Recombinant measles virus requiring an exogenous protease for activation of infectivity

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Proteolytic cleavage of the fusion protein (F) is an important control mechanism of the biological activity of paramyxoviruses. The sequence R-R-H-K-R(112) at the cleavage site of the F protein of measles virus (MV) was altered by site-directed mutagenesis to R-N-H-N-R(112), which is not recognized by the ubiquitous cellular protease furin. When transiently expressed in cell cultures standard F protein was cleaved, whereas the mutant remained in the uncleaved form. Syncytium formation by the mutant was analysed after coexpression with haemagglutinin protein dependent on the presence of trypsin. Recombinant MV containing the mutation required trypsin activation for fusion and infectivity in cell culture. Intranasal infection of transgenic mice susceptible to MV infection (Ifnar<sup>−/−</sup>-CD46<sup>Ge</sup>) resulted in a moderately productive infection and inflammation of the lung. In contrast to parental virus, intracerebral inoculation did not induce neural disease. The possible effects of the change in cleavage activation on tissue tropism and pathogenicity are discussed.

Introduction

Measles virus (MV) is one of the most contagious human pathogens. Vaccination with attenuated live virus has greatly reduced the number of cases, but measles is still a major cause of serious disease and infant mortality in developing countries due to low vaccine coverage and the inability to vaccinate infants in the first 6 months of life. The virus is transmitted by the respiratory route. Entry and initial replication occur in the conjunctiva and the respiratory mucosa. Infection and inflammation of the lower respiratory tract and the lung follow. Viraemia and systemic infection inevitably occur before host defence mechanisms control virus replication and clear infected cells. Recently, a genetically modified mouse was established to study MV spread in an animal model. Mice expressing human CD46 and lacking the interferon receptor type I were shown to be productively infected after intranasal and intracerebral inoculation (Mrkic <i>et al.</i>, 1998).

MV is the prototype member of the morbillivirus genus in the <i>Paramyxoviridae</i> family of negative-stranded RNA viruses. Virions have an envelope with two virus-encoded integral membrane glycoproteins, the viral attachment protein haemagglutinin (H) and the fusion protein (F), which form spike-like projections on the outer surface. The H protein is responsible for binding to cellular receptors, such as CD46 (Dürg <i>et al.</i>, 1993; Naniche <i>et al.</i>, 1993; Schneider-Schaulies <i>et al.</i>, 1995), and is essential as a cofactor for fusion (Wild <i>et al.</i>, 1991). The F protein is synthesized as an inactive precursor molecule F<sub>0</sub>, which is cleaved intracellularly by host proteases to generate two polypeptide subunits, F<sub>1</sub> and F<sub>2</sub>, held together by disulfide bonds. Infected cells exposing cleaved F protein on the surface fuse with adjacent cells at neutral pH, thereby causing syncytium formation. The multibasic cleavage site at which the F protein of MV is activated consists of five basic amino acids, R-R-H-K-R, at positions 108–112. Correct proteolytic cleavage after arginine 112 is essential, because changing this residue to leucine was shown to result in aberrant cleavage and loss of fusion ability (Alkathib <i>et al.</i>, 1994).

The major cellular protease responsible for correct cleavage of the F<sub>0</sub> precursor protein is furin, a subtilisin-like endo-
protease in the trans-Golgi network (Watanabe et al., 1995; Bolt & Pedersen, 1998). Furin has also been shown to be responsible for the cleavage of several other viral glycoproteins and, as a ubiquitous protease, is an important determinant for the systemic infection caused by these viruses. Viruses encoding F proteins with monobasic cleavage sites are activated by proteases restricted to specific tissues. These viruses therefore cause only local infection (for a review, see Klenk & Garten, 1994). Except for the F proteins of Sendai virus and apathogenic Newcastle disease virus (NDV), the F proteins of all other paramyxoviruses have multibasic cleavage sites and can be activated ubiquitously. For most paramyxoviruses, including all morbilliviruses, natural or cell culture-derived avirulent isolates with monobasic cleavage sites have not been found. Nevertheless, we tried to generate an MV which is no longer activated by ubiquitous intracellular proteases but depends upon extracellular F protein cleavage. For this purpose, mutations were introduced into the cleavage site, rendering the F protein of the Edmonston strain insensitive to furin. In contrast to standard F protein, intracellular cleavage of transiently expressed mutant F protein did not occur and syncytium formation after coexpression with H protein completely depended on the addition of trypsin to the medium, indicating that MV F protein with a monobasic cleavage site can be activated by exogenous proteases. We then used a genetic approach (Radecke et al., 1995) to analyse the effect of the F mutation on the biological properties of recombinant MV. We generated a recombinant virus which required trypsin to become infectious. In genetically modified mice susceptible to MV infection, a productive lung infection with moderate syncytia were picked for infection of a Vero cell monolayer in the presence of trypsin. When the cytopathic effect (CPE) reached 90%, the cells were scraped into 1 ml of the cell culture medium and subjected to freezing and thawing. The cleared supernatants were considered as ‘plaque-purified’ recombinant virus (MV-Edm, MV-Fcm). To produce virus stocks, cleared supernatants were taken to infect subconfluent Vero cell monolayers. During infection at 33 °C, the cells were kept in DMEM–trypsin. Infected cells showing 90–100% CPE were considered as ‘plaque-purified’ recombinant virus (MV-Edm, MV-Fcm). To produce virus stocks, cleared supernatants were taken to infect subconfluent Vero cell monolayers. During infection at 33 °C, the cells were kept in DMEM–trypsin. Infected cells showing 90–100% CPE were considered as ‘plaque-purified’ recombinant virus (MV-Edm, MV-Fcm).

