The products of the UL10 (gM) and the UL49.5 genes of Marek’s disease virus serotype 1 are essential for virus growth in cultured cells

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The role of the products of the UL10 and the UL49.5 homologous genes of Marek’s disease virus serotype 1 (MDV-1) in virus replication was investigated. Deletion of either open reading frame in an infectious bacterial artificial chromosome clone (BAC20) of MDV-1 resulted in progeny viruses that were unable to spread from cell to cell. After transfection of UL10- or UL49.5-negative BAC20 DNA into chicken or quail cells, only single infected cells were observed by indirect immunofluorescence analysis. In contrast, plaque formation was restored when mutant BAC DNAs were co-transfected with the corresponding expression plasmid encoding either the UL10-encoded gM or the UL49.5 gene product. These data demonstrate that gM and its putative complex partner, the UL49.5 homologous protein, are essential for MDV-1 growth in cultured cells. Thus, MDV-1 represents the first example of a member of the family Herpesviridae for which the highly conserved membrane proteins are indispensable for cell-to-cell spread.
however, analysis of a library of human cytomegalovirus (HCMV) mutants suggested that gM is essential for growth of this betaherpesvirus (Hobom et al., 2000). Although the EHV-1 and PRV gM and UL49.5 products are nonessential, concomitant deletion of gM and gE–gI led to significantly reduced virus titres and plaque sizes, which were caused by an inefficient secondary envelopment (Brack et al., 1999; Seyboldt et al., 2000). The same observation of reduced growth properties caused by inefficient secondary envelopment was made if the UL49.5 gene product of EHV-1 was absent (Rudolph et al., 2002). These results suggested similar, albeit not strictly overlapping, functions of two glycoprotein complexes of the Alphaherpesvirinae (Brack et al., 1999, 2000; Seyboldt et al., 2000; Rudolph et al., 2002). The essential function of both gE and gI for MDV-1 and of at least gE in its closest relative, VZV, demonstrated that this interpretation does not hold for highly cell-associated members of the subfamily Alphaherpesvirinae. In the case of both MDV-1 and VZV, the function of the gE–gI complex cannot be compensated for by the putative UL10–UL49.5 complex (Schumacher et al., 2001; Cohen & Nguyen, 1997; Mallory et al., 1997).

In an effort to systematically analyse the complex action of membrane and tegument proteins in MDV-1 cell-to-cell spread, the aim of this study was to explore the effect of the deletion of UL10 (gM) and UL49.5. Virus mutants were constructed using an infectious MDV-1 bacterial artificial chromosome (BAC) clone and recE/T-based mutagenesis (Muyrers et al., 1999; Narayanan et al., 1999; Zhang et al., 1998). Both, UL10- and UL49.5-negative MDV-1 were unable to grow in cultured cells; however, growth of the mutants was restored after co-transfection of an expression plasmid harbouring the respective gene. The results demonstrate that both gM and the UL49.5 gene product are indispensable for MDV-1 replication.

UL10 and UL49.5 deletion mutants were generated from the infectious MDV-1 BAC20 clone (Schumacher et al., 2000, 2001; Dorange et al., 2002). The UL10 (20gM) and the UL49.5 (20∆49.5) deletion mutants were generated by recE/T cloning in Escherichia coli DH10B cells, essentially as described previously (Muyrers et al., 1999; Narayanan et al., 1999; Schumacher et al., 2000; Zhang et al., 1998). The respective ORF was replaced with the kanamycin resistance gene (kan) amplified from plasmid pACYC177 (MBI Fermentas) by PCR using appropriate primers (Table 1). For recombination of the linear fragment into BAC20, 300 ng of the purified PCR product was electroporated using standard parameters (1-25 kV/cm, 200 Ω and 25 μF). Cells were grown in 1 ml LB for 90 min and plated onto LB agar plates containing 30 μg/ml chloramphenicol and 30 μg/ml kanamycin. Extrachromosomal DNA of double-resistant colonies was prepared by column chromatography (Qiagen). Mutant BAC DNA was analysed by restriction enzyme digestion using BamHI or HindIII, Southern blot analysis and cycle sequencing of the recombination sites (Schumacher et al., 2000, 2001). The results of these investigations demonstrated that the respective ORF was deleted successfully from clones 20gM and 20∆49.5 (Fig. 1).

