Two functionally linked amino acids in the stem 2 region of measles virus haemagglutinin determine infectivity and virulence in the rodent central nervous system


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INTRODUCTION

Measles virus (MV) strain CAM/RB, adapted to growth in brains of newborn rodents, is highly neurovirulent, whereas other MV strains, such as the laboratory strain Edmonston and a variant of CAM adapted to growth in cell culture (CAM/Vero), are not neurovirulent (Moeller et al., 2001). To investigate the molecular basis of neurovirulence, we have previously created recombinant MVs bearing the haemagglutinin (H) gene of the rodent-adapted strain CAM/RB and found that, in newborn mice, the H gene alone can determine whether the virus is neurovirulent or not (Duprex et al., 1999b). The viral attachment H protein interacts with cellular receptors mediating virus uptake and spread from cell to cell, and also influences budding and virus release. Distinct amino acid exchanges in the H protein responsible for antibody escape and neurovirulence have been characterized in Lewis rats (Moeller et al., 2001). We have demonstrated that the amino acids associated with antibody escape are not identical to those mediating neurovirulence, as had been suggested previously (Liebert et al., 1994). Most epitopes interacting with neutralizing antibodies are located in the globular head of the H protein, whereas the two amino acids associated with neurovirulence are situated in the stem 2 region of the H protein at positions 195 (Gly) and 200 (Ser). The surface of this area is proposed to be parallel to the vertical axis of the H molecule and could affect the overall structure of the H protein or interactions with molecules inserted in the same membrane, such as the F protein (Langedijk et al., 1997).

The biological consequences of the mutation at position 195 (Gly→Arg) are unknown, although from a biochemical perspective, the change introduces a positive charge. The change at position 200 (Ser→Asn) introduces a potential glycosylation site into the H protein. Here, we investigate the functional consequences of alterations at
these positions in the H protein, both individually and in combination, on virus infectivity and neurovirulence.

**METHODS**

**Antibodies and cells.** The mouse monoclonal anti-MV nucleocapsid (N) protein antibody (mAb F227; Baczko et al., 1986) and anti-MV H antibody (mAb L77; ter Meulen et al., 1981) were produced from hybridomas using RPMI 1640 medium containing 10 % (v/v) fetal calf serum (FCS) and purified over protein G-Sepharose. As secondary antibodies for immunohistological analyses, we used biotinylated rabbit anti-mouse serum (DAKO). The rabbit polyclonal anti-MV-H serum, recognizing the conserved peptide NH2-SPQRDRINAFYKDN(C)-COOH of the cytoplasmic domain (Buchholz et al., 1996), was produced by Eurogentec. Activated caspase-3 was detected by using a rabbit polyclonal antibody against cleaved caspase-3 (New England Biolabs; dilution 1:100). We used biotinylated or horseradish peroxidase (HRP)-conjugated swine anti-rabbit secondary antibodies (DAKO).

Primary mixed brain cells, containing neurons and glial cells, were prepared from whole brains of newborn Lewis rats. Brains were washed with ice-cold Hanks’ balanced salt solution (HBSS; Biochrom) and meninges and visible blood vessels were removed mechanically. Left and right hemispheres and cerebellums were pressed through a metal grid to dissociate the tissue. Cells were washed by centrifugation at 200 g with HBSS and seeded into Dulbecco’s modified Eagle’s medium containing 10 % (v/v) FCS on poly-l-lysine (Sigma)-coated eight-chamber slides (LabTekII; Nunc). Human astroglia U251 and U373, glioblastoma D54 (Bigné et al., 1981) and neuroblastoma IMR-32 cell lines (ATCC), murine astrocytoma delayed brain tumour (DBT) cells and rat glioblastoma G6 (ATCC) and pheochromocytoma PC-12 (ATCC) cells were cultivated in minimum essential medium (MEM) containing 10 % (v/v) FCS. African green monkey kidney (Vero) cells were cultured in MEM containing 5 % (v/v) FCS.

