Proteolytic cleavage of the murine coronavirus surface glycoprotein is not required for fusion activity

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A cDNA copy of the murine coronavirus [otherwise known as murine hepatitis virus (MHV)] surface (S) glycoprotein gene was isolated and expressed in DBT cells by using a recombinant vaccinia virus system. The expressed S protein induced extensive syncytium formation at neutral pH. Oligonucleotide mutagenesis was used to engineer an S protein gene in which codons for the proteolytic cleavage site, Arg-Arg-Ala-Arg-Arg, were replaced with an equal number of codons for amino acids with aliphatic or aliphatic hydroxyl side-chains. The mutated S protein was stably expressed in DBT cells and, in contrast to the wild-type protein, was not proteolytically cleaved. Nevertheless, the non-cleaved protein induced extensive syncytium formation. These results clearly indicate that the non-cleaved form of the MHV S protein is able to mediate cell membrane fusion. Thus proteolytic cleavage is not an absolute requirement for fusion activity.

Introduction

The coronaviruses are a group of enveloped RNA viruses that infect vertebrates, including man. They are mainly associated with respiratory or gastrointestinal illness (for a review of coronavirus biology, see Spaan et al., 1990). The murine coronaviruses are generically called murine hepatitis virus (MHV), although different MHV strains have quite different organ tropisms and cause a variety of different diseases (Wege et al., 1982; Barthold, 1986). The MHV genome is a positive-strand RNA of about 32 kb (Pachuk et al., 1989; Lee et al., 1991) which encodes four major structural proteins: the nucleocapsid protein N (50000 to 60000 Mr), the membrane protein M (23000 to 26000 Mr), the haemagglutininesterase protein HE (65000 Mr) and the surface protein S (180000 Mr) (Siddell, 1982).

The S protein of MHV has been intensively studied for a number of reasons. First, it is able to mediate attachment of the virus to the cell surface and the fusion of cell and virus membranes (Collins et al., 1982; Vennema et al., 1990a). Recent evidence suggests that these functions also involve a cell surface receptor, a member of the carcinoembryonic antigen family (Dveksler et al., 1991; Williams et al., 1991) and that post-translational modifications of the receptor, possibly glycosylation, are required (Pensiero et al., 1992; see also Schultze et al., 1991; Schultze & Herrler, 1992). Second, the MHV S protein is the major viral immunogen. The majority of protective neutralizing antibodies produced during an MHV infection are S protein-specific (Collins et al., 1982; Wege et al., 1984) and S protein determinants are clearly involved in T cell-mediated immune reactions (Körner et al., 1991; Mobley et al., 1992). Third, the S protein has been shown to carry determinants which are associated with dramatic changes in viral pathogenicity (Dalziel et al., 1986; Wege et al., 1988).

As the name suggests, the MHV S protein forms the projecting spikes or peplomers on the surface of the virus particle. The S protein is synthesized as a co-translationally glycosylated precursor which then undergoes oligomerization and carbohydrate processing. The polypeptide may also be proteolytically cleaved by a host cell enzyme which recognizes a basic sequence, Arg-Arg-Ala-Arg/His-Arg-Ser, located approximately in the middle of the molecule (Luytjes et al., 1987; Schmidt et al., 1987). Thus the mature spike structure consists of two S protein molecules, each of which is a heterodimer composed of two non-covalently bound subunits. The C-terminal S\(_2\) subunit is anchored in the virion membrane and the N-terminal S\(_1\) subunit forms the bulbous head of the projection (de Groot et al., 1987; Vennema et al., 1990b; see also Delmas & Laude, 1990).

The process of coronavirus S protein-mediated membrane fusion is poorly understood. The information which is available generally relates to one of three questions. First, is an exposure to acid pH required to activate fusion? With respect to the initiation of the infection process, it has been shown that MHV infection

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does not depend on functional endocytosis and MHV can mediate fusion from without at neutral pH. The logical conclusion is that the fusion of viral and cell membranes takes place in a non-acidified environment, probably at the plasmalemma (Sturman et al., 1985; Kooci et al., 1991). Similarly, MHV-mediated syncytium formation, i.e. the fusion of cell membranes from within, is not dependent upon exposure to acid pH (Frana et al., 1985; Sturman et al., 1990). The answer to this question, therefore, seems to be no. Interestingly, however, Gallagher et al. (1991) have been able to isolate MHV 4 variants which are entirely dependent upon exposure to acid pH to fuse cell membranes and, furthermore, they have identified the S protein residues responsible for this altered phenotype.