To analyse the growth properties of mutant MDV-1, 2 μg BAC20, 20gM or 20∆49.5 BAC DNA was transfected by the calcium phosphate precipitation method into 1 × 10⁶ CEF cells that had been grown to between 60 and 80% confluency in 6-well plates (Morgan et al., 1990; Schumacher et al., 2000, 2001). Whereas approximately 80 MDV-1-specific plaques were visible from day 2 after transfection of BAC20 DNA, no plaques were formed after the transfection of mutant BAC DNA lacking either the UL10 or the UL49.5 ORF. Only single infected cells were detected after indirect immunofluorescence (IIF) analysis using an anti-pp38 monoclonal antibody (mAb), H19 (Cui et al., 1990) (Fig. 2A). To verify the absence of plaque formation at later time points, CEF cells transfected with BAC20, 20gM or 20∆49.5 DNA were trypsinized at 5 days after transfection and co-seeded with freshly prepared CEF cells (Schumacher et al., 2001). Whereas propagation of MDV-1 reconstituted from BAC20 DNA was successful, only single infected cells and no amplification of MDV-1-infected cells could be identified in CEF cells co-seeded with 20gM- or 20∆49.5-transfected cells (data not shown). A cell type-specific growth defect of the two MDV-1 mutants was excluded by transfection of quail muscle QM7 cells (Dorange et al., 2002; Schumacher et al., 2001). As described for CEF cells, transfection of UL10- or UL49.5-negative BAC DNA resulted in single infected cells only (Fig. 2A). An effect on the UL49 homologous gene, which is essential for the growth of MDV-1 (Dorange et al., 2002), by deletion of the immediately adjacent UL49.5 gene in 20∆49.5 DNA was also viewed as possible, because a polycistronic mRNA containing the UL48 to UL49.5 ORFs was detected in MDV-1-infected cells (Dorange et al., 2000). Expression of VP22, the UL49 product, was analysed by IIF analysis using VP22-specific mAb L13 (Dorange et al., 2000). CEF cells were transfected with the UL49-negative 20Δ49.5 DNA and single infected cells were readily detected with the VP22-specific mAb (Fig. 2B). From these results, it was concluded that expression of the UL49 gene was not affected by the deletion of the UL49.5 gene.

Two revertant viruses from the UL10- and UL49.5-negative viruses were generated and the deletions introduced previously by the insertion of kan into E. coli cells by recE/T cloning were repaired. DNA of 20gM or 20∆49.5 mutants was co-transfected with the BamHI C fragment or the EcoRI C fragment (Fig. 1). Co-transfection of CEF cells with mutant BAC DNA and the respective genomic DNA fragment resulted in the formation of MDV-1-specific plaques in both cases. These MDV-1-specific plaques could be serially propagated by co-seeding with uninfected CEF cells, indicating that virus growth was fully restored in both revertant viruses (Fig. 2D).

The results of the transfection experiments using mutant MDV-1 BAC20 DNA and the generation of revertant viruses, in which plaque formation in CEF cells was restored, indicated
that growth of MDV-1 in cultured cells required both the UL10 and the UL49.5 gene products. To confirm this assumption further, mutant 20ΔgM and 20ΔUL49.5 DNA was co-transfected with 10 µg of the expression plasmids pcMgM or pcM49.5, respectively (Table 1). The plasmids contained the respective ORFs cloned into vector pcDNA3 (Invitrogen), where expression is controlled by the HCMV immediate early promoter/enhancer (Osterrieder, 1999). Co-transfection of 20ΔgM with pcMgM, which provided the deleted gM gene in trans, resulted in the formation of MDV-1-specific plaques, as visualized by the anti-pp38 mAb, H19. Similarly, co-transfection of mutant 20ΔUL49.5 BAC DNA with pcM49.5 also resulted in MDV-1-specific plaques (Fig. 2C). The results of the co-transfection experiments eventually demonstrated that cell-to-cell spread of MDV-1 requires the presence of both the UL10-encoded gM and the UL49.5 gene product.