**MV strains and recombinants.** MV CAM/RB was passaged by MV strains and recombinants. A panel of H protein mutants was assembled in a eukaryotic expression backbone to allow the functionality of the protein to be assessed in transfected cells.

**Cloning and rescue of MVs expressing H mutations.** Full-length, mutated constructs were assembled in an H gene insertion vector (pMVins-H2), the construction of which has been described previously (Duprex et al., 1999b). Briefly, this vector contains two unique restriction sites (Pad and AatII) that permit the directional cloning of complete H genes obtained either by PCR amplification using H-specific, Pad- or AatII-containing oligonucleotides uniH+ and uniH2− or directly as DNA fragments isolated from pCG-based eukaryotic expression clones used to transiently express H proteins. This vector was originally used to generate plasmid pJMVCAMH, which contains the H gene of CAM/RB in the Edmonston background. The pMVins-H2 plasmid was used to construct a set of full-length MV plasmids containing nucleotide changes at positions 603 (A→G) and 619 (A→G), corresponding to amino acid changes at positions 195 (Arg→Gly) and 200 (Asn→Ser), respectively. The sequences of the resulting plasmids were confirmed as described above and recombinant viruses were rescued from these constructs following transfection of MVA-T7-infected HeLa cells mediated by Lipofectamine 2000 (Invitrogen) as described previously (Duprex et al., 1999b).

**Co-transfection of H and F expression vectors and cell fusion assay.** Recombinant H genes were subcloned into the pCG vector and co-transfected with pCG-MV-F1 expressing the fusion gene of MV Edmonston strain (a gift from Dr R. Cattaneo, Mayo Clinic College of Medicine, Rochester, NY, USA) in Vero cells using Lipofectamine 2000 (Invitrogen). Phase-contrast photomicrographs were taken 48 h after transfection, and the mean number of nuclei in syncytia was determined by counting at least 10 random fields of syncytia.

**Glycosylation analysis.** Vero cells were infected with MVs at an m.o.i. of 0.01. Six hours p.i., the medium was changed and substituted with 1 mM 1-deoxymannojirimycin (DMJ; Calbiochem). Infected cells were grown in the presence of DMJ until syncytia were observed, typically 3 days p.i. Cells in PBS were lysed by a single freeze–thaw at −70 °C, and cell debris was pelleted by centrifugation. Cell lysates (50 μl) containing 20 μg protein were incubated with 20 μl endoglycosidase H (20 μl EndoH; Sigma) for 2 h at 37 °C. Probes were mixed with Laemmlı buffer for SDS-PAGE, and separated by using 10 % polyacrylamide gels. Proteins were semidry-blotted onto nitrocellulose filters. Western blots were blocked with 5 % (v/v) dried milk in PBS containing 0.05 % (v/v) Tween 20 and incubated with polyclonal anti-H serum (1:1000) and HRP-conjugated secondary antibodies (1:2000; Immunotech). Bands were visualized by using the ECL system (Amersham Biosciences).

**Animal infection and histology.** Timed pregnant Lewis rats were purchased from Harlan–Winkelmann. One- to two-day-old pups were infected i.c. in the left hemisphere with 2×106 p.f.u. recombinant viruses, or 2×105 p.f.u. of the more virulent CAM/RB). The body masses of animals were measured at a number of time points p.i. Animals were infected for no longer than 7 days, anaesthetized and sacrificed by decapitation. The brains were removed and fixed in 4 % (w/v) paraformaldehyde in PBS without Ca2+ and Mg2+, pH 7.4, for at least 2 days before frontal brain

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Two amino acids in MV-H determine neurovirulence
sections were embedded in paraffin. Tissue sections were routinely stained with haematoxylin and eosin (H&E; Sigma) and Luxol fast blue.

