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Functional F11L and K1L genes in modified vaccinia virus Ankara restore virus-induced cell motility but not growth in human and murine cells

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ABSTRACT

Modified vaccinia virus Ankara (MVA) was generated by serial passaging in chicken embryo fibroblasts. During this attenuation, MVA lost the capacity to productively grow in human and most other mammalian cell lines, as well as acquiring a multitude of deletions and mutations in the MVA genome. This means that the precise molecular basis for the MVA host-range restriction is still unknown. The vaccinia virus (VACV) genes F11L and K1L are mutated or truncated in MVA. F11L was previously implicated in VACV-induced cell motility and virion maturation. Here, we demonstrate that the restoration of F11L gene expression in MVA rescued virus-induced cell motility, but had no impact on MVA virion maturation and host-range restriction. Additional insertion of the K1L gene, which restores MVA replication in RK-13 cells, was not sufficient to extend MVA growth capacity to other mammalian cells.

Introduction

Modified vaccinia virus Ankara (MVA) is a highly attenuated strain of vaccinia virus (VACV) that originally served as a candidate vaccine in Germany to improve the safety of smallpox vaccination (Stickl and Hochstein-Mintzel, 1971; Stickl, 1974). MVA was obtained by serial passaging of the ancestor chorioallantois vaccinia virus Ankara (CVA) in chicken embryo fibroblasts (CEF) (Mayr and Munz, 1964; Mayr, 1976) and its genome contains substantial genetic alterations adding up to a total loss of about 30 kb of DNA (Meyer et al., 1991). Deletions and mutations affect many regulatory virus genes likely involved in VACV host cell interaction and immune evasion (Antoine et al., 1998). The phenotypic consequence is that MVA is replication defective in most human and other mammalian cell lines (Carroll and Moss, 1997; Drexler et al., 1998). While the cascade-like pattern of viral early, intermediate and late gene expression, including DNA replication, is still active, viral multiplication is arrested at a late stage leading to accumulation of immature particles (Sutter and Moss, 1992). This unique phenotype, which allows high expression of recombinant antigens without virus replication, makes MVA a safe and efficient candidate vector for therapeutic vaccines against infectious diseases and cancer (Sutter and Staib, 2003). However, the mechanism and genetic basis underlying MVA's restricted replication are not understood.

VACV has a broad host-range and can productively infect cells from many vertebrate animal species. VACV host tropism is linked to the function of so-called viral host-range genes, which when inactivated cause host-range restrictions. A number of VACV genes have been associated with host-range phenotypes, the most important being the genes K1L, and C7L (Werden et al., 2008). Restoration of K1L in MVA or its expression in MVA infected cells expands the host-range of MVA to rabbit kidney-13 (RK-13) cells, but not to other mammalian cell lines analyzed so far (Carroll and Moss, 1997).

Both K1L and C7L are important for growth of VACV in mammalian cells, since mutant viruses with inactivated K1L and C7L genes replicate abortively in many mammalian cells. However, each gene can compensate for the other and rescue the growth capacity of deletion mutant viruses (Perkus et al., 1990). The molecular functions of these VACV host-range gene products are largely unknown. The K1L gene encodes a 32 kDa ankyrin repeat protein that was previously shown to prevent activation of host NFκB (Shisler and Jin, 2004), and to interact with ACAP2, a GTPase activating protein (GAP) for ADP-ribosylation factor 6 (Arf6) (Bradley and Terajima, 2005). Recently, K1 was also described to prevent protein kinase R (PKR) mediated phoshorylation of eukaryotic translation initiation factor 2 alpha (eIF2α), which is a typical interferon effector function (Backes et al., 2010; Willis et al., 2009). However, neither interaction nor inhibition of the PKR-eIF2α pathway appears to be essential for the host-range determining function of K1L (Meng and Xiang, 2006), which is possibly associated with inhibition of other type I interferon response effector(s) (Meng et al., 2009).

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K1L is fragmented in the MVA genome, but the open reading frame (ORF) for C7L is fully conserved (Antoine et al., 1998). Thus, (an)other unknown viral host-range regulator(s) must be impaired to result in the MVA growth restriction in mammalian cells. In an early attempt to address this question, Wyatt et al. (1998) performed genome-wide marker rescue experiments using DNA from the ancestor VACV Ankara and identified a left-side region of the MVA genome as containing additional fragmented host-range genes, specifically suggesting the ORFs F5L, F11L and O1L as candidates.

