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MUTATIONAL ANALYSIS OF FUSION PEPTIDE-LIKE REGIONS IN THE MOUSE HEPATITIS VIRUS STRAIN A59 SPIKE PROTEIN

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1. ABSTRACT

The coronavirus peplomer protein S is responsible for attachment and fusion during viral entry as well as for the induction of cell to cell fusion.While several regions within S have been shown to influence the ability to induce fusion, the region of the protein actually responsible for fusion, the fusion peptide, has not yet been identified. We identified two hydrophobic peptides (peptides #1 and #2) within MHV-A59 S2 as possible fusion domains. This was based on hydrophobicity, conservation among coronavirus S proteins and the prediction of a sided helix conformation. Using site directed mutagenesis and an *in vitro* cell to cell fusion assay we showed that substitution of hydrophobic amino acids with charged amino acids, within the predicted hydrophobic face of either of these two peptides eliminated fusion. Within peptide #1 substitution of the same hydrophobic amino acids with other hydrophobic amino acids or substitution of polar amino acids with charged or polar amino acids had little effect on fusion. Thus peptides #1 and #2 remain likely candidates for the MHV fusion peptide. A third previously identified peptide within S2 (Chambers *et al.,* 1990) is unlikely as a fusion peptide as it is not well conserved among coronaviruses and substitution within the hydrophobic face with charged amino acids does not effect fusion.

2. INTRODUCTION

Studies on viral fusion proteins indicate that their fusion peptides have certain common characteristics: (i) a relatively hydrophobic stretch of 16–26 amino acids which generally shows an amphipathic pattern if modeled as an alpha helix; (ii) they are rich in

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alanine and/or glycine; (3) they are found in a membrane anchored subunit and often near (but not within) the heptad repeat regions; and 4) they are highly conserved within but not between virus families (White, 1992; White, 1990). Fusion peptides can be divided into two groups based on their locations relative to the N-terminus of the membrane anchored subunit: amino terminal and internal fusion peptides. Fusion proteins such as paramyxovirus F proteins, influenza HA proteins, and the envelope protein of several retroviruses have amino terminal fusion peptides (White, 1990). Putative internal fusion peptides have been documented for togaviruses Sindbis and SFV and the retrovirus RSV (White, 1990). Besides having the common features of viral fusion peptides, these internal fusion peptides contain a proline residue in the center and a charged residue on both N- and C-ends. Sequence analysis of coronavirus fusion proteins suggests that they possess internal rather than aminoterminal fusion peptides. The coronavirus fusion peptide is presumed to be within S2 because S2 is highly conserved among coronaviruses and contains the membrane anchor and heptad repeats domains (White, 1992; White, 1990).

A prevalent hypothesis regarding the role of fusion peptides in the mechanism of viral fusion is that these peptides act as sided insertional helices (White, 1992; White, 1990). A typical feature of such helices is that distribution of the bulky hydrophobic residues is asymmetric. The more hydrophobic face of the helix is believed to insert into the membrane with an oblique orientation with respect to the lipid-water interface, thus inducing a local disorder of the bilayer structure.The other face of the helix is hydrophilic and contains polar and charged residues. We have analyzed the sequence of S2 of MHV-A59 and we have identified two conserved peptides that fit the description of fusion peptides as sided helices (White, 1992; White, 1990). These are amino acids 971–989 within heptad repeat #1 and 1098–1114 between the heptad repeat regions (see Figure 1). We are using site directed mutagenesis to analyze the effects of mutation of these peptides on the ability of S to induce fusion.

Mutagenesis studies presented here are consistent with either of these functioning as a fusion domain.We have tentatively ruled out a third peptide (amino acids 911–948), suggested previously to be a candidate fusion domain (Chambers *et al.,* 1990). This region, encoded just upstream of heptad repeat #1, predicted earlier to be a fusion peptide because this domain is less conserved and mutation of this domain does not seem to affect the ability of S to induce cell to cell fusion in an *in vitro* assay.