Immunohistology for detection of MV nucleocapsids was performed by using mAb F227 (2 μg ml\(^{-1}\)). In brief, slides were rehydrated and pretreated for 10 min in a microwave oven (600 W) in 10 mM sodium citrate, pH 6.0. Incubations with 1% (v/v) hydrogen peroxide to block endogenous peroxidase and with normal swine serum to block non-specific binding sites were performed for 15 min each. mAb F227 was applied overnight at 4 °C. Antibody binding was visualized with biotinylated secondary antibody (rabbit anti-mouse), streptavidin and biotinylated HRP complex (StreptABComplex/HRP; Dako) and diaminobenzidine (DAB; Fluka) as the chromogen. Sections were counterstained with Mayer’s haematoxylin.

To analyse apoptosis, cells displaying (i) typical nuclear morphology of apoptosis with intense, uniform nuclear basophilia, chromatin condensation with nuclear shrinkage (pyknosis) or fragmentation of the nucleus into several rounded and uniformly dense basophilic masses (karyorrhexis), and/or (ii) cytoplasmic immunoreactivity for activated caspase-3, the final executioner in the apoptotic cascade, were considered to be apoptotic (Edwards et al., 1997; Nakajima et al., 2000). Activated caspase-3 was detected by using a 1 : 100 dilution of rabbit polyclonal antibody against cleaved caspase-3 (New England Biolabs). The immunostaining procedure for the cleaved caspase-3 antibody was identical to that described above for mAb F227, except that a biotinylated swine anti-rabbit antibody was applied as the secondary antibody. Immunolabelling for F227 and caspase-3 was performed on serial sections.

**RESULTS**

**Functional analysis of mutated H proteins**

Critical amino acids in the CAM-H protein were found at positions 195 and 200, with the combination Gly/Ser being neurovirulent and Arg/Asn non-virulent. We prepared a variety of eukaryotic H protein expression plasmids with different amino acid combinations at these positions and assessed their capacity in co-transfection assays with MV-F expression plasmids to induce cell–cell fusion (Table 1). The original combinations Gly/Ser and Arg/Asn (as found in CAM/RB and CAM/Vero, respectively) both induced massive areas of fusion in cultures of Vero cells, whereas the combinations Arg/Ser and Gly/Asn induced small syncytia, and Gly/Ala, Gly/Arg, Gly/Glu, Gly/Thr and Gly/Tyr showed expression only in single cells and thus were functionally restricted (Table 1). In order to investigate the contribution of the single amino acid exchanges to neurovirulence, we decided to rescue two additional recombinant viruses containing the amino acid combinations that were able to induce syncytium formation with Arg/Ser and Gly/Asn at positions 195 and 200. The amino acid combinations with restricted capacity to induce syncytia were not used for further experiments.

**Rescue and growth analysis of the MV recombinants MV-14 and MV-15**

One recombinant virus based on EdtagCAMH with a Gly→Arg exchange at position 195 (nucleotide exchange G→A at position 603) was named EdtagCAMH-14 (henceforth referred to as MV-14), and one with a Ser→Asn exchange at position 200 (nucleotide exchange G→A at position 619) was named EdtagCAMH-15 (referred to as MV-15; Table 1; Fig. 1a). Sequencing of RT-PCR products of the complete H genes generated from mRNA of Vero cells infected with rescued viruses confirmed that the mutations were present in the viral genomes.

Both recombinant viruses MV-14 and MV-15 replicated well in Vero cells. Cell-free and cell-bound viruses were titrated separately (Fig. 1b and c, respectively). Interestingly, MV-15 released approximately 10–50% fewer particles than MV-14.

