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Location of a Highly Conserved Neutralizing Epitope in the F Glycoprotein of Human Respiratory Syncytial Virus

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Trypsin digestion of the purified F protein from human respiratory syncytial virus (Long strain) generated a set of fragments in the amino-terminal third of the F1 subunit which contained the epitope 47F involved in neutralization. Sequencing of five escape mutant viruses selected with monoclonal antibody 47F allowed us to map precisely two amino acid residues (262 and 268) of the F1 subunit which are essential for the integrity of this important epitope. The results are discussed in terms of the mechanisms involved in virus neutralization and the design of potential synthetic vaccines.

Human respiratory syncytial virus is the most important cause of lower respiratory tract infections in infants and young children (8). Trials with conventional vaccines had to be abandoned because of inadequate protection (1, 13) or because vaccinees developed more severe disease following their next natural infection (6). Thus, research has now focused on elucidating the molecular basis of a protective immunity to respiratory syncytial virus.

We recently described the preparation of a panel of monoclonal antibodies raised against the respiratory syncytial virus Long strain (4). One antibody (47F), which reacted with the fusion (F) glycoprotein, showed the highest neutralization index. This epitope was conserved in 12 strains tested, including viruses of the A and B subgroups. However, we could isolate five neutralization escape mutant viruses which had lost the capacity to bind antibody 47F. We now report the location of this epitope in the F-protein primary structure and the identification of two amino acid residues essential for the integrity of the epitope.

As an initial step, 47F antibody was tested by Western (immuno-) blot against extracts of cells infected with either Long virus or the escape mutants. Antibody 1P, directed against the viral phosphoprotein (P), was included in the assay to assess the extent of infection. A similar P band was present in all the extracts (Fig. 1A). In contrast, the large F1 subunit was detected only in the Long strain-infected cells but not in cells infected with the mutant viruses. This result located the epitope 47F in the large subunit of the Long strain F protein and confirmed the absence of antibody binding in the mutant molecules. A minor band of 20.5 kilodaltons (kDa) present in the extract of Long strain-infected cells was later identified as a proteolytic fragment of the F1 subunit.

To narrow down the location of epitope 47F, purified F protein was treated with trypsin, and the digestion products were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and tested against 47F antibody in Western blots. Two major fragments of 20.5 and 19 kDa were visualized after Coomassie blue staining (Fig. 1B, lane h), but other minor components were also observed. Both major fragments reacted with monoclonal antibody 47F (Fig. 1C, lane h'). The 20.5-kDa fragment, which also appeared as a minor component of the Long strain extract and the purified F protein, was probably generated to some extent during the purification process. Two other minor bands of 15 and 13 kDa, reacting with 47F antibody, were also identified among the trypsin digestion products (Fig. 1C, lane h'). However, the amount of the 15-kDa band, which was also

