number of amino acids (aa) lost or changed in the mutated G proteins is indicated at the right.

electrotransferred to nitrocellulose paper (27). The G bands were then visualized with MAb 63G (Fig. 3). Antibody 79N, directed against the viral NP protein, was also included in the assay to standardize the amount of viral antigen in each lane. A wide band of 90 kDa was detected in the lanes corresponding to Long, R25/7/12, and R25/8/3 viruses. The G bands of the other mutants showed slight increases in electrophoretic mobility, in agreement with the truncations predicted for the G polypeptides. Thus, the G band with the highest electrophoretic mobility corresponded to mutant R/27/8/3 (Fig. 3, lane 4), which has 42 amino acids deleted at the C-terminal end; the G bands of the other mutants showed progressive retardation in the gel as the number of amino acids deleted decreased. It should be pointed out that the increase in electrophoretic mobility could be a consequence of both the shortening of the amino acid backbone and the loss of potential glycosylation sites in the mutant proteins.

The information gained from the escape mutants is relevant to an understanding of the antigenic and structural properties of the RS virus G protein and the genetic mechanisms which operate in the generation of mutants resistant to anti-G antibodies.

The most unexpected finding was that the majority of the new escape mutants contained premature in-frame stop codons which predicted the truncation of the G polypeptide of between 11 and 42 amino acids. Premature stop codons

FIG. 3. Electrophoretic mobilities of G proteins from escape mutants. Proteins from purified virions were blotted with a mixture of MAbs 63G and 79N. Viruses in lanes 1 to 8 correspond to Long, R25/7/12, R25/8/3, R27/8/3, R64/8/2, R62/6/1, R62/C2/10, and R74/1/1, respectively. Positions of G and NP bands are indicated at the right. Molecular weight markers, shown at the left, are expressed in thousands.
have been also described for other RNA viruses under different circumstances. For instance, the selection of in-frame stop codons in the transmembrane glycoprotein of simian immunodeficiency virus was observed during its adaptation to human cells in culture (3, 10, 11, 13). In influenza virus, nonsense mutations affecting the lengths of the NS1 protein were detected among natural isolates (20). Nevertheless, the RS virus mutants described here represent, to our knowledge, the first example of premature stop codons selected in viruses resistant to neutralization by MAbs.

Not all RS virus mutants which escape neutralization by MAbs were derived by the same mechanism. Viruses selected with MAb 25G contained two amino acid substitutions, those selected with MAb 63G contained frameshift mutations (9), and viruses resistant to neutralization with an anti-F MAb contained single amino acid substitutions (16). It remains to be determined whether different mutants selected with the same antibody show consistently the same type of genetic alteration. It is also intriguing that premature stop codons were placed at different sites of the G gene in mutants selected with different anti-G antibodies when, for instance, the mutant with the shortest G protein (R27/8/3) was also resistant to MAbs 64G, 62G, and 74G.

The sequence changes detected in the escape mutants (Table 1 and Fig. 2), together with the ELISA results of Fig. 1, have important implications for the antigenic structure of the G molecule.

(i) The amino acid change Leu→Pro at residue 274 selected in the mutants resistant to MAb 25G is also present in the A2 strain of RS virus, whereas both the Long and A2 strains contain Phe at residue 265. Since MAb 25G reacts with the Long strain but not with A2 (8), it seems that amino acid 274 is essential for the integrity of epitope 25G. This conclusion is also supported by the data from mutants which contained the premature stop codon.

(ii) Epitope 68G is tentatively located between residues 205 and 256 of the G polypeptide, as evidenced by the lack of reactivity of antibody 68G with mutant R63/2/4/8, which has a frameshift after amino acid 204, and its reactivity with mutant R27/8/3, which has a stop codon at residue 257.

(iii) Epitope 63G has been previously shown to include some or all of amino acids 205 to 211 changed in mutant R63/2/4/1 (9). Thus, the only three epitopes of the G molecule which can be tentatively ascribed to specific sequences are located in its carboxy-terminal third.

(iv) The viruses which contain frameshift mutations or premature stop codons have drastic antigenic changes, reflected in the absence of reactivity with most anti-G antibodies. A provisional conclusion could be that the majority of G epitopes are clustered in the last 11 amino acids, deleted in mutant R74/1/1. However, it is also feasible that truncated G proteins are not folded in the same manner as in the wild type during intracellular traffic, resulting in different modifications which affect the antigenic integrity of the mature protein. In any case, the carboxy-terminal part of the G protein seems to play an essential role in determining the antigenic structure of the mature G molecule.

The behaviors of mutants containing either frameshift changes or in-frame stop codons indicate that RS virus G protein can tolerate deletions in the carboxy-terminal end without affecting RS virus infectivity in tissue-cultured cells. This extends previous observations, made with simian virus 40 recombinants, indicating that truncated G proteins were transported normally to the cell surface (19).

It is tempting to speculate that the genetic mechanisms which operate in the selection of the antibody escape mutants could also operate to generate the antigenic and genetic variation detected among natural isolates (2, 5, 8). In this respect, premature stop codons might be responsible for the size differences detected between the G proteins of human subgroup A and B viruses and between the proteins of human and bovine RS viruses. Thus, the G mRNA of strain CH18537 (human subgroup B) is four nucleotides longer than Long G mRNA (human subgroup A), yet the former strain encodes a polypeptide six amino acids shorter than the Long polypeptide (12). More strikingly, the G mRNA of bovine strain 391-2 encodes a polypeptide 41 amino acids shorter than the Long polypeptide, but its noncoding 3′ end is 38 nucleotides longer (14). It remains to be seen whether the frameshift changes detected in the escape mutants are also observed among natural isolates and the relative frequency of single base substitutions versus other genetic changes during RS virus propagation in humans.

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