Second, is the fusion process influenced by the cell membrane? Sturman and coworkers (Frana et al., 1985) have found that there are significant differences in the susceptibility of murine cell lines to MHV-mediated fusion from without. Similarly Anderson and coworkers (Mizzen et al., 1983; Daya et al., 1989) conclude that host cell permissiveness to MHV infection correlates with cellular susceptibility to membrane fusion. Also, Kooci et al. (1991), Cervin & Anderson (1991) and Roos et al. (1990) have shown that the susceptibility of cells to MHV-induced fusion can be altered by modification of the membrane lipid composition. These data seem to indicate that the nature of the cell membrane is indeed critical.

Third, is cleavage of the S protein required to activate fusion? The lines of evidence which have been used to argue that this is in fact the case are as follows. First, the addition of exogenous protease to the growth medium can enhance plaque and syncytium formation by some strains of MHV (Yoshikura & Tejima, 1981) as well as other coronavirus (Storz et al., 1981; Payne & Storz, 1988). Second, MHV virions grown in 17Cl-1 cells contain approximately equal amounts of cleaved and uncleaved S protein and are unable to mediate fusion from without. After treatment with trypsin in vitro (which converts the majority of the S protein to the cleaved form), they acquire this ability (Sturman et al., 1985). Third, the addition of protease inhibitors, leupeptin or TPCK, to the medium of MHV-infected cells causes a delay in the onset of cell fusion (Frana et al., 1985). And finally, by analogy to other enveloped viruses, cleavage of viral fusion proteins is generally thought to be necessary to activate their fusion potential (White et al., 1983; McCune et al., 1988; Morrison, 1988; Lobigs & Garoff, 1990).

However, none of these arguments are conclusive. The data on MHV are consistent with the idea that cleavage of the S protein is required for fusion activity but it is equally possible that cleavage only ‘enhances’ the ability of the S protein to fuse membranes. The fact that a number of coronavirus S proteins do not undergo post-translational cleavage and are nevertheless able to initiate infection and induce syncytium formation (Spana et al., 1988; de Groot et al., 1989; Vennema et al., 1990a) prompted us to reinvestigate the relationship of MHV S protein cleavage to fusion. The experiments reported here lead us to the conclusion that proteolytic cleavage is not an absolute requirement for fusion activity.

**Methods**

**Cells and viruses.** HeLa cells (ATCC CCL2), DBT cells (Kumanishi, 1967), Sac(−) cells (Weiland et al., 1978) and thymidine kinase-negative (TK−) 143B cells (ATCC CRL 8303) were grown in monolayers in MEM (041 01385, Gibco) containing 10% heat-inactivated foetal calf serum, glutamine, antibiotics and non-essential amino acids. The MHV strain used in this study is the MHV Wb1 isolate described in Schwarz et al. (1990). Infections of Sac(−) and DBT cells were performed as described by Siddell et al. (1980). Vaccinia virus (WR strain) and the recombinant vaccinia virus vTF7-3 (Fuerst et al., 1986) were plaque-purified twice and grown to stocks of approximately 2 × 10⁸ p.f.u./ml in HeLa cells as described by Mackett et al. (1985).

**Recombinant DNA.** Plasmid purification, agarose gel electrophoresis and standard recombinant DNA procedures were performed as described by Sambrook et al. (1989). Colony hybridizations were done as described by Woods (1984). Oligonucleotides were synthesized using phosphoramidite chemistry on a Cyclone DNA synthesizer (Milligen) and were purified by gel electrophoresis. DNA sequencing was done on single- and double-stranded DNA templates using the dideoxynucleotide chain termination method of Sanger et al. (1977).