**Methods**

**Plasmid constructs.** Cloning of the viral glycoprotein (H and F protein) genes into the expression vector pCG under the control of the cytomegalovirus early promoter has been described by Cathomen et al. (1995). The F cleavage mutant (pCG-Fcm) with substitutions in the furin recognition motif (Fig. 1A) was prepared by introduction of site-specific mutations with the complementary primers Fcm1 (5’ GCTTCAAGT-AGGAACAAAGCATTTGGCGG 3’) and Fcm2 (5’ CCCGCA-AATCTGTGGTTCTACTTGAACG 3’) in the double-stranded pCG-F plasmid using the QuickChange site-directed mutagenesis kit (Stratagene). After dideoxy sequencing of the complete F gene, the mutated plasmid was used for transfection of 293 and Vero cells.

For the generation of recombinant MV, a derivative [p(+)MVNe: Singh et al., 1999] of the cDNA clone containing the full-length MV Edmonston B-based genome described by Radecke et al. (1995) was used in this work. To construct a full-length MV genome with a mutated F protein, the F protein was mutagenized in the shuttle vector pEF1 (Radecke et al., 1995) using the primer pair Fcm1 and Fcm2. A NdeI–FclI fragment containing the F gene with the mutated cleavage site was subcloned into p(+)MVNe and completely sequenced by the dideoxy method using an automatic sequencer (Perkin Elmer). The parental cDNA clone [p(+)MVNe] as well as the mutated clone [p(+)MV-Ncm] were used to generate recombinant MV.

**Cells.** Vero (African green monkey) cells and 293 (human embryonic kidney) cells were grown in Dulbecco’s modified minimal essential medium (DMEM, GIBCO-BRL) supplemented with 10% foetal calf serum (FCS, GIBCO-BRL), 100 U/ml penicillin and 100 µg/ml streptomycin. To maintain the selection pressure, 1 mg/ml G418 was added to the growth medium of the helper cell line 293-3-46. These cells stably express MV nucleoprotein (NP) and phosphoprotein (P) as well as T7 RNA polymerase (Radecke et al., 1995).

**Transient expression.** For analysis of transiently expressed MV H, F and Fcm proteins, Vero cells or 293 cells were transfected using the calcium phosphate technique essentially as described by Prill et al. (1993). For coexpression of H and F proteins, 1×10^6 Vero cells were cotransfected with 20 µg pCG-F or pCG-Fcm in addition to 20 µg pCG-H. Transfected 293 cells were grown for 47 h in DMEM supplemented with 10% FCS and then used for Western blot analysis. Transfected Vero cells were grown for 16 h and incubated for 8 h in DMEM or DMEM containing 1 µg/ml TPCK-treated trypsin (Sigma) (DMEM–trypsin) before performing the fusion assay.