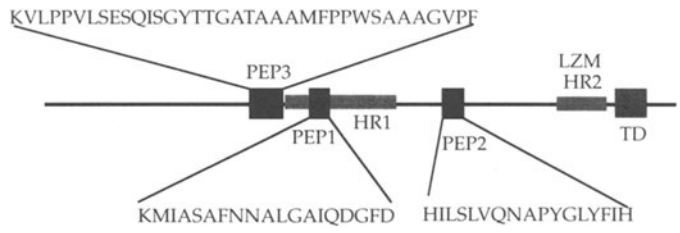

Figure 1. Schematic diagram of the S2 subunit of the MHV-A59 spike protein. Distinctive structural motifs in the S2 subunit are shown as boxes whereas the rest of S2 is shown as a thin line. PEP1, PEP2, PEP3: candidate fusion peptide # 1, 2, 3; HR1, HR2: heptad repeat region 1 and 2; LZM: leucine zipper motif; TD: transmembrane domain. The primary sequence of each candidate fusion peptide is shown.

3. METHODS

3.1. Plasmid Construction and Mutagenesis

For construction of a plasmid from which to express the S protein, the entire wild type MHV-A59 S gene was amplified by RT/ PCR from MHV-A59 intracellular RNA, using a 5' primer containing a SacI site and 3' primer containing a Bam HI site. This DNA was cloned into SacI/BamHI cut pBluescript II KS+ behind a T7 promoter and called pJG10. This plasmid was modified by Dr. Paul Masters to correct a mutation in the cleavage site and to introduce a silent mutation at nucleotide 517 which destroyed an internal HindIII site. The final clone, pINT 2, encodes an S protein with the identical amino acid sequence as our wild type MHV-A59 S (Hingley *et al.,* 1994). For mutagenesis, we followed published procedures (Landt *et al.,* 1990) in which the mutation (either point mutation or deletion) is introduced via a primer and PCR is used to amplify a DNA fragment containing the mutation. The product of the PCR amplification is gel purified, and subcloned into pBS II KS+. The presence of specific mutations in the resulting plasmid clones is verified by DNA sequencing.

3.2. Fusion Assay

We adapted the assay of Nussbaum et al., (1994) in which expression of the E. coli LacZ gene is used as the "readout" to measure S protein induced cell fusion. In this assay a group of DBT cells are infected with vaccinia vTF7–3 (expressing the T7 RNA polymerase), followed by transfection of the wild type (pINT2) or mutant S cDNA clone in the presence of Lipofectin. Another group of DBT cells is transfected also in the presence of Lipofectin with pG1NTβGal, a plasmid that expresses β galactosidase under control of the T7 promoter. After four hours the cells containing the S plasmid and vTF7–3 are removed from the plastic and plated onto a monolayer of the pG1NTβGal transfected cells and incubated overnight.Thus when the cells express fusion competent S, fusion occurs between the two cell types and the LacZ gene is expressed in the syncytia. Cells are fixed with 2% formaldehyde/0.2% glutaraldehyde and incubated in the presence of X-gal. A blue color is observed in the syncytia Fusion can be measured quantitatively by lysing the cells, adding the lysates to a solution of chlorophenol red-β-D-galactopyranoside (CPRG) and measuring the rate of hydrolysis compared to that of purified E. coli β galactosidase, in a microplate absorbance reader. The rate of hydrolysis is then converted to the amount of β galactosidase in ng/well background (after subtraction of the amount of activity for vTF7–3 infection alone) and then expressed as a percentage of wild type.When we quantitate fusion we carry out three independent transfections and assay each of these in duplicate. In Figure 2, the results of the fusion assay are designated: - (<10%); + (10–50%); and ++ (>50%) where wild type is 100%.

4. RESULTS

4.1. Identification of Putative Fusion Peptides

Using the computer program, Clustal, to analyze MHV-A59 S2, we have identified two peptides that fit the description of fusion peptides as they can be modeled as sided helices (White, 1992; White, 1990). The peptides identified are KMIASAFNNALGAIQDGFD (amino acids 971–989) and HILSLVQNAPYGLYFIH (amino acids 1098–1114). (See Figure