**cDNA cloning.** Poly(A)-containing RNA was isolated from MHV-infected Sac(−) cells and cDNA was synthesized essentially by the method of Gubler & Hoffman (1983) using the MHV-specific oligonucleotide 5′ TTC TGT CTT TCC AGG AGA GG 3′ [complementary to positions 3748 to 3767 in Schmidt et al. (1987)] as a first-strand primer. The synthesized double-stranded cDNA was size-fractionated on Sephacryl S1000, treated with T4 DNA polymerase, ligated into Smal-linearized Bluescript pSKII+ DNA and used to transform competent Escherichia coli TG1 cells. Recombinant clones were identified by colony hybridization with two MHV-specific oligonucleotides, the first-strand primer described above and 5′ TAA AGA CGA ACA GCA T 3′ [complementary to positions 31 to 46 in Schmidt et al. (1987)]. The cDNA of one clone, pBS/S+, which gave a positive signal with both oligonucleotides in the colony hybridization was sequenced. The cDNA insert of pBS/S+ was excised with XbaI and HindIII, treated with the Klenow fragment of DNA polymerase I and ligated into the BamHI site of the vaccinia virus transfer vector pTF7-5 (Fuerst et al., 1987). The correct clone, pTF7-5/S+, was identified by colony hybridization, restriction endonuclease digestion and sequence analysis.

**Oligonucleotide-directed mutagenesis.** Mutagenesis was performed with a kit from Amersham (RPN 1523) based upon the method of Nakamaye & Eckstein (1986). Briefly the cDNA insert of pBS/S+ was excised with XbaI and HindIII and ligated to M13mp19 replicative form (RF) DNA which had been cut with the same enzymes. This construct, M13mp19/S+, was used to transform E. coli TG1 cells and single-strand phage DNA was isolated. The oligonucleotide 5′ AGA AAC TGA CGG CGG GCT CGA TGA AT 3′ was annealed to the phage DNA and then extended and ligated in the presence of thiocynucleotide. After removal of ssDNA, the ‘non-
mutated strand of the duplex was nicked with NcoI and partially digested with exonuclease III. The ‘mutated’ strand was then used as a template to reconstruct the closed circular molecule, which was used to transform *E. coli* TG1 cells.

After plaque isolation, RF DNA of the phage M13mp19/Smut was digested with MscI and Sp6I and a 632 bp fragment encompassing the cleavage site mutation was recovered and exchanged for the equivalent DNA fragment in pBS/S\(^{+}\). The cDNA insert of this construct, pBS/Smut, was excised with XhoI and HindIII and cloned into the BamHI site of pTF7-5 as described above. The correct clone, pTF7-5/Smut, was identified by colony hybridization, restriction endonuclease digestion and sequence analysis.

*Isolation of vaccinia virus recombinants.* HeLa cells were infected with vaccinia virus (WR strain) at an m.o.i. of 0.01 p.f.u./cell. The virus-containing medium was removed 2 h after infection and replaced with 10\(\mu\)g of pTF7-5/S\(^{+}\) DNA or 10\(\mu\)g of pTF7-5/Smut DNA coprecipitated with calcium phosphate as described by Sambrook *et al.* (1989). Six hours later the precipitate was replaced with medium and 30 to 40 h later the progeny virus was harvested. Progeny virus was plaque-purified three times on TK-143B cells in the presence of 25\(\mu\)g 5-bromodeoxyuridine (BUDR)/ml. Recombinant vaccinia viruses vTF7-5/S\(^{+}\) and vTF7-5/Smut were identified by indirect immunofluorescence (see below) and stocks with a titre of approximately 2 \(\times\) 10\(^8\) p.f.u./ml were grown in HeLa cells using standard procedures.

*Virus infection and cell lysates.* DBT cells were mock-infected or infected with wild-type or recombinant vaccinia viruses at an m.o.i. of 5 p.f.u./cell. After 12 h, the cells were washed with ice-cold PBS, scraped into PBS and pelleted at 800 \(g\) for 2 min. The cells were then lysed at 4 °C in 50 mM-Tris-HCl pH 7.5, 150 mM-NaCl, 0.2% NP40, 500 units of aprotinin/ml. The cytoplasmic lysates were centrifuged at 10000 \(g\) for 2 min and the supernatants were stored at -70 °C.