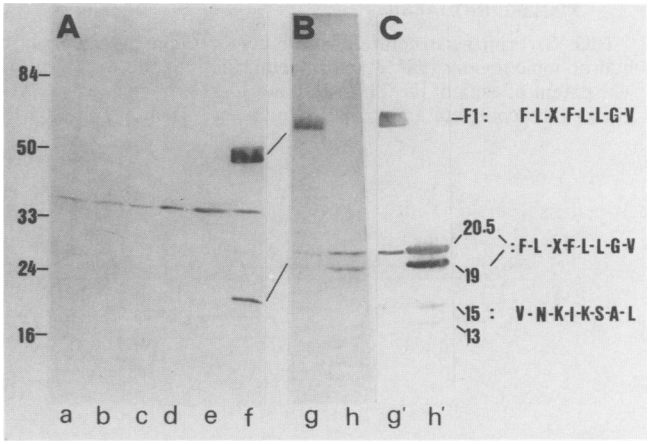

FIG. 1. Identification of the F-protein subunit and trypsin peptides reacting with 47F antibody. (A) A 50-μg sample of protein from extracts of HEp-2 cells infected with escape mutant R/47/4 (lane a), R/47/7 (lane b), R/47/16 (lane c), R/47/25 (lane d), or R/47/27 (lane e) or with Long strain virus (lane f) was tested by Western blot (10) against a mixture of 47F and 1P antibodies, as described previously (4). Molecular mass markers, shown at left, are expressed in kilodaltons. (B) A 30-μg sample of F protein, purified by immunoaffinity chromatography (4), was digested with 30 μg of trypsin (Sigma Chemical Co.) for 4 h at 37°C in a final volume of 50 μl of phosphate-buffered saline. The same amount of untreated (lane g) and trypsin-digested (lane h) material was applied to a 12% sodium dodecyl sulfate-polyacrylamide gel and stained with Coomassie blue. (C) Parallel lanes of the gel shown in panel B were transferred to nitrocellulose paper with buffer (2) (3 mM Na_2CO_3–10 mM $NaHCO_3$ [pH 9.9] in 20% methanol) and reacted with 47F antibody. Other strips of the same nitrocellulose paper, unreacted with the antibody, were matched to panel C and used to sliced out the F1 band from the untreated F protein and the 20.5-, 19-, and 15-kDa bands from the trypsin-digested material. The pieces of paper were mounted in the reaction chamber of a pulse liquid-phase sequenator (model 477A; Applied Biosystems) and subjected to eight cycles of automated Edmann degradation (5, 12). The sequence determined for each band is shown at the right by single-letter code. An X indicates undetermined amino acid at that cycle.

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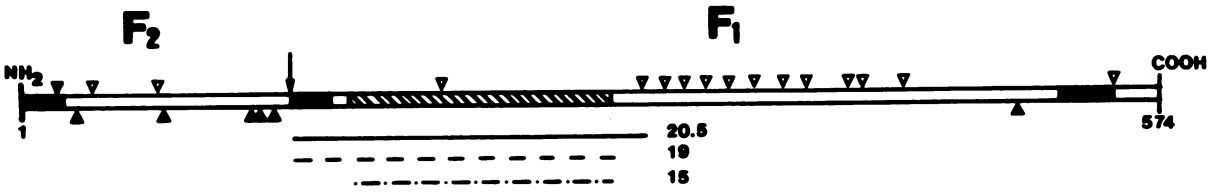


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MELPILKANA ITTILAAVTF CFASSQNITE EFYQSTCSAV SKGYLSALRT GWYTSVITIE LSNIKENKCN GTDAKVKLIK    80