The results presented here demonstrate that growth of MDV-1, i.e. the formation of virus plaques by direct cell-to-cell spread, requires both the UL10 and the UL49.5 gene products. Being a strictly cell-associated virus, MDV-1 appears to depend on a different set of (glyco)proteins for growth in cultured cells when compared to other members of the Alphaherpesvirinae. Previously, we demonstrated that both gE and gI are essential for the growth of MDV-1. These results indicated that the partially redundant functions of the gE–gI complex and the gM–UL49.5 complex, as was described for EHV-1 and PRV, could not be confirmed in the case of MDV-1 (Schumacher et al., 2001). The findings of the essential nature of MDV-1 gM and the UL49.5 gene product, or the putative complex between these transmembrane proteins, indicate that their action cannot be substituted for by another virus protein. Therefore, the gE–gI and the putative gM–UL49.5 complexes of MDV-1 act in different and probably nonoverlapping steps of cell-to-cell spread. Hence, the mechanism of MDV-1 cell-to-cell spread appears to be different from that of other members of the subfamily Alphaherpesvirinae. These observations are not entirely surprising, because MDV-1 expresses neither gD nor gG in cultured cells and is unable to produce free infectious virus (Lee et al., 2000; Tan et al., 2001; Tulman et al., 2000; Biggs, 2001). It is conceivable that in the absence of gD, which is essentially involved in cell-to-cell spread of most members of the subfamily Alphaherpesvirinae, the presence of other players involved in this process is more important. This interpretation is supported by the fact that the expression of gE and gI is required for the efficient growth of VZV, which also does not encode a gD homologue and is highly cell associated (Cohen & Nguyen, 1997; Mallory et al., 1997, 1998). The high cell association in cultured cells and most target cells in vivo of both MDV-1 and VZV, which produce free infectious virus only in the feather follicle epithelium (MDV-1) and in epithelial cell pustules in the acute rash of chicken pox or recurrent infections (VZV), may be reflected by a different evolution of gE and gI.

In light of the obviously different requirements for virus spread, it is important to note also that a different set of tegument proteins is necessary for the growth of MDV-1 when compared to other members of the subfamily Alphaherpesvirinae. Recently, the essential nature of the MDV-1 VP22 (UL49) homologous protein, which is dispensable for the growth of HSV-1 and PRV, was demonstrated. Conversely, the MDV-1 VP16 homologue is nonessential for virus assembly, as had been reported for HSV-1, and the expression

Table 1. Primers used for the generation of expression plasmids and construction of MDV-1 mutant and rescue viruses

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′ → 3′)</th>
<th>Fragment/plasmid generated</th>
</tr>
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<tbody>
<tr>
<td>gM-a</td>
<td>TAAGgattccGTGGCTATGGCCATGTCG</td>
<td>1.2 kb pcMgM</td>
</tr>
<tr>
<td>gM-b</td>
<td>ACTctcagCTAATCCATTCCATCG</td>
<td>1.2 kb pcMgM</td>
</tr>
<tr>
<td>49.5-a</td>
<td>GAAGgattccGATGGGACCTATGGACATTC</td>
<td>300 bp pcM49.5</td>
</tr>
<tr>
<td>49.5-b</td>
<td>GATgaattcTTTACCTTCTTCTTAAAC</td>
<td>300 bp pcM49.5</td>
</tr>
<tr>
<td>gM-kan-a</td>
<td>GCGTTGTTCCTACGATTCTTTCGCCACC</td>
<td>kan for gM deletion</td>
</tr>
<tr>
<td>gM-kan-b</td>
<td>TGACTACCCCCCCCTTATAAAACTCCGCTCATAATTGCTTATGCTGGTGTTTT</td>
<td>kan for gM deletion</td>
</tr>
<tr>
<td>49.5-kan-a</td>
<td>TTCCTGACATGGGCTGATCAATGCTTTTTTTCACTGGGTCTGCTGCTGT</td>
<td>kan for UL49.5 deletion</td>
</tr>
<tr>
<td>49.5-kan-b</td>
<td>GTCAAAAATCTCTTTATGAAAGAGATAAGATTGTTGCTCCAGCCACATAA</td>
<td>kan for UL49.5 deletion</td>
</tr>
</tbody>
</table>