**Virus rescue and preparation of recombinant virus stocks.** Transfection and rescue of MV were performed mainly as described by Radecke et al. (1995). Briefly, 293-3-40 helper cells mediating both artificial T7 transcription and NP and P functions were transfected with 8 µg of either p(+)MVNe or p(+)MV-Fcm in the presence of 5 ng of plasmid encoding the MV polymerase (pEMC-La). At 2 days post-transfection, cells were expanded. To induce syncytium formation in p(+)MV-Fcm-transfected cells, cells were washed and activated for 2 h at 37 °C with DMEM–trypsin. After activation, cell growth was allowed to proceed in DMEM supplemented with 10% FCS. At 4 days post-transfection, cells were scraped into Optimem (GIBCO-BRL) with trypsin (1 µg/ml) and adsorbed to Vero cell monolayers. After washing, infected Vero cells were kept in DMEM–trypsin because they tolerate a trypsin concentration up to 1.2 µg/ml without detectable (in contrast to 293 cells). At 5 days post-transfection, multiple syncytia indicated the successful rescue of p(+)MVNe or p(+)MV-Fcm (MV-Edm). First syncytia in the p(+)MV-Fcm rescue appeared 7–8 days post-transfection. Single syncytia were picked for infection of a Vero cell monolayer in the presence of trypsin. When the cytopathic effect (CPE) reached 90%, the cells were scraped into 1 ml of the cell culture medium and subjected to two rounds of freezing and thawing. The cleared supernatants were considered as ‘plaque-purified’ recombinant virus (MV-Edm, MV-Fcm). To produce virus stocks, cleared supernatants were taken to infect subconfluent Vero cell monolayers. During infection at 33 °C, the cells were kept in DMEM–trypsin. Infected cells showing 90–100% CPE were scraped into the medium, frozen and thawed, aliquoted and stored at −80 °C. Infectivity was determined by 50% end-point dilution assay in the presence of 2 µg/ml trypsin (TCID_{50}).

**SDS–PAGE and Western blot analysis.** Transiently expressing or virus-infected cells were lysed in electrophoresis buffer (50 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS) either lacking 2-mercaptoethanol (nonreducing conditions, −ME) or containing 2% 2-mercaptoethanol (reducing conditions, +ME). The samples were boiled for 5 min and directly subjected to SDS–PAGE. The gel was blotted to nitrocellulose and probed with glycoprotein-specific antibodies (anti-Fcyt and anti-Hcyt) described by Cathomen et al. (1998). Bound antibodies were stained by subsequent incubation with biotin-labelled anti-rabbit immunoglobulins (Amersham) and horseradish peroxidase-conjugated streptavidin (Amersham). Peroxidase was detected with the enhanced chemiluminescence system (ECL, Amersham).
Fusion assay. Because of the sensitivity of 293 cells to trypsin, fusion activity was analysed in transiently transfected or infected Vero cells, respectively. For syncytium formation, Vero cells were cotransfected with the standard or mutant F gene (pCG-F, pCG-Fcm) and the standard H protein gene (pCG-H). At 16 h post-transfection, the cells were washed twice with PBS to remove the FCS-containing medium and were further incubated with DMEM. To one monolayer from each set of duplicate samples TPCK-treated trypsin (Sigma) to a concentration of 1 µg/ml was added. At 24 h post-transfection, the transiently expressing cells were fixed with ethanol and stained with 1:10 diluted Giemsa’s staining solution (Merck). To analyse the biological activity of recombinant MV with a mutated F protein, subconfluent Vero cells were infected with parental (MV-Edm) or mutant MV (MV-Fcm) at an m.o.i. of 0.01–0.1. The infected cells were cultivated at 37 °C in DMEM in the absence or presence of 1 µg/ml trypsin. At 24 h post-infection (p.i.), the infected cells were fixed and stained as described.

Immunostaining. Subconfluent Vero cells (1 × 10⁵ cells) were grown on coverslips and infected with trypsin-activated MV-Fcm at an m.o.i. of 5 for 2 h at 37 °C. The cells were intensively washed with PBS overlaid with DMEM or DMEM–trypsin and incubated for 28 h at 33 °C to allow one-step growth in the presence or absence of trypsin. To quantify the number of infected cells, immunostaining was performed. After fixation and permeabilization at −20 °C with methanol–acetone (1:1), MV-positive cells were detected with a polyclonal rabbit antiserum (DAKO). The samples were mounted in Mowiol and 10% triethylenediamine. For quantification of infectious cell-free virus, the cell supernatant was collected before immunostaining of the infected cells (28 h p.i.). The supernatant (300 µl) was directly used to infect fresh Vero cells grown to subconfluency on coverslips. To activate cell-free virus grown in the absence of trypsin, 1 µg/ml TPCK-treated trypsin was added to the supernatant. As a control, 300 µl of the supernatant without trypsin addition was used for infection. Virus adsorption in the absence or presence of trypsin was allowed to proceed for 4 h at 37 °C. Then, the cells were washed several times with PBS, overlaid with DMEM and incubated at 33 °C. To quantify the number of infected cells, the cells were fixed at 42 h p.i. and immunostaining was performed as described.