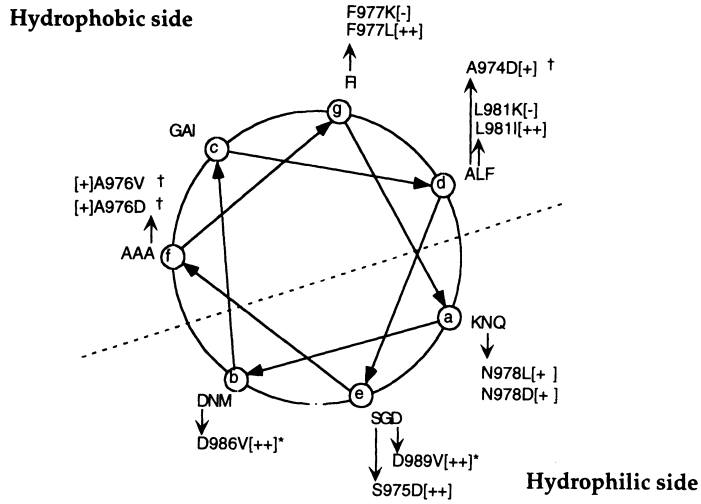

Figure 2. Candidate fusion peptide # 1 shown as a hypothetical α helix. The dotted line indicates the sidedness of the helix. Amino acid substitutions performed inside this region are indicated. The effect of each amino acid substitution on the fusogenic ability, as determined by measuring β-gal activity, is shown in the brackets as percentage of the wild type activity. "++": >50%; "+": 10–50%; "-": <10%; "*"; results shown are referred to the D986V-D989V double mutant; "†": Results shown are referred to the corresponding single mutants. Results for double mutants A974D-A976D and A974D-A976V are [-].

1 for location within S2.) The hydrophobicity indices of the predicted hydrophobic faces of peptides #1 and #2 are 0.91 and 0.96, respectively. These peptides are conserved among coronaviruses, an important criterion for fusion peptides (White, 1992; White, 1990) and nearly identical among several stains of MHV (data not shown). There is another region of S2 (peptide #3, amino acids 911–948, see Figure 1) that was suggested previously to be a candidate fusion domain based on its hydrophobicity and its proximity to the heptad repeats (Chambers *et al.,* 1990). We think this is a less likely candidate for a fusion peptide because it is less conserved than either peptide #1 and #2. It furthermore is not predicted to form a sided helix.

4.2. Effects of Mutagenesis of Putative Fusion Peptide Domains on Cell to Cell Fusion

4.2.1. Fusion Assay. In order to use site directed mutagenesis to map the fusion peptide we needed both a plasmid encoding S and a quantitative *in vitro* assay to measure cell to cell fusion. We constructed a cDNA clone (pINT2) with which to express the S protein under control of the T7 RNA polymerase promoter (see Materials and Methods). Using pINT2, transfected into cells infected with vaccinia virus expressing T7 polymerase (vTF7–3), we confirmed the results of others (Taguchi *et al.,* 1992; Gallagher *et al.,* 1991) that expression of S protein alone is sufficient to induce cell to cell fusion (data not shown). However for the analysis of S mutants, we wanted to establish a more quantitative assay. Thus we adapted the assay of Nussbaum et al., (1994) in which expression of the E. coli LacZ gene is used as the "readout" to measure S protein induced cell fusion. This is described in the Materials and Methods section. Using this assay, fusion mediated by S

can be assayed qualitatively using an *in situ* assay by staining for LacZ activity or quantitatively by measuring the amount of LacZ activity compared to a standard curve for bacterial β-galactosidase all as described in the Materials and Methods.

4.2.2. Mutagenesis of Peptide #1. We analyzed the effects of mutations primarily in peptide #1. Deletion of the entire peptide eliminated cell fusion in this assay. We then introduced charged residues into the predicted hydrophobic face of peptide #1. These results are shown in Figure 2, in which peptide #1 is modeled as a sided helix. Replacement of hydrophobic residues (for example L981K or F977K) reduces fusion to background level. Replacement of the same residues with other hydrophobic residues (L981I or F977L), however, does not reduce the amount of fusion. These results suggest that maintenance of the hydrophobicity in these residues is important for fusogenicity, consistent with the sided helix model. We also introduced mutations into two alanine residues as alanine is a common feature of fusion peptides (White, 1992; White, 1990). While mutation of A976 or A974 alone reduced somewhat the levels of fusion, replacement of both of these residues eliminates fusion completely, suggesting that alanines may be important for fusion.

In general, substitutions within the predicted hydrophilic portion of the helix had less impact on fusion. For example, mutation of the polar residue N978 to either a charged (N879D) or hydrophobic (N879L) residue reduced but did not eliminate fusion and mutation of S975D had little, if any, effect on fusion. The double replacement of charged for hydrophobic residues (D986V/D989V) once again reduced but did not eliminate fusion.