While O1L and F5L are VACV genes with unknown functions, F11L encodes a protein able to inhibit host RhoA downstream signaling pathways and induce motility of VACV infected cells (Valderrama et al., 2006; Arakawa et al., 2007). Interestingly, knock-down of F11L expression in VACV stain Western Reserve (VACV WR) infected HeLa cells resulted in accumulation of immature virions resembling those observed in MVA infected HeLa cells (Sutter and Moss, 1992; Valderrama et al., 2006). In addition, a mutant VACV WR F11L-deletion virus showed a smaller plaque size phenotype upon infection of monkey BSC-40 cells (Morales et al., 2008) or BS-C-1 cells (Cordeiro et al., 2009). These observations prompted us to assess whether impaired F11L function contributes to the restricted host-range of MVA.

After removing the fragmented F11L gene locus from the MVA genome we reinserted a functional F11L gene alone, or in combination with K1L, and tested the resulting viruses upon infection of permissive and non-permissive cells. Restoration of F11L rescued the motility of MVA infected cells, confirming that the reinserted F11L gene product functioned properly. However, infections with recombinant MVA-F11L, MVA-F11L-K1L or wild-type MVA revealed highly comparable replicative capacities and indistinguishable phenotypes of virion morphogenesis. This demonstrates that restoring F11L alone or combined with K1L does not suffice to relieve the host-range restriction of MVA.

Results

Reinsertion of the F11L gene into the MVA genome

To test whether the absence of functional F11L is responsible for MVA's replication defect in mammalian cells, we reintroduced an intact F11L ORF into the MVA genome. Large parts of the F11L coding sequence are still present in the MVA genome, but due to a point mutation and two further deletions the gene is defective and probably translates a small aberrant protein (Fig. 1A).

We first generated a MVA deletion mutant lacking most of the genomic F11L sequence but preserving 89 bp at its 5′-end to retain the adjacent late promoter sequence of the essential F10L gene (Lin and Broyles, 1994). This virus is referred to as MVAΔF11L (Fig. 1B). In a second step, a wild-type copy of F11L including its authentic promoter sequence, amplified by PCR from VACV WR DNA, was inserted into the deletion III region of MVAΔF11L, resulting in the recombinant virus MVA-F11L (Fig. 1B).

Restoration of the VACV host-range gene K1L extends MVA growth capacity to rabbit RK-13 cells (Meyer et al., 1991; Sutter et al., 1994). Thus, we additionally generated a recombinant virus carrying the complete sequences for both F11L and K1L (MVA-F11L-K1L) inserted into the deletion III region (Fig. 1B) in order to test the possibility that both genes are needed to reconstitute MVA replication competence in mammalian cells. A comparable recombinant MVA virus carrying only the sequences for K1L, (MVA-K1L) served as a control. We checked the genetic features of these mutant/recombinant MVA viruses by PCR analyses of viral DNA prepared from infected HeLa cells (Fig. 1C). Primers annealing to sequences in F11L that are deleted in the MVA genome (Fig. 1A) confirmed insertion of the full-length F11L gene into the genome of MVA-F11L-K1L and MVA-F11L (Fig. 1C, top panel). Using flanking primers binding upstream and downstream of the original F11L locus, we confirmed deletion of residual F11L sequences in MVAΔF11L and the related viruses MVA-F11L-K1L and MVA-F11L (Fig. 1C, middle panel). Finally, co-insertion of the VACV K1L gene was analyzed with primers annealing within the K1L ORF present in MVA-F11L-K1L and MVA-K1L (Fig. 1C, bottom panel).

To monitor for synthesis of the corresponding proteins, we tested lysates from infected HeLa cells by Western blotting using antibodies directed against VACV F11 and K1 proteins (Fig. 1D). As expected, the MVA recombinant viruses produced F11 and K1 proteins in amounts comparable to the control virus. Sequence analysis (Poxvirus Bioinformatics Resource Center, www.poxvirus.org) suggested conservation of the 95N-terminal amino acids of the MVA F11 protein including one of the peptides we had used for immunization to generate F11 specific antibodies. However, we failed to detect a corresponding small aberrant F11 protein in Western blot analysis of lysates from MVA infected cells (data not shown) implying that this protein is instable or not recognized by our antibodies.