Mice infections. The Ifnar<sup>−/−</sup>-CD46Ge mice used in this study have a targeted mutation (tm) inactivating the interferon receptor type I (Ifnar). Since a yeast artificial chromosome covering about 400 kilobases of human genome surrounding the CD46 gene (CD46Ge) was transferred to mice, these animals express CD46 with human-like tissue specificity (Mrkic et al., 1998). Stock virus for animal infection was grown in the presence of trypsin and isolated as described except for removing the trypsin-containing media before harvesting the virus. Age-matched mice were used for infections at the age of 6–7 weeks. For intranasal inoculation a total volume of 50 µl of appropriate virus stocks was administered into both nares. Intracerebral inoculations were done along the midline by using a 27-gauge needle. The inoculum consisted of 30 µl stock virus diluted in PBS.

Histology and in situ hybridization assay. Assays were basically performed as described previously (Mrkic et al., 1998). Briefly, mice were euthanized with CO₂, the lungs were removed and fixed in 4% PBS-buffered formaldehyde. Paraffin-embedded tissues were cut at 2–3 µm sections. For general histological analysis the sections were stained with haematoxylin–eosin staining solution. Detection of MV N mRNA in situ was performed with a digoxigenin (DIG)-labelled N RNA probe (30 pg/µg) followed by immunological staining with a DIG-nucleic acid detection kit (Boehringer Mannheim). The sections were counterstained with haematoxylin solution.

Results

Intracellular cleavage and biological activity of transiently expressed mutant F protein

The cleavage site of the MV F protein consists of five basic amino acids (108–112) and is intracellularly cleaved by the
host cell protease furin (Alkathib et al., 1994; Watanabe et al., 1995) to generate a biologically active molecule (F<sub>1</sub>/F<sub>2</sub>) from the inactive precursor molecule (F<sub>0</sub>). To prevent intracellular activation of F<sub>0</sub>, the furin consensus site in the F protein sequence was altered by changing the arginine at position 109 and the lysine at position 111 to asparagine. Fig. 1(A) shows the amino acid sequence of the cleavage site of the standard and mutant F protein. Both proteins were transiently expressed in 293 cells by transfection of the recombinant expression plasmids pCG-F and pCG-Fcm. To assay for the susceptibility of the cleavage site in the mutant F protein to activation by host cell proteases, transfected cell lysates were subjected to Western blot analysis (Fig. 1 B). Under nonreducing conditions (–ME) disulfide bonds remained intact, and cleaved standard F protein (F) as well as potentially uncleaved mutant F protein (Fcm) migrated on the SDS gel with an apparent molecular mass of about 60 kDa (F<sub>0</sub>). SDS–PAGE under reducing conditions (+ ME) resulted in the dissociation of the F<sub>1</sub> and F<sub>2</sub> subunits of cleaved F protein. Since the blot was probed against a synthetic peptide corresponding to the carboxy terminus of the F protein (anti-FcYt), both the F<sub>0</sub> precursor and the F<sub>1</sub> subunit of the cleaved F protein, but not the F<sub>2</sub> subunit, were detected. In cells transiently expressing standard F protein a significant portion of the protein (20–30%) was found as F<sub>1</sub> subunit with a molecular mass of about 40 kDa. In cells expressing mutant F protein only uncleaved F<sub>0</sub> was found, indicating that alteration of the furin recognition motif completely abolished the susceptibility to host cell proteases and, therefore, intracellular cleavage of mutant F protein.

To analyse the ability of the mutant F protein to mediate membrane fusion, Vero cells were transfected with recombinant pCG-F or pCG-Fcm plasmids in combination with the standard H gene (pCG-H). As determined by immunofluorescence equivalent amounts of both F proteins were expressed. To activate uncleaved F protein expressed on the cell surface, 1 µg/ml trypsin was added to each set of duplicate samples at 16 h post-transfection (+ trypsin). Cells were fixed and stained at 24 h post-transfection. As shown in Fig. 1(C), cotransfection of standard MV glycoproteins (H+F) induced syncytium formation in the absence (–trypsin) and presence (+trypsin) of trypsin, confirming that cell-to-cell fusion to form syncytia was completely dependent on the presence of both viral glycoproteins, but independent of exogenous trypsin addition. In contrast, coexpression of standard H protein and mutant F protein (H+Fcm) only induced cell fusion in the presence of trypsin (+trypsin), indicating that mutant F protein was transported to the cell surface where it could be biologically activated by trypsin cleavage to cause syncytium formation.