In these initial experiments we only assayed the ability of mutant proteins to induce fusion. For fusion negative proteins, we need to determine whether these proteins are misfolded and never reach the surface of the cell or whether they are present on the cell surface and are defective in the fusion process itself. We have begun to examine the synthesis and processing of these mutant S proteins to determine whether they do, in fact, arrive at the surface of the cell. We have detected full length intracellular proteins for several of the fusion impaired mutants (D986V/D989V, A976D/A974D and A976V/A974D). Preliminary results of fluorescent activated cell sorting (FACS) analysis suggest that A974D, S975D, F977K and A976D/A974D do reach the cell surface at similar levels to wild type S.

4.2.3. Mutagenesis of Peptide #2. We have shown that deletion of peptide #2 eliminates fusion activity. We have introduced substitutions of hydrophobic residues with charged lysine residues in several positions within the predicted hydrophobic face of peptide #2. These substitutions all eliminate fusion. Furthermore substitution of the central proline residue with lysine also eliminates fusion. Thus this peptide remains a candidate fusion peptide.

4.2.4. Mutagenesis of Peptide #3. Mutation of a nonpolar methionine to either leucine of lysine had no effect on fusion. Furthermore mutation of an internal proline to leucine or lysine did not effect fusion. These results along with the fact that this region of S2 is not very conserved among coronaviruses makes it unlikely that this is indeed a fusion peptide.

5. DISCUSSION

Both peptides #1 and #2 are conserved, hydrophobic regions of S that can be modeled into sided helices. Both of these peptides have charged residues at the boundaries, an-

other predicted common feature of internal fusion peptides (White, 1992; White, 1990). We tested the hypothesis for each of these peptides that the hydrophobic residues forming the predicted face are necessary for the induction of cell fusion.

We concentrated on peptide #1. We constructed a group of plasmids to express S proteins with substitution of the nonpolar amino acids predicted to form the hydrophobic face with charged amino acids such as aspartic acid or lysine; the prediction would be that these substitutions would greatly decrease or eliminate fusion. In the case of the HIV fusion peptide one valine to glutamic acid mutation completely eliminated fusion (Pereira *et al.,* 1995). The data in Figure 2 (for example F977K, L981K, A974D/A976D) conform to this prediction. We also mutated amino acids believed to be on the hydrophilic side of the helix; mutations in this region were less detrimental to the induction of fusion. However the substitution of two hydrophobic amino acids for two charged amino acids (D986V/D989V) did substantially reduce fusion. Thus the most drastic effect on fusion was the replacement of bulky hydrophobic residues within the predicted hydrophobic face with charged residues, consistent with the requirement for a hydrophobic face for the induction of fusion. Thus peptide #1 remains a likely candidate fusion peptide.

Peptide #2 has an additional feature thought to be common to internal fusion peptides, that is the central proline residue White, 1990; White 1992). Mutation of this residue or several of the hydrophobic residues on the predicted hydrophobic face of the helix with charged residues eliminates fusion. Thus from the limited analysis we have carried out, peptide #2 conforms to the sided helix model and remains a candidate fusion peptide.

It is not clear, however, that all fusion peptides form sided helices. Indeed, studies on the fusion peptides of PH-30, measles virus, and HIV have suggested that fusion peptides can assume conformations other than alpha-helix when inserted into lipid bilayers and it has been suggested that fusion peptides may be more generally hydrophobic regions (Gallaher *et al.,* 1992). By this criterion, as well, peptides #1 and #2 are likely candidate fusion peptides in that they are the most conserved, hydrophobic regions of S2 other than the transmembrane domain. We have included in our analysis, a third possible fusion peptide (amino acids 911–948), suggested previously to be a candidate fusion domain (Chambers *et al.,* 1990). Mutation of a methionine or central proline residue to either charged or polar residues had no effect on fusion. We will carry out other mutations within this peptide. However, based on our limited analysis and the fact that this region is not highly conserved among coronavirus S protein, this is not a likely fusion domain.

The analysis we have carried out so far examines fusion as an end point. Some of the fusion mutants may well be conformational mutants that never reach the cell surface. We are in the process of distinguishing processing mutants from those defective in fusion. Among those defective in fusion there will be some likely in domains other than the fusion peptide that effect fusion. For example, It has been shown previously that mutations in other domains of S, for example the heptad repeats (Gallagher *et al.,* 1991), hypervariable domain (Gallagher *et al.,* 1990) and the cleavage site (Gombold *et al.,* 1993; Bos *et al.,* 1996) affect cell to cell fusion. Thus once candidate fusion peptide are defined by mutagenesis, they must be assayed in an *in vitro* assay with liposomes (Pereira *et al.,* 1995) to conclusively show that they are involved in the fusion process.

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