MVA-F11L infection induces cell motility

F11L gene function is necessary to support motility of VACV WR-infected cells as demonstrated in tissue culture by a wound healing assay (Valderrama et al., 2006). This assay determines the capacity of a virus to induce migration of infected cells into a defined cell-free area generated by scratching a confluent cell monolayer with a pipette tip to create a “wound”. Confluent BS-C-1 cell monolayers were infected at a multiplicity of infection (MOI) of 10 with MVA-F11L, MVA-F11L-K1L, VACV WR and MVA. Defined wound areas were selected and documented by photography 0 and 24 hours post infection (hpi). Clearly, more BS-C-1 cells moved into the wound area when infected with recombinant MVA carrying a functional F11L gene (MVA-F11L and MVA-F11L-K1L) compared to MVA infected cells, but VACV WR infection resulted in even higher levels of cell motility (Fig. 2A). These differences were statistically significant, and suggest that despite regaining F11L function the motility of MVA infected cells remains slightly impaired, while additional expression of K1L had no influence on motility (Fig. 2B).

To assess another implication of F11 function, we monitored the rearrangement of actin filaments after VACV infection. Recently, it was observed that F11L expression coincides with loss of central actin stress fibers within the first few hours post VACV infection (Morales et al., 2008). In MVA or mock infected cells the whole cell body is crossed by actin stress fibers, whereas in VACV WR-infected cells actin filaments are concentrated at the cell's edge. We infected HeLa cells with MVA, MVA-F11L and VACV WR at a MOI of 20 and stained the actin cytoskeleton with phalloidin–rhodamine (Fig. 2C). DAPI staining of viral and cellular DNA was used to identify virus-infected cells. In VACV WR and MVA-F11L infected cells we observed the characteristic loss of central actin stress fibers, as described above. As expected, the actin cytoskeleton remained unchanged upon MVA or mock infection. These findings not only concur with the known function of F11, i.e. binding and inhibiting RhoA signaling, but also further confirm that this gene function was successfully reintroduced into the MVA-F11L recombinant viruses.

F11L expression does not extend the cellular host-range of MVA

The growth deficiency in mammalian cells is a characteristic feature of VACV MVA. Carroll and Moss (1997) suggested classifying cell lines as non-permissive, semi-permissive and permissive for MVA replication based on the increase of infectious virus 72 h after a low MOI infection. To test whether expression of F11L alone or in combination with K1L can alter the replicative capacity of MVA, we infected confluent monolayers of CEF (permissive), BS-C-1 cells (semi-permissive), and human HeLa or mouse NIH-3T3 cells (non-permissive) with MVA-F11L, MVA-F11L-K1L, MVA-K1L, MVA and