**Rescue of recombinant MV with mutant F protein**

Characterization of transiently expressed mutant F protein had shown that the alterations in the furin recognition site prevented intracellular cleavage and resulted in the synthesis of a biologically inactive molecule that could be activated by exogenous trypsin. An MV particle with such a mutant F protein should be a noninfectious virus that can be converted into an infectious particle by the addition of trypsin. We attempted to rescue such a virus using a reverse genetics system (Radecke et al., 1995). We constructed full-length antigenic cDNA carrying the same mutation as the pCG-Fcm expression plasmid (Fig. 1 A). This plasmid [p (+)MV-Fcm] or the standard plasmid containing the authentic MV-Edm F protein [p (+)MVNSe] was transfected into 293-3-46 helper cells in the presence of plasmid encoding the MV polymerase. Parental MV (MV-Edm) was rescued with the standard protocol at 5 days post-transfection (Radecke et al., 1995). For the successful rescue of mutant MV (MV-Fcm) at 8 days post-transfection, trypsin had to be added at different steps of the rescue protocol as described in Methods. To analyse the susceptibility of the virus-encoded mutant F protein to cleavage by intracellular proteinases, Vero cells were infected with MV-Edm or MV-Fcm at an m.o.i. of 0.01–0.1 and cultivated in the absence (–trypsin) or presence (+trypsin) of 1 µg/ml trypsin. At 24 h p.i., infected cells were subjected to Western blot analysis, demonstrating a clear difference between standard and mutant F protein (Fig. 2 A, anti-F). About 95% of the standard protein was found as F<sub>1</sub> subunit independently of trypsin addition, whereas 100% of the mutant F protein migrated as precursor molecule F<sub>0</sub> in the absence of trypsin. Only when mutant F protein was synthesized in infected cells cultivated in the presence of trypsin could a significant amount of the cleavage product F<sub>1</sub> be detected. This indicates that virus-derived mutant F protein, in contrast to standard F protein, was not susceptible to intracellular cleavage by host cell proteases but was transported to the cell surface where it could be cleaved by trypsin. In Fig. 2(A) (anti-H), samples were probed with antibodies raised against the MV H protein and showed no differences in the H protein expression in infected cells, indicating that cell infection by MV-Edm and MV-Fcm was comparable and that H protein expression was not influenced by trypsin addition during 24 h of infection.

**Restoration of fusion competence by trypsin**

For infectious MV it is not sufficient to possess a cleaved F protein; F has to be fusion-competent. In order to analyse this biological activity, cells infected with parental MV or trypsin-activated MV-Fcm were cultivated in the absence or presence of trypsin and tested for syncytium formation at 24 h p.i. (Fig. 2 B). As expected, MV-Edm-infected cells showed trypsin-independent cell-to-cell fusion. Cells infected with MV-Fcm did not show any syncytium formation when cultivated without trypsin (–trypsin), but infection in the presence of trypsin resulted in cell fusion (MV-Fcm, +trypsin). Although trypsin cleavage could restore the biological activity of mutant F protein, the fusion process in MV-Fcm-infected
cells was delayed. Syncytium formation reached completion at 48 h p.i., whereas in MV-Edm-infected cells maximal fusion was already observed at 30 h p.i. This phenomenon is probably due to a delayed F activation by trypsin in the media compared to intracellular activation of F in parental virus infection.