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**Table 1. Nucleotide sequences and combinations of amino acids at positions 195 and 200 in the H protein, and cell-to-cell fusion induced by co-transfection of H and F expression plasmids in Vero cells**

<table>
<thead>
<tr>
<th>Nucleotides at position*</th>
<th>Amino acid at position</th>
<th>Extent of cell fusion†</th>
<th>Corresponding recombinant viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td>603–605</td>
<td>618–620</td>
<td>195</td>
<td>200</td>
</tr>
<tr>
<td>GGA AGC</td>
<td>Gly</td>
<td>Ser</td>
<td>+ + + +</td>
</tr>
<tr>
<td>AGA AAC</td>
<td>Arg</td>
<td>Asn</td>
<td>+ + + +</td>
</tr>
<tr>
<td>AGA AGC</td>
<td>Arg</td>
<td>Ser</td>
<td>+ +</td>
</tr>
<tr>
<td>GGA AAC</td>
<td>Gly</td>
<td>Asn</td>
<td>+</td>
</tr>
<tr>
<td>GGA GCC</td>
<td>Gly</td>
<td>Ala</td>
<td>+</td>
</tr>
<tr>
<td>GGA CGC</td>
<td>Gly</td>
<td>Arg</td>
<td>+</td>
</tr>
<tr>
<td>GGA GAA</td>
<td>Gly</td>
<td>Glu</td>
<td>+</td>
</tr>
<tr>
<td>GGA ACC</td>
<td>Gly</td>
<td>Thr</td>
<td>+</td>
</tr>
<tr>
<td>GGA TAC</td>
<td>Gly</td>
<td>Tyr</td>
<td>+</td>
</tr>
</tbody>
</table>

*Mutagenesis was based on the CAM/RB-H sequence with Gly and Ser at positions 195 and 200.
†Cell fusion and expression of the H protein were analysed microscopically by immunofluorescence staining with mAb L77 to MV-H and propidium iodide staining of the nuclei. Ranges of the number of nuclei in syncytia are indicated as follows: +, 3–10; ++, 11–40; + + + +, >80.
infectious virus particles than MV-14 and the recombinants EdtagCAMH and EdtagCAMH-6 (MV-6). Such differences were not observed with cell-bound infectious particles, which were produced at similar titres.

**Glycosylation analysis of recombinant MVs**

In order to investigate whether the potential glycosylation site at position 200 was actually used (Fig. 1a), we compared the molecular masses of the H proteins of the recombinants and natural MVs. Vero cells were infected, lysates were prepared and the molecular masses of the H proteins were determined from Western blots (Fig. 2). The H proteins of the neurovirulent viruses EdtagCAMH and CAM/RB (Fig. 2a, lanes 2 and 6) expressing Ser at position 200 had significantly lower molecular masses than the H proteins of the non-neurovirulent viruses Edtag, EdtagCAMH-6 and CAM/Vero (Fig. 2a, lanes 1, 3, and 7) with Asn at position 200 (approx. 80 kDa). The differences in molecular mass correspond to the loss of one N-linked carbohydrate moiety. Recombinant MV-14 (lane 4) encoded an H protein with the same apparent molecular mass as that of the neurovirulent EdtagCAMH parent virus, whereas MV-15 (lane 5) had an apparently larger H protein due to the introduction of Asn at position 200. When the cells were treated with DMJ, the differences were more pronounced due to the better-focused bands (Fig. 2b). Additional EndoH treatment reduced the apparent molecular masses of neurovirulent and non-neurovirulent H proteins to the same size (Fig. 2c and d, lanes 3 and 6), indicating that the differences in the molecular masses were associated with differences in glycosylation at position 200 and not with amino acid alterations in the recombinants. Thus, MV-14 is not glycosylated at position 200, and the consequence of the single mutation at position 200 (Ser → Asn) in MV-15 is gain of an actually used glycosylation site.