*Transient expression.* DBT cell monolayers were infected with the vaccinia virus recombinant vTF7-3 at an m.o.i. of 30 p.f.u./cell. The virus-containing medium was removed 2 h after infection and replaced with 20 \(\mu\)g of pTF7-5/S\(^{+}\) DNA or 20 \(\mu\)g of pTF7-5 DNA coprecipitated with calcium phosphate as described by Sambrook *et al.* (1989). The infected/transfected cells were incubated at 37 °C for 10 h and photographed using a Leitz Laborveit microscope.

*SDS–PAGE and immunoblotting.* SDS–PAGE was done under reducing conditions on 7.5% polyacrylamide gels according to the method of Laemmli (1970). Western blotting was carried out as described by Samson *et al.* (1986). Briefly, blots were stained with undiluted tissue culture supernatant from the MHV S protein-specific hybridoma 11F (Routledge *et al.*, 1991) or the MHV N protein-specific hybridoma, 556 (H. Wege, unpublished results) followed by peroxidase-conjugated rabbit anti-mouse immunoglobulin and the substrate 4-dehydrogenase (36500 M\(_{r}\)). ovalbumin (42700 M\(_{r}\)) and lactate dehydrogenase (55400 M\(_{r}\)).

*Indirect immunofluorescence.* DBT cell monolayers on glass coverslips were infected with recombinant vaccinia viruses at an m.o.i. of 1 p.f.u./cell. After 2 h, the virus-containing medium was replaced with medium or medium containing 0.5% ascites fluid. The ascites fluid contained monoclonal antibody (MAb) 11F (Routledge *et al.*, 1991) or a MAb specific for the MHV Sb protein (Schwarz, 1991). At 14 h post-infection, the cells were washed twice with ice-cold PBS, fixed with acetone–methanol (1:1) at 4 °C for 2 min, washed again with ice-cold PBS and incubated for 16 h at 4 °C with tissue culture supernatant containing a mixture of 12 MAb which recognize linear or discontinuous determinants on the MHV S protein (Wege *et al.*, 1984; Routledge *et al.*, 1991). The cells were then washed extensively at room temperature with PBS and incubated for 2 h with fluorescein-conjugated goat anti-mouse immunoglobulin (115 095 003, Dianova) (0.5% in PBS containing 5% goat serum). After extensive washing with PBS, the coverslips were mounted in 90% glycerol (pH 8.0) and fluorescence micrographs were taken on a Leitz Aristoplan microscope.

**Results**

The cDNA insert of pBS/S\(^{+}\) was sequenced and found to extend from a position 29 nucleotides downstream of the S protein gene to a position 63 nucleotides upstream of the initiation codon. The sequence upstream of the initiation codon corresponds to the MHV leader sequence, showing that the cDNA was copied from mRNA 3 (Skinner & Siddell, 1983; Schmidt *et al.*, 1987). Within the S protein gene coding region, this cDNA differs from the S gene sequence published by Schmidt *et al.* (1987) at positions 793, G → C; 794, C → G (an alanine to arginine substitution at amino acid 255); 1855, A → G (an asparagine to aspartic acid substitution at amino acid 609); and 3281, C → T (an alanine to valine substitution at amino acid 1084).

To demonstrate the fusogenic activity of the S protein encoded by this cDNA, we performed a transient expression assay using a system based on a recombinant vaccinia virus that synthesizes T7 RNA polymerase (Fuerst *et al.*, 1986). As shown in Fig. 1(b), DBT cells which were infected with vTF7-3 and transfected with pTF7.5/S\(^{+}\) DNA showed extensive syncytium formation. In contrast, cells which were infected with vTF7-3 and transfected with pTF7-5 DNA (a) showed no syncytium formation, although cell rounding, and c.p.e. characteristic of vaccinia virus (strain WR) infection at neutral pH (Gong *et al.*, 1990), were evident. This result shows that the S protein gene used in these studies is functional and is able to induce fusion from within in the absence of other viral proteins. Metabolic labelling, immunoprecipitation and MAb analysis indicate that, in the vaccinia virus system, the cDNA-encoded S protein is synthesized, processed and compartmentalized in a manner similar, if not identical, to the authentic S protein expressed in MHV-infected cells (data not shown).