**Infectivity of cell-free MV-Fcm particles**

As both MV-Fcm and MV-Edm are released from infected cells and do not significantly differ in the protein composition of the virus particles (data not shown), we wanted to demonstrate that MV-Fcm grown in the absence of trypsin is actually noninfectious and activation of cell-free virus completely depends upon extracellular proteases. In Fig. 3, subconfluent Vero cells were infected with trypsin-activated MV-Fcm at an m.o.i. of 5 for 28 h in the presence (+ trypsin) or absence (− trypsin) of trypsin. To monitor the efficiency of infection, the infected cells were stained with an antiserum raised against purified MV. As expected, all cells were MV-positive, and cells infected in the presence of trypsin showed almost complete fusion, whereas cells infected in the absence of trypsin did not show any syncytium formation. To test if infectious virions were released into the culture media, supernatants were used to infect fresh Vero cells. The
Fig. 3. Trypsin activation of cell-free MV-Fcm grown in the absence of trypsin. Vero cells were infected for 28 h at 33 °C with MV-Fcm (one-step growth) in the absence (–trypsin) or presence of 1 µg/ml trypsin (+trypsin). Immunostaining of the infected cells was performed with a polyclonal anti-MV serum and an FITC-conjugated second antibody. The supernatant of the infected cells was either used directly (SP) or complemented with 1 µg/ml trypsin (SP + trypsin) and used for the infection of fresh Vero cells. After virus adsorption for 4 h at 37 °C, the cells were intensively washed and incubated in the absence of trypsin. Immunostaining was performed as described at 42 h p.i.

supernatant of MV-Fcm grown without trypsin was either used untreated (SP) or complemented with trypsin to a final concentration of 1 µg/ml (SP + trypsin). The supernatants were allowed to adsorb for 4 h at 37 °C and then removed by extensive washings. Since further infection was performed without trypsin, no virus spread occurred, and the number of MV-infected cells directly reflects the number of infectious particles. At 42 h p.i., MV-positive cells were detected by immunostaining (Fig. 3, lower panel). No MV-Fcm-positive cell was detected after infection with supernatant of cells infected in the absence of trypsin. Addition of trypsin to this ‘noninfectious’ supernatant (SP + trypsin) during virus adsorption resulted in the infection of about 30% of the cells, clearly demonstrating that the supernatant contained cell-free MV-Fcm particles that could be activated by trypsin. The infectivity of these viruses is reduced compared to the supernatant of MV-Fcm grown in the presence of trypsin, indicating that incubation with trypsin after virus release only partially restored the infectivity of a virus suspension. Even if the supernatant was incubated with trypsin for more than 4 h no increase in the infectivity could be observed. If trypsin is present during 28 h of infection Fcm is cleaved directly after reaching the cell surface, and infectious viruses that were capable of infecting 100% of the second cell monolayer were released (Fig. 3, left panel). To analyse the infectivity of trypsin-activated MV-Fcm, Vero cells were infected with mutant and parental virus either in the absence or presence of trypsin. The growth curves shown in Fig. 4 confirm that MV-Fcm is actually noninfectious when grown without trypsin in the media. No major differences could be observed between

Fig. 4. Time-course of cell-free virus production. Vero cells were infected at an m.o.i. of 0:1 in the absence or presence of trypsin either with MV-Edm (without trypsin ——; with trypsin ———) or with trypsin-activated MV-Fcm (without trypsin ————; with trypsin ————). Virus titres were determined by 50% end-point dilution of the supernatants at the indicated time-points p.i. (detection limit, 10^2 TCID_{50}/ml).
MV with restricted F protein cleavage

Fig. 5. Histological analysis of mouse lungs after intranasal infection with recombinant MV. Ifnar\textsuperscript{tm}-CD46\textsuperscript{Ge} mice were infected with $3 \times 10^5$ TCID\textsubscript{50} of MV-Edm (A, C) or MV-Fcm (B, D, E). Four days p.i., the lungs were collected and tissue sections processed for general histological analysis (A, B) or for MV-N-specific \textit{in situ} hybridization assay (C, D, E). Magnification, $\times 320$.

the virus titres obtained after infection with MV-Fcm grown in the presence of trypsin and parental virus grown with or without protease. This indicates that trypsin can completely restore the infectivity of an MV-Fcm virus suspension when permanently present during virus growth, a phenomenon that was not observed after trypsin activation of released viruses (Fig. 3).