QELDKYKNAV TELQLLMQST PAANNRARRE LPRFMNYTLN NTKKTNVTLS KKRKRRFLGF LLGVGSAIAS GTAVSKVLHL   160

EGEVNKIKSA LLSTNKAVVS LSNGVSVLTS KVLDLKNYID KQLLPIVNKQ SCRISNIETV IEFQQKNNRL LEITREFSVN   240

AGVTTPVSTY MLTNSELLSL INDMPITNDQ KKLMSNNVQI VRQQSYSIMS IIKEEVLAYV VQLPLYGVID TPCWKLHTSP   320

LCTTNTKEGS NICLTRTDRG WYCDNAGSVS FFPQAETCKV QSNRVFCDTM NSLTLPSEVN LCNVDIFNPK YDCKIMTSKT   400

DVSSSVITSL GAIVSCYGKT KCTASNKNRG IIKTFSNGCD YASNKGVDTV SVGNTLYYVN KQEGKSLYVK GEPIINFYDP   480

LVFPSDEFDA SISQVNEKIN QSLAFIRKSD ELLHHVNAGK STTNIMITTI IIVIIVILLS LIAVGLLLYC KARSTPVTLS   560

KDQLSGINNI AFSN                                                                          574
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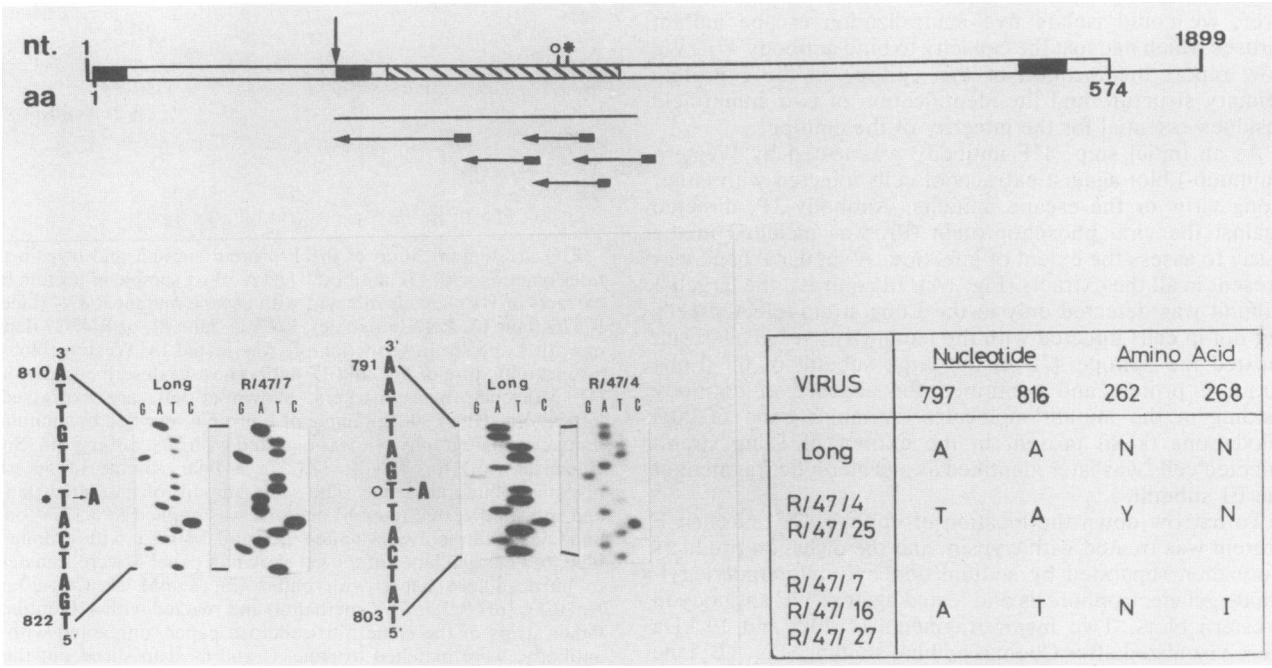
FIG. 2. Primary structure of the F glycoprotein. A scheme of the F protein structure is shown (top), with the length in amino acids, the hydrophobic regions (■), the proteolytic activation site (↓), the glycosylation sites (▲), and the positions of cysteine residues (▽) indicated. The extent of each of the 20.5-, 19-, and 15-kDa fragments is represented below. The overlapping segment of these peptides is also indicated (▨). The complete amino acid sequence (7) is shown (bottom), denoting the extent of the F1 fragments.



VIRUS	Nucleotide		Amino Acid	
	797	816	262	268
Long	A	A	N	N
R/47/4 R/47/25	T	A	Y	N
R/47/7 R/47/16 R/47/27	A	T	N	I

FIG. 3. Sequences of F mRNAs from Long strain and escape mutant viruses. The structure of the F mRNA is represented (top), with the nucleotide (nt) length, the regions encoding the hydrophobic domains (■), the positions of the nucleotide changes observed in the escape mutants (○, *), and the extent of peptide overlapping (▨), as shown in Fig. 2. The region of the F mRNA which was sequenced by the dideoxy method is shown below (——), indicating the oligonucleotides used and the extent of sequence determined with each primer (■→). Two examples of sequencing gels are shown (bottom), in which partial sequences of Long strain and mutant R/47/7 (left) and Long strain and mutant R/47/4 (right) are compared. The sequence changes detected in each mutant virus, compared with Long strain, are shown at the right. aa, Amino acid.

present in the undigested material, was not increased after trypsin digestion. This band was later characterized as a degradation product of the F1 subunit generated by other proteases (see below).

The bands of the F1 subunit and the 20.5-, 19-, and 15-kDa fragments were sliced out from unstained nitrocellulose strips of the same blot as that shown in Fig. 1C and subjected to eight cycles of automated Edmann degradation. The results (Fig. 1) identified the same amino-terminal sequence in both the F1 band and the 20.5- and 19-kDa fragments. This sequence could only be matched with the first eight residues of the F1 subunit in the deduced primary structure of the entire F molecule (Fig. 2) (3, 7). Considering the size of the trypsin fragments and that the last amino acid should be either lysine or arginine, we have tentatively located the carboxy termini of the 20.5- and 19-kDa peptides at residues 293 and 315, respectively, of the F protein (Fig. 2).