Restriction enzyme sites are given in lower case bold letters, whereas sequences in italic letters represent the gM and UL49.5 sequences that allowed homologous recombination for recE/T-mediated deletion of the genes.
Fig. 1. (A) Schematic illustration of the MDV-1 (BAC20) genome and the structure of the generated mutant viruses and plasmids. The organization of the approximately 183 kb BAC20 genome and the BamHI restriction map is shown. The unique long (UL), unique short (US) and repeat regions (TRL, TRS, IR, and IRS) as well as the UL10- (gM) and UL49.5-encoding ORFs are indicated. Genomic DNA fragments used for the generation of revertant viruses are also shown (BamHI-C and EcoRI-C).

(B) The UL10 and UL49.5 genes were deleted by recE/T mutagenesis, which resulted in the insertion of the kanamycin resistance gene (kan) instead of the respective ORF. Scales in bp or kb are given. Restriction enzyme sites are abbreviated as follows: B, BamHI; E, EcoRI; and H, HindIII. (C) The resulting mutants 20ΔgM and 20Δ49.5 were tested by agarose gel electrophoresis and Southern blotting and compared with parental BAC20 DNA. DNA was isolated from E. coli, digested with the restriction enzymes BamHI or HindIII and separated using 0.8% agarose gels. Fragments caused by the insertion of kan by recE/T cloning and reactive with the kan probe are indicated by arrows. A 1 kb molecular mass marker is also included (Gibco-BRL).
MDV-1 gM and UL49.5 are essential

Fig. 2. (A) Growth of BAC20 mutant viruses on CEF or QM7 cells. Cells were transfected with BAC20, 20ΔgM or 20Δ49.5 DNA. IIF analysis using the anti-pp38 mAb H19 was performed 5 days after transfection. After transfection of BAC20 DNA, plaque formation in CEF and QM7 cells was readily observed but only single infected cells were seen after transfection of 20ΔgM or 20Δ49.5 into CEF or QM7 cells. Individual panels represent views of 650 × 450 µm in size. (B) Analysis of MDV-1 VP22 expression by gM (UL10)- and UL49.5-negative MDV-1. CEF cells were transfected with BAC20, 20ΔgM or 20Δ49.5 DNA. At 5 days after transfection, IIF analysis using the anti-VP22 (UL49) mAb L13 was performed. Individual panels represent views of 300 × 200 µm in size. (C) Trans-complementation of mutant viruses by expression plasmids. Mutant 20ΔgM or 20Δ49.5 BAC DNA was co-transfected with pcMgM or pcM49.5. Five days after transfection, cells were fixed with acetone and IIF analysis using the anti-pp38 mAb H19 was performed. Cell-to-cell spread of 20ΔgM and 20Δ49.5 was rescued by the corresponding expression plasmid. Individual panels represent views of 300 × 200 µm in size. (D) Plaque formation of revertant viruses 20ΔgMR and 20Δ49.5R on CEF and QM7 cells. 20ΔgM or 20Δ49.5 DNA was transfected with the BamHI C (20ΔgM) or EcoRI C (20Δ49.5) fragment. IIF analysis using the anti-pp38 mAb H19 was performed 3 days after co-seeding of 20ΔgMR or 20Δ49.5R-infected cells with fresh CEF or QM7 cells. Individual panels represent views of 650 × 450 µm in size.

of the UL48 gene product, VP16, was barely detectable in MDV-1-infected cells (Dorange et al., 2000). Further studies will therefore concentrate on the interaction between MDV-1 gM–UL49.5 and gE–gI complexes with tegument proteins. In addition, the nature of the putative gM–UL49.5 complex will be analysed by the generation of specific antibodies.
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