MV-Fcm infection \textit{in vivo}

As MV-Fcm, in contrast to parental MV-Edm, depends on activation by extracellular proteases, we wanted to know how this reflects replication \textit{in vivo}. A group of eight Ifnar\textsuperscript{tm}-CD46\textsuperscript{Ge} mice were infected intranasally with $3 \times 10^5$ TCID\textsubscript{50} of trypsin-activated virus to study the pathogenic effects of MV-Fcm replication after uptake through the respiratory route. As a control, six mice were infected with MV-Edm. The lungs were removed for histological analysis and \textit{in situ} hybridization at 4 days p.i., when high levels of parental virus replication were observed. We reported previously that parental MV-Edm causes acute lung inflammation, extensive hyperaemia and diffuse haemorrhage in large areas of the lung (Mrkic \textit{et al}., 1998). Although less pronounced than in parental virus infection (Fig. 5A), mutant MV also caused pathological effects. MV-Fcm-infected mice revealed an increased cellular density and infiltration of inflammatory cells, particularly in the perivascular regions (Fig. 5B). To demonstrate virus replication, MV-infected cells were detected by MV N-specific \textit{in situ} hybridization assay. After parental virus infection, MV-positive cells were mainly found close by or in the alveolar epithelium, mainly in cell groups, indicating virus spread by cell-to-cell fusion (Fig. 5C). In MV-Fcm-infected mice, single virus-positive cells were distributed all over the whole lung tissue, but the majority were found in the alveolar walls (Fig. 5D). Cells of the bronchiolar epithelium were occasionally infected (Fig. 5E). No virus-positive cell groups were detected,
indicating that virus spread by cell-to-cell fusion probably did not occur. The number of virus-positive cells was rather small, suggesting that MV-Fcm replication was not as efficient as replication of parental MV-Edm. However, MV-Fcm was able to induce lymphatic infiltration in the lung. In parallel, lung sections from mice infected with UV-inactivated MV-Edm revealed no MV RNA-specific signals and only minor pathological changes (data not shown).

We next tested the sensitivity of Ifnar<sup>−/−</sup>-CD46Ge mice to intracerebral infection with trypsin-activated MV-Fcm. When infected with MV-Edm five of six animals showed clinical signs of neural disease and died within 1 week after infection. In contrast, all mice infected with MV-Fcm survived and did not develop any signs of disease (Table 1). Thus, MV-Fcm was not pathogenic in mice when inoculated into the brain.

**Discussion**

We describe here the generation and characterization of a recombinant MV containing a mutated F protein with a monobasic cleavage site requiring an exogenous protease for activation of infectivity. Previous efforts to obtain MV with nonactivated F protein have failed, since residual F cleavage was observed when virus was grown in furin-defective Lovo cells (Watanabe et al., 1995) or in lymphoblastoid cell lines also markedly defective in intracellular cleavage activity (Fujinami & Oldstone, 1981). An attempt to generate uncleaved F protein by in vitro mutagenesis failed because the Leu112 mutant (cleavage site R-H-K-L) was aberrantly cleaved in transfected cells (Alkathib et al., 1994). Furthermore, there are no natural cleavage site variants of MV available as are observed with other viruses, such as NDV or influenza virus. Our construct is therefore a unique tool in the study of the effect of F cleavage on virus assembly, fusion activity, infectivity, tissue tropism and pathogenicity.

Our MV F mutant was completely resistant to furin or furin-like proteases, but was susceptible to activation by trypsin. Thus, MV-F belongs to the group of viruses, such as influenza virus or NDV, in which differences in the pathogenicity could be attributed to differences in cleavability of the glycoprotein responsible for virus-cell fusion (Klenk & Garten, 1994). As shown for the influenza virus H protein and the NDV F protein (Vey et al., 1992) conversion of a multibasic to a monobasic cleavage site by site-directed mutagenesis caused restricted protease sensitivity of the MV F protein. This finding was not necessarily expected because the F protein of another paramyxovirus, simian parainfluenza virus 5 (SV5), could no longer be activated by proteolysis after similar mutations at the cleavage site. When the five arginine residues in the wild-type SV5 F protein were reduced to two or three, cleavage could still be accomplished by trypsin, but no longer resulted in fusion activation unless an additional mutation was introduced into the fusion peptide (Ward et al., 1995). Moreover, when only one arginine was left, the SV5 F protein became resistant to trypsin cleavage (Paterson et al., 1989). These data suggest that mutations at the cleavage site may affect the conformation of the F protein of SV5 in such a way that it is no longer susceptible to proteolytic activation. The F mutant of MV, on the other hand, can be activated by trypsin, indicating that the mutations we introduced did not result in a detrimental conformational change. Furthermore, MV-Fcm was readily released from infected cells and the virions were indistinguishable from parental virus in their protein composition (data not shown), demonstrating that the mutations at the F cleavage site did not interfere with virus assembly and release. As noninfectious virus is released in the absence of extracellular protease, F protein cleavage is not necessary for glycoprotein incorporation into mature virions.