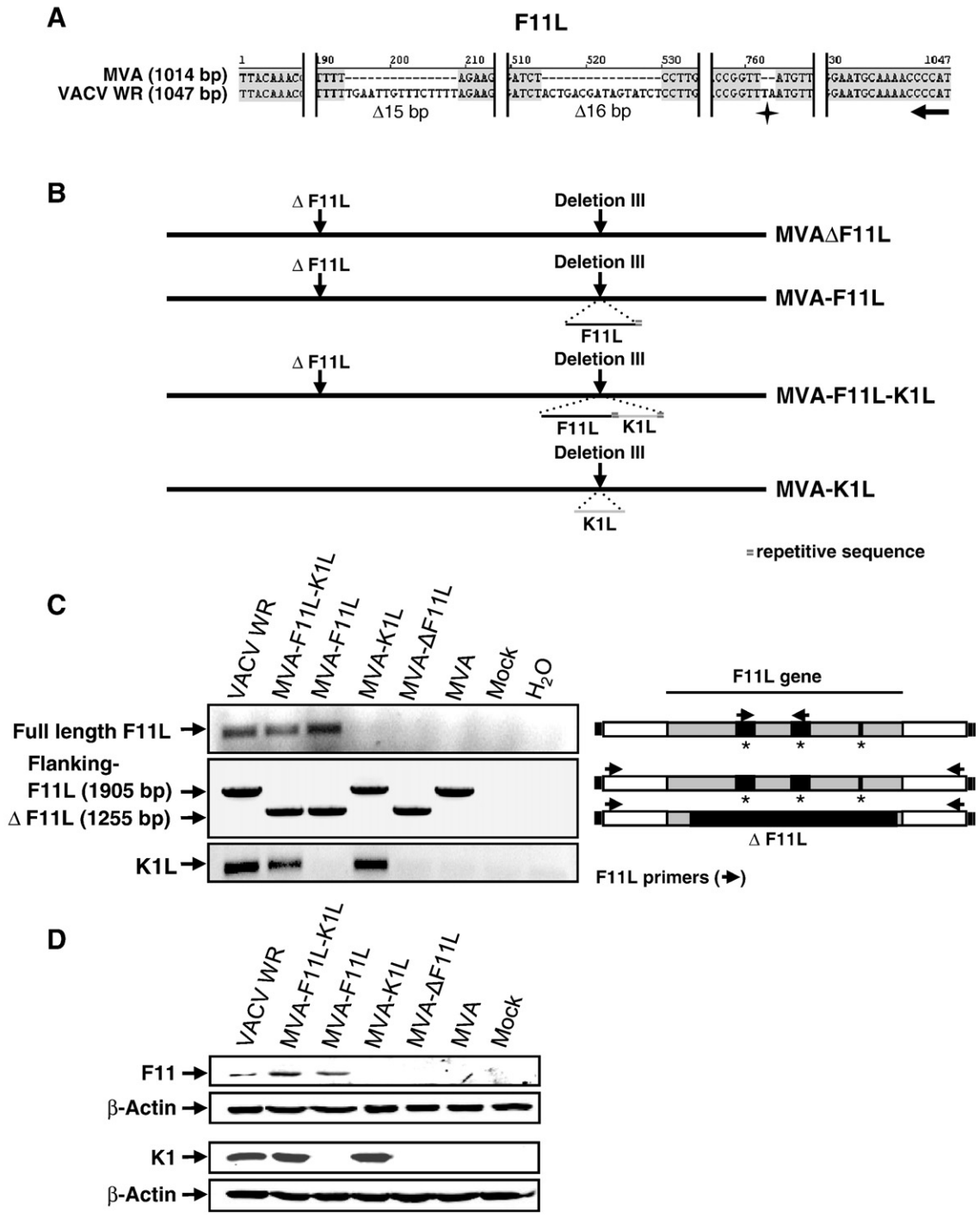


Fig. 1. (A) Alignment of F11L ORF in MVA and VACV WR. The sequence identity is 96%. The F11L ORF of MVA is truncated by 33 bp compared to VACV WR, due to the indicated 15 bp and 16 bp deletions, plus the 2 bp marked by a star that generate a nonsense mutation. Arrow: start codon. (B) Schematic illustration of the recombinant viral genomes. The F11L locus was deleted as described in Materials and methods, and deletion III already exists in the MVA genome. Inserted F11L (black) and K1L (light grey) were derived via recombination between vectors carrying cloned PCR-amplified VACV sequences and viral genomes. Dark grey boxes: repetitive LacZ sequences flanking K1L. (C) PCR analysis of DNA from cells infected with the indicated viruses. Top panel: full-length F11L fragment, generated using primers annealing to regions of F11L deleted in MVA (see A). Middle panel: genomic MVA F11L locus amplified with flanking primers annealing 479 bp upstream and 412 bp downstream of the F11L gene. Bottom panel: K1L-specific PCR fragments. Right: schematic representation of the F11L gene and the positions of the primers (arrows) used for PCR. The black regions indicate deletions, and those marked with asterisks are already deleted in the F11L sequences of MVA (see A). (D) Western blot analysis of F11L and K1L. HeLa cells were mock infected or infected with indicated viruses at a MOI of 5. Protein lysates were prepared 5 hpi. β-actin detection served as a control for equal loading.

VACV WR using a MOI of 0.05. In addition, we infected RK-13 cells to monitor whether MVA replication is rescued by reinsertion of the K1L host-range gene.