**Determination of the neurovirulence of MV-14 and MV-15**

Knowing that non-neurovirulent viruses have (in the context of other mutations) an additional glycosylation site at amino acid position 200 that is not present in neurovirulent MV strains, we hypothesized that this glycosylation at residue 200 may mask a neurovirulence determinant. We therefore analysed the neurovirulence of the single amino acid exchange mutants at position 195 and 200, recombinants MV-14 and MV-15. Whilst the parental recombinant expressing the CAMH protein (EdtagCAMH) induced symptoms typical of the acute encephalitis and death 3–5 days after infection, surprisingly, both new recombinants, MV-14 and MV-15, were non-neurovirulent. Animals infected with MV-14 and MV-15 gained body mass similarly to uninfected animals or animals infected with the non-neurovirulent mutant MV-6 (Fig. 3). In addition, the brains of infected animals (n=3) were analysed at days 3, 5 and 7 by RT-PCR using P-gene-specific primers (Barrett et al., 1993) and showed no sign of virus replication in the cases of MV-14 and MV-15 and other non-neurovirulent viruses, whereas virulent viruses produced high levels of detectable mRNAs at all three time points (results not shown). Therefore, introduction of a glycosylation site is only one possibility for abolishing neurovirulence, and the H protein of MV-14
without glycosylation, but with an altered amino acid at position 195, is also non-neurovirulent. Thus, neurovirulence is associated only with H proteins bearing a certain combination of amino acids, Gly/Ser at positions 195 and 200, respectively, and both single amino acid changes, i.e. Gly→Arg or Ser→Asn, abolished neurovirulence (Table 2).

Histological analysis

To demonstrate the basis of neurovirulence, we investigated the distribution of viral nucleocapsid (N) protein in infected brains, and whether viral infection is able to induce apoptosis. In brains infected with the neurovirulent virus EdtagCAMH (Fig. 4a), cortical and infracortical cell death occurred in the left and also frequently in the right hemisphere, revealing numerous pycnotic nuclei and a perifocal oedema. MV-N-positive neurons were detected in the cortex and infracortical layers of the left and right hemispheres. Thus, in animals infected with the neurovirulent virus, numerous cells exhibited an apoptotic-like morphology. Furthermore, many cells in these brains expressed the apoptotic marker cleaved caspase-3. The vast majority of apoptotic cells were localized in the same regions as N-positive cells, indicating a correlation of virus spread and induction of apoptosis (Fig. 4a). In mock-infected controls, only a few, scattered caspase-3-positive cells were observed in both hemispheres (data not shown).

In contrast, in animals infected with the non-neurovirulent recombinants, no N-positive neurons were observed in the brain 3, 5 or 7 days p.i. In these brains, rare caspase-3-positive cells were distributed similarly to those found in uninfected brains of newborn rats (Nakajima et al., 2000) (results not shown). Both MV-14 and MV-15 produced the same inconspicuous histomorphology as was found for other non-neurovirulent mutants, with no detectable virus-positive cells (Fig. 4b). Thus, analysis of the brains revealed a close correlation between the neurovirulence of certain MV recombinants, numbers of infected of neurons and induced neuronal apoptosis.

Infection of primary mixed brain-cell cultures with recombinant MVs reflects their neurovirulence

In order to assess the infectivity of neural cells with the MV recombinants, various human and rat neural cell lines (U251, U373, IMR, D54, DBT, C6 and PC-12) and primary mixed brain-cell cultures from newborn Lewis rats were infected and the cytopathogenic effects were observed by microscopy. All CD46-positive human cell lines were infected about as well as Vero cells, whereas the rodent cell lines DBT, C6 and PC-12 could not be infected with the MV strains and recombinants used in this work (as
Two amino acids in MV-H determine neurovirulence