The data on MV-Fcm growth in cultured cells suggested that spread of infection in the organism may be restricted. Indeed, this hypothesis is supported by our findings after intranasal and intracerebral infection of susceptible Ifnar<sup>−/−</sup>-CD46Ge mice. Although less efficient than infection with MV-Edm, we observed a productive infection when trypsin-activated MV-Fcm was administered by the respiratory route. It is not yet clear whether the limited spread of MV-Fcm is due to the cleavage of mutant F protein by extracellular proteases in the lung. There are various candidates for in vivo activation of F proteins with monobasic cleavage sites. Tryptase Clara cells are prominent among proteases of this type, since it was shown to activate respiratory viruses such as human influenza viruses and Sendai virus in their natural setting. The protease is secreted into the airway lumen from bronchiolar epithelial Clara cells, a subset of nonciliated cells that are different from other bronchiolar and alveolar cells in which virus replication occurs (Kido et al., 1992; Tashiro et al., 1992<sup>b</sup>). Unlike the situation in mice infected with parental MV-Edm, MV-positive giant cells were not detected in MV-Fcm-infected mice. This can be explained by the restriction of the activating protease to the apical side and, therefore, the lack of activated F protein on the basolateral surface, which is required for fusion of epithelial cells (Maisner et al., 1998; Tashiro et al., 1992<sup>a</sup>). In contrast to the productive replication found in the lung, our cleavage

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**Table 1. Susceptibility of Ifnar<sup>−/−</sup>-CD46Ge mice to intracerebral infection with recombinant MV**

Six-week-old animals were injected intracerebrally with 10<sup>5</sup> TCID₅₀ of MV-Edm or MV-Fcm and observed daily for 28 days.

<table>
<thead>
<tr>
<th>MV Strain</th>
<th>No. of mice dead/no. of mice infected</th>
<th>Average time to death (days)</th>
<th>Mortality (%)</th>
</tr>
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<tbody>
<tr>
<td>MV-Edm</td>
<td>5/6</td>
<td>6.8</td>
<td>83</td>
</tr>
<tr>
<td>MV-Fcm</td>
<td>0/6</td>
<td>&lt; 28&lt;sup&gt;∗&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>∗</sup> No clinical symptoms or death during observation period.

The data on MV-Fcm growth in cultured cells suggested that spread of infection in the organism may be restricted. Indeed, this hypothesis is supported by our findings after intranasal and intracerebral infection of susceptible Ifnar<sup>−/−</sup>-CD46Ge mice. Although less efficient than infection with MV-Edm, we observed a productive infection when trypsin-activated MV-Fcm was administered by the respiratory route. It is not yet clear whether the limited spread of MV-Fcm is due to the cleavage of mutant F protein by extracellular proteases in the lung. There are various candidates for in vivo activation of F proteins with monobasic cleavage sites. Tryptase Clara cells are prominent among proteases of this type, since it was shown to activate respiratory viruses such as human influenza viruses and Sendai virus in their natural setting. The protease is secreted into the airway lumen from bronchiolar epithelial Clara cells, a subset of nonciliated cells that are different from other bronchiolar and alveolar cells in which virus replication occurs (Kido et al., 1992; Tashiro et al., 1992<sup>b</sup>). Unlike the situation in mice infected with parental MV-Edm, MV-positive giant cells were not detected in MV-Fcm-infected mice. This can be explained by the restriction of the activating protease to the apical side and, therefore, the lack of activated F protein on the basolateral surface, which is required for fusion of epithelial cells (Maisner et al., 1998; Tashiro et al., 1992<sup>a</sup>). In contrast to the productive replication found in the lung, our cleavage
mutant was unable to cause neural disease. These observations indicate that pathogenicity of MV-Fcm is reduced. MV spreads systemically and induces suppression of immune responses that lasts for weeks to months after the onset of acute disease and, therefore, plays a major role in morbidity and mortality associated with measles (Borrow & Oldstone, 1995; Schlender et al., 1996). Our data support the concept that the cleavage site mutant, unlike wild-type and the presently used vaccine strains, is unable to cause systemic infection after uptake through the respiratory route. Furthermore, it can be assumed that MV-Fcm has lost its immunosuppressive properties. This notion is supported by recent findings that F protein cleavage is an absolute requirement for virus-induced immunosuppression in vitro (A. Weidmann, A. Maisner, W. Garten, M. Seufer, V. ter Meulen & S. Schneider-Schaulies, unpublished results). These properties may make the cleavage site mutant an interesting vaccine candidate, especially for immunocompromised hosts.

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References


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