Having confirmed that the recombinant MHV S protein has fusion activity, we addressed the question of whether cleavage at the motif Arg–Arg–Ala–Arg–Arg (amino acids 624 to 629) is necessary to activate this function. To this end, we used oligonucleotide mutagenesis to engineer an S protein gene in which the codons for the cleavage site amino acids were eliminated and replaced by an equal number of codons for amino acids with aliphatic or aliphatic hydroxyl side-chains. Fig. 2(b and c) shows the relevant DNA sequence of the non-mutated (pTF7-5/S\(^{+}\)) and mutated (pTF7-5/Smut) S
genes in the vaccinia virus transfer vectors. These constructs were then used to generate the vaccinia virus recombinants vTF7-5/S+ and vTF7-5/Smut.

To confirm that the mutations we had introduced prevented cleavage but did not have any dramatic effect on, for example, the stability of the expressed S protein we performed a Western blot analysis of the S protein in MHV-infected and recombinant vaccinia virus-infected cells. Fig. 3 shows the results. First, in MHV-infected DBT cells (lane 4) the S protein was present in both cleaved and uncleaved forms. We estimate that in the steady state about 50% of the molecules were present in each form, which is in agreement with the data of Frana et al. (1985). Second, the S protein expressed from the vaccinia virus recombinant vTF7-5/S+ (lane 8) accumulated to approximately the same level as the S protein expressed in MHV-infected cells and undergoes essentially the same degree of proteolytic cleavage. Third, the S protein expressed from the recombinant vTF7-5/Smut was present only in the uncleaved form (lane 7). We have not been able to detect any cleaved form of the S protein in vTF7-5/Smut-infected cells using radioimmunoprecipitation or Western blotting with a variety of MAbs.

The Western blots shown in Fig. 3, lanes 4 to 8, use MAb 11F, which recognizes a determinant located in the S1 region of the protein. Similar results have been obtained with MAb 10G, directed towards an epitope located at the C terminus (i.e. S2 subunit) of the protein (Routledge et al., 1991; data not shown). Also shown in Fig. 3 are a number of controls which demonstrate the specificity of the immunological reactions which were detected. These include Western blotting of lysates from cells infected with vTF7-5/S+ or vTF7-3 alone (lanes 5 and 6) and Western blotting with a MAb specific for the MHV nucleocapsid protein (lanes 1, 2 and 3).
The final series of experiments reported here were aimed to test the fusogenic activity of the S protein expressed from the vTF7-5/Smut recombinant. DBT cells which had been infected with vTF7-3 as well as vTF7-5/S+ or vTF7-5/Smut were examined by indirect immunofluorescence 14 h post-infection. The primary antibodies used were specific for the MHV S protein and detected both linear and discontinuous determinants. Fig. 4(a and b) shows that there was no great difference in the fusogenic activity of the S protein expressed from pTF7-5/S+ (i.e. partially cleaved) and pTF7-5/Smut (i.e. uncleaved). In both infections, approximately the same levels of expression were seen and extensive syncytium formation was evident.

To confirm that the syncytium formation in these cell monolayers was indeed mediated by the expressed MHV S proteins, we added MAb 11F to the culture. This antibody is able to block MHV-induced syncytium formation (Routledge et al., 1991). Fig. 4(c and d) show that this treatment significantly inhibited syncytium formation and essentially only individual S protein-expressing cells were detected. As a control, the same experiment was performed with another MAb directed against the MHV 5b protein and no inhibition of syncytium formation was observed (Fig. 4 e and f). These results clearly indicate that the uncleaved form of the MHV S protein was able to mediate cell membrane fusion.

**Discussion**

The results presented here and those of earlier studies (Frana et al., 1985; Sturman et al., 1985; Routledge et al., 1991; Gallagher et al., 1991; Kooi et al., 1991) indicate that the fusion activity of the coronavirus MHV S protein does not fit easily into the paradigms established for the fusion proteins of other enveloped RNA viruses (Marsh & Helenius, 1989). For example, in contrast to alphaviruses and orthomyxoviruses, the fusion activity of the MHV S protein does not require activation by conformational changes induced by exposure to low pH (Kielian & Helenius, 1985; Steinhauer et al., 1992). Sturman et al. (1990) have postulated that conformational changes are indeed required (possibly involving the rearrangement of thiol bonds) but that they are induced at mildly alkaline pH. In this respect, the MHV S protein may more closely parallel the fusion proteins of lentiviruses and paramyxoviruses (McClure et al., 1988; Morrison, 1988).