Table 2. Amino acid exchanges and neurovirulence of recombinant MVs

The recombinants Etag, EtagCAMH and MV-6 and the natural variants CAM/RB and CAM/Vero have been described previously (Moeller et al., 2001).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Additional mutations in the H gene*</th>
<th>Amino acid at position</th>
<th>Neurovirulent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>195</td>
<td>200</td>
</tr>
<tr>
<td>Etag</td>
<td>Several</td>
<td>Arg</td>
<td>Asn</td>
</tr>
<tr>
<td>EtagCAMH</td>
<td>No</td>
<td>Gly</td>
<td>Ser</td>
</tr>
<tr>
<td>MV-6</td>
<td>No</td>
<td>Arg</td>
<td>Asn</td>
</tr>
<tr>
<td>MV-14</td>
<td>No</td>
<td>Arg</td>
<td>Ser</td>
</tr>
<tr>
<td>MV-15</td>
<td>No</td>
<td>Gly</td>
<td>Asn</td>
</tr>
<tr>
<td>CAM/RB</td>
<td>No</td>
<td>Gly</td>
<td>Ser</td>
</tr>
<tr>
<td>CAM/Vero</td>
<td>No</td>
<td>Arg</td>
<td>Asn</td>
</tr>
</tbody>
</table>

*In comparison to the CAM-H gene.

determined by immunofluorescence; results not shown). In contrast, the primary rat brain-cell cultures containing neurons and glial cells could be infected with the neurovirulent viruses EtagCAMH and MV-1, as described previously (Moeller et al., 2001), whereas non-neurovirulent viruses MV-14 and MV-15 did not infect these primary brain cells, reflecting the in vivo effect in tissue culture (Fig. 5a–e). Control cultures and cultures treated with non-neurovirulent MV strains contained smooth, round, healthy neurons with long processes attached to the surface of glial cells (Fig. 5h, i). In contrast, a cytopathic effect was observed in the cultures infected with neurovirulent recombinants, with cells showing a shrivelled morphology and large cellular aggregates, loss of the processes and several giant cell ‘bubbles’ (Fig. 5a, b, f, g). These results indicate that only H proteins with the amino acids Gly/Ser at positions 195 and 200, as present in the neurovirulent MV variants, are able to mediate the infection of primary rodent neurons.

Fig. 4. Expression of MV nucleocapsids and activated caspase-3 in rat brains 5 days post-i.c. infection. Schematic drawings of frontal brain sections illustrate extent and distribution of MV-N-positive (mAb F227, red) and cleaved caspase-3-positive cells (caspase-3, blue) in animals infected with EtagCAMH (a) or with non-neurovirulent recombinants MV-14 and MV-15 (b). The frame in each schematic drawing indicates the cortical region shown in the figures immunolabelled for F227 and caspase-3 (low magnifications). (a) In animals infected with EtagCAMH, numerous F227-positive cells (brown) were detected in both hemispheres, predominantly in the cortex of the left hemisphere. In these animals, a strong increase in the numbers of apoptotic-like cells occurred, as detected by apoptotic-like nuclear morphology (H&E) and caspase-3 immunoreactivity (brown), as indicated. (b) Infection with the recombinant strains MV-14 (left panels) and MV-15 (right panels) did not lead to detection of F227-positive cells (n=5).
Here, we demonstrated that two amino acids in stem 2 of the viral H protein, Gly and Ser at positions 195 and 200, respectively, are required in combination to allow infection, virus spread and pathogenesis in the brains of newborn Lewis rats. Glycosylation of Asn at position 200, or introduction of the positively charged amino acid Arg at position 195 alone, abolished neurovirulence and apoptosis. As the in vivo findings are reflected by the capability of the neuroviral viruses to infect primary mixed brain-cell cultures, the immune system obviously does not play a decisive role in the described differential properties of the MV recombinants. In Vero cells, which are CD46-positive, the neuroviral and non-neuroviral recombinants replicate similarly well, whereas in CD46-negative rodent primary neurons, the amino acids at positions 195 and 200 determine the capacity of the H protein to mediate infection. It is therefore likely that the receptor-mediated attachment and fusion helper function of the H protein is the basis of successful or unsuccessful infection of rodent neurons.