The amino-terminal sequence determined for the 15-kDa band corresponded to eight residues of the F1 subunit, starting at position 164 of the F-protein primary structure (Fig. 2). The preceding amino acid in the F sequence was glutamic acid, thus confirming that this fragment had not been generated by the trypsin digestion of the F protein. This prevented the assignment of any amino acid at the carboxy-terminal end; however, the size of the 15-kDa fragment was compatible with ending, as was the 19-kDa band, near residue 293 of the F sequence.

The results shown in Fig. 1 thus identified a set of peptides in the amino-terminal third of the F1 subunit which contained the epitope 47F. Considering the extent of peptide overlapping, this epitope was located primarily between residues 164 (valine) and 293 (lysine) of the F sequence.

In order to make a more precise assignment of the amino acids included in this epitope, we turned to sequencing studies of the neutralization-resistant viruses selected with the antibody 47F. Poly(A)$^+$ RNAs isolated from cells infected with either Long strain virus or each of the mutants were used to sequence the F mRNA segment which encoded the largest (20.5-kDa) trypsin fragment. These sequences were determined by the dideoxy method (9), using oligonucleotide primers synthesized according to the previously reported F-protein sequence of the Long strain (7). Figure 3 shows parts of the gels in which the Long strain sequence is compared with those of the R/47/4 and R/47/7 escape mutants. A single transversion (A to T) at nucleotide 797 was detected in mutant R/47/4, compared with the Long virus. This nucleotide substitution changes amino acid 262 from asparagine to tyrosine. The same change was also observed in mutant R/47/25 (Fig. 3, right). The other three mutants (R/47/7, R/47/16, and R/47/27) contained a single transversion (A to T) (shown only for R/47/7) in nucleotide 816, which leads to a change from asparagine to isoleucine at amino acid 268 (Fig. 3, right). No other sequence differences with the Long strain were detected in the F segments of the mutant viruses which encode the 20.5-kDa trypsin peptide.

Since antibody 47F reacted in Western blots with both the F1 subunit and proteolytic fragments, it is likely that the antibody-binding site is determined by a linear sequence of contiguous amino acids in which residues 262 and 268 of the F1 subunit play an essential role.

The five escape mutant viruses were independently isolated from a cloned stock of the Long strain (4). However, only two different nucleotide substitutions were identified in the resistant viruses, suggesting that the five mutants originated from two minor subpopulations of the parental virus. None of these changes implied drastic alterations of the F-protein primary structure. In addition, the five mutants grew efficiently in tissue culture, indicating that there were no major functional alterations of the F protein. In spite of these arguments, the epitope 47F is conserved in 12 human (4) and 2 bovine strains tested so far (data not shown). Hence, this epitope offers new, interesting possibilities for the design of synthetic vaccines.

We have previously shown (4) that the mutant viruses had also lost the binding site of another nonneutralizing antibody (49F), which competed reciprocally with 47F in enzyme-linked immunosorbent assays. Antibody 49F immunoprecipitates the F protein from extracts of infected cells but does not react with the F subunits in Western blots. Thus, 49F defines a conformational epitope, partially determined by amino acid residues 262 and 268 of the F1 subunit. It is interesting that the same residues might contribute to the integrity of two epitopes of very different characteristics.

The results shown in Fig. 1B suggest that the amino-terminal third of the F1 subunit is rather resistant to trypsin digestion. Since this segment contains a number of lysine and arginine residues, trypsin inaccessibility is probably due to the three-dimensional conformation of the segment. This F1 segment is confined by the hydrophobic sequence located after the activation site and the cysteine-rich region in the middle of the F1 sequence (Fig. 2). Another highly conserved neutralizing epitope (7C2) (11) has been mapped with synthetic peptides to residues 221 to 232, also contained in the amino-terminal third of the F1 subunit. Thus, the elucidation of the infectious cycle steps inhibited by antibodies such as 47F and 7C2 would be important to understand the functional role of this part of the F protein in respiratory syncytial virus infectivity.

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