The experiments reported here make it clear that proteolytic cleavage of the MHV S protein is not an absolute requirement for the activation of its fusion
Fig. 4. Fusion activity of the wild-type and mutated MHV S proteins. Monolayers of DBT cells were infected with recombinant vaccinia viruses at an m.o.i. of 1 p.f.u./cell. The infected cells were incubated in medium or medium containing 0.5% ascites fluid (MAb 11F or a MAb directed against the MHV 5b protein). Fourteen hours post-infection the cells were fixed and stained with hybridoma tissue.
proteolytic processing ‘enhances’ the fusion activity of heterologous system. It is quite conceivable that the membrane fusion, i.e. fusion from within, and that the S have used analyses only S protein-mediated cell-to-cell suggested recently, they may even be located in other components of the oligomeric spike structure (Paterson et al., 1989; Lobigs & Garoff, 1990). In the case of the MHV S protein, it appears that if such hydrophobic domains exist, and at the present time there is no direct evidence for this, then they are able to function without conformational changes related to proteolytic cleavage.

Although we conclude that proteolytic cleavage of the MHV S protein is not required for its fusion activity, we do not wish to imply that this processing event is without significance. The cleavage motif is well conserved amongst MHV strains (Schmidt et al., 1987; Luytjes et al., 1987) and processing of the S protein precursor occurs in a wide variety of murine cell lines. One possibility, which we consider quite likely, is that proteolytic processing ‘enhances’ the fusion activity of the MHV S protein. The selective advantage of an enhanced fusogenic potential may be manifest in the extensive development of syncytia in vivo and the spread of virus from cell to cell without exposure to the humoral immune response. This interpretation would also be consistent with the data of Yoshikura & Tejima (1981) and Storz et al. (1981) which show that proteases enhance the formation of plaques and syncytia by MHV and bovine coronavirus. In our experiments, there was no obvious indication of a dramatic difference in the fusion activity of the cleaved and uncleaved MHV S proteins. However, the assay we used can only be considered qualitative. Possible differences in the levels of expression, processing and transport of the mutated and non-mutated proteins were not investigated. It would be of interest to develop an assay in which the stoichiometry and kinetics of the fusion reaction were measured accurately.

Finally, it should be remembered that the system we have used analyses only S protein-mediated cell-to-cell membrane fusion, i.e. fusion from within, and that the S protein that we have analysed has been expressed in a heterologous system. It is quite conceivable that the fusion event necessary to initiate an infection, i.e. virus-to-cell membrane fusion, is quite different with respect to the local concentration and topography of the S protein. Recently, it has been shown that human immuno-
deficiency virus type 2 that has been manipulated such that it is severely compromised for syncytium formation nevertheless exhibits only slightly lower levels of infectivity (Steffy et al., 1992). The idea that the ‘fusogenicity’ of the S protein, with or without cleavage, may play a role in determining the accessibility of different cell types to MHV infection and thus be a factor governing tropism and pathogenesis is obviously worthy of further investigation. In our opinion, however, answers to questions such as these require the development of an infectious MHV cDNA clone or a system of targeted recombination (see Koetzner et al., 1992). With such a system, it would be possible to introduce specific changes into the MHV genome and analyse their effect on the virus phenotype in vitro and in vivo.

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References


Franke, M. F., Behnke, J. N., Stirman, L. S. & Holmes, K. V. (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: culture supernatant (a mixture of 12 S protein-specific MAbs) and fluorescein-conjugated goat anti-mouse immunoglobulin. (a) vTF7-5/S-infected, medium; (b) vTF7-5/Smut-infected, medium; (c) vTF7-5/Smut-infected, medium with MAb 11F; (d) vTF7-5/Smut-infected, medium with MAb 5b; (e) vTF7-5/Smut-infected, medium with MAb 5b.
host dependent differences in proteolytic cleavage and cell fusion.


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