Interestingly, the two key amino acids in stem 2 (Fig. 6) are not located in the globular head of the H protein, which is known to interact with the cellular receptors CD46 or CD150 (Dörig et al., 1993; Naniche et al., 1993; Tatsuo et al., 2000; Erlenhofer et al., 2001; Hsu et al., 2001; Masse et al., 2004; Vongpunsawad et al., 2004). Thus, it appears likely that these two amino acids influence the conformation of the complete H protein. The surface of the stem 2 area of the H protein has been proposed to be parallel to the vertical axis of the H molecule and could affect interactions with molecules in the same membrane as H (Langedijk et al., 1997). This may affect the interaction with cellular receptors on mouse neurons and/or homotypic interactions with other H proteins (tetramers) or interactions with the F protein (trimers), which are required for the fusion process (Plemper et al., 2000). It is not clear what structural consequences the Gly→Arg mutation at position 195 may have, except that it potentially introduces a new positive charge on the surface.

**Fig. 5.** Infection of rat primary brain cells with MV recombinants. Rat mixed brain-cell cultures were infected at an m.o.i. of 0.5 for 48 h with EdtagCAMH (a), MV-1 (b), MV-14 (c) or MV-15 (d) or left uninfected (e), and were stained with MV-N-specific and fluorescein isothiocyanate-conjugated secondary antibodies (×40 objective). Phase-contrast micrographs (×25 objective) are presented of cells infected with EdtagCAMH (f), MV-1 (g), MV-14 (h) and MV-15 (i) to illustrate the effects of these strains on cell morphology.

**Fig. 6.** Localization of aa 195 and 200 in the structural model of MV-CAM-H [with alterations according to Langedijk et al. (1997)].
of the non-neurovirulent molecule, and how they may correspond to the Ser→Asn mutation at position 200, which generates an additional glycosylation site. Alterations in H protein structure may mask novel receptor-binding sites, leading to less of the capacity to infect neurons and to spread in the brain. The cooperativity of the two amino acids Gly and Ser required at these positions in order to gain neurovirulence suggests that a certain structure is necessary to interact with host receptors in the brains of newborn rodents. From using Vero cells with different receptors, these structural alterations obviously have no effect on virus replication. The hypothesis that a receptor-mediated mechanism is affected by the mutations at positions 195 and/or 200 is further supported by the observation that, when the MV receptor CD46 is provided on the surface of neurons in neuron-specific enolase-CD46 transgenic mice, MV-strain Edmonston is also neurovirulent (Rall et al., 1997). Thus, providing the proper receptor for Edmonston on neurons had the same functional consequence as mutation of aa 195 and 200 to Gly and Ser in the H protein.

Little is known about mechanisms of virus spread in the brain. Two apparently contradictory phenomena in the brains of rodents or neuronal cell cultures have been described by several authors: cell-to-cell spread without the requirement for receptors, and requirement for a receptor in order to enable brain infection (Meissner & Koschel, 1995; Allen et al., 1996; Rall et al., 1997; Urbanska et al., 1997; McQuaid et al., 1998; Mrkic et al., 1998; Duprex et al., 1999a, 2000; Evlashev et al., 2000; Lawrence et al., 2000; Ehrengruber et al., 2002). These findings are not necessarily contradictory. Results obtained with transgenic animals and our data presented in this work support the view that a certain structure of the H protein is required for the initial receptor-mediated infection of neurons. The subsequent cell-to-cell spread of virus in the brain may not require the presence of these receptors (Makhortova et al., 2007).

Analysis of virus growth in tissue culture may provide pointers for the functional consequences of the mutations. Interestingly, MV-15-infected Vero cells produced less cell-free virus than was found in the supernatants of Vero cells infected with the other recombinants, whereas the infectivities of the viruses were similar when cell-bound viruses were titrated. This indicates that the amino acid combination Gly/Asn at positions 195 and 200 results in impaired virus release, which is compensated by exchange of either one of the amino acids leading to the combinations Arg/Asn or Gly/Ser, which are both fully functional in virus release. Because titres of cell-bound viruses are not affected by these mutations, it is likely that protein synthesis, folding and processing are not disturbed. It remains to be demonstrated which step(s) of virus assembly or budding impair virus release of MV-15.

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