Disappointingly, after immunostaining the infected cell monolayers, we observed that virus plaques formed by MVA expressing F11L were almost identical to MVA wild-type virus plaques. Under permissive conditions we detected similar plaque sizes and morphologies. Reinsertion of F11L with or without K1L seemed to have no influence on MVA spreading in HeLa and NIH-3T3 cells. Only single infected NIH-3T3 or HeLa cells were detected 72 hpi with MVA-F11L or MVA-F11L-K1L, as seen with the parental MVA (Fig. 3). Also on semi-permissive (BS-C-1) or permissive cells (CEF) we observed no

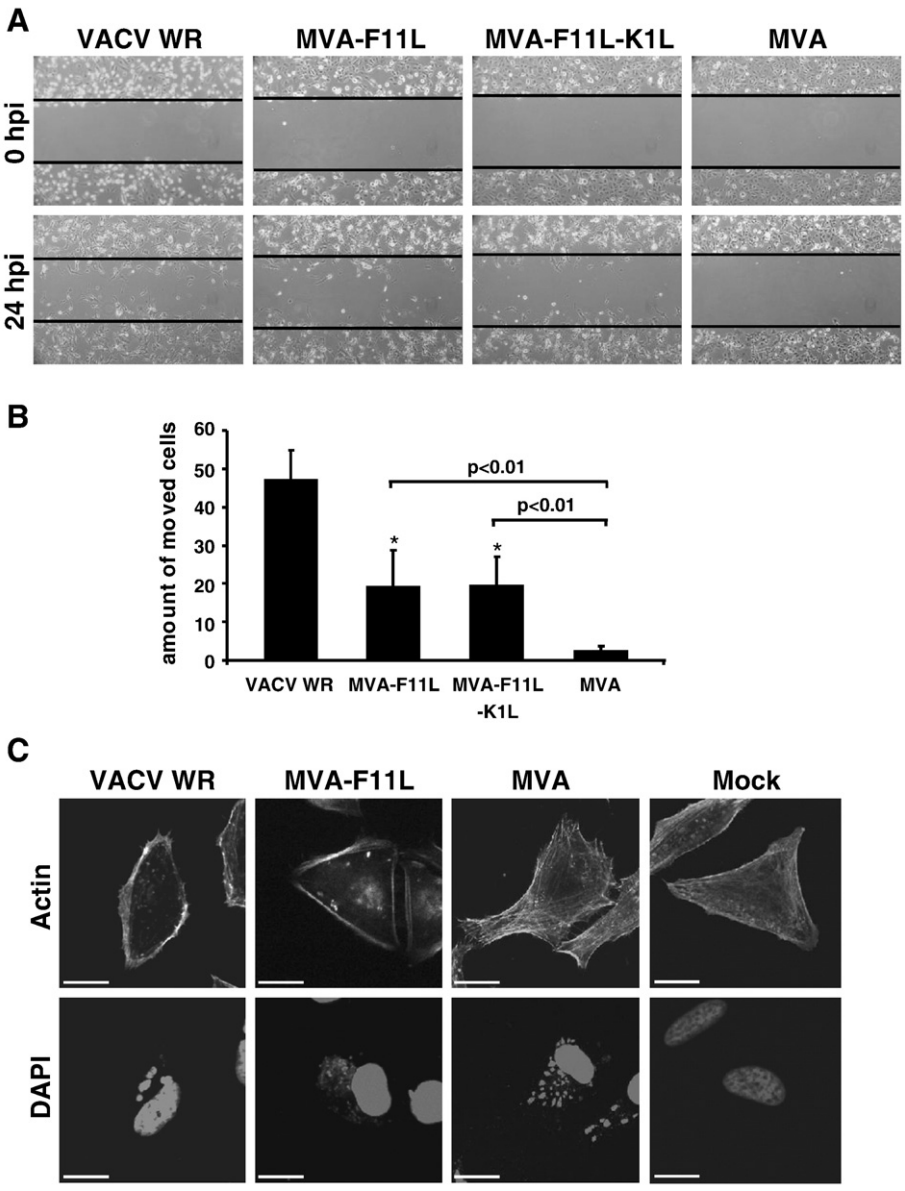


Fig. 2. (A and B) Wound healing assays. Confluent BS-C-1 cell monolayers, starved for two hours, were infected at a MOI of 10 with different MVA viruses, plus VACV WR as a positive control. Wounds were then scratched into the monolayer. (A) Each wound was photographed 0 and 24 hpi with the indicated viruses. Bars indicate the initial wound width. (B) Average number of cells entering the wound area 24 hpi. The plotted numbers represent eight independent experiments. (C) VACV induced rearrangements of the actin cytoskeleton. HeLa cells were infected with the indicated viruses at a MOI of 20. Cells fixed 4 hpi were stained for actin using phalloidin–rhodamine, and viral and cellular DNA using DAPI. Typical cells are shown. Bars: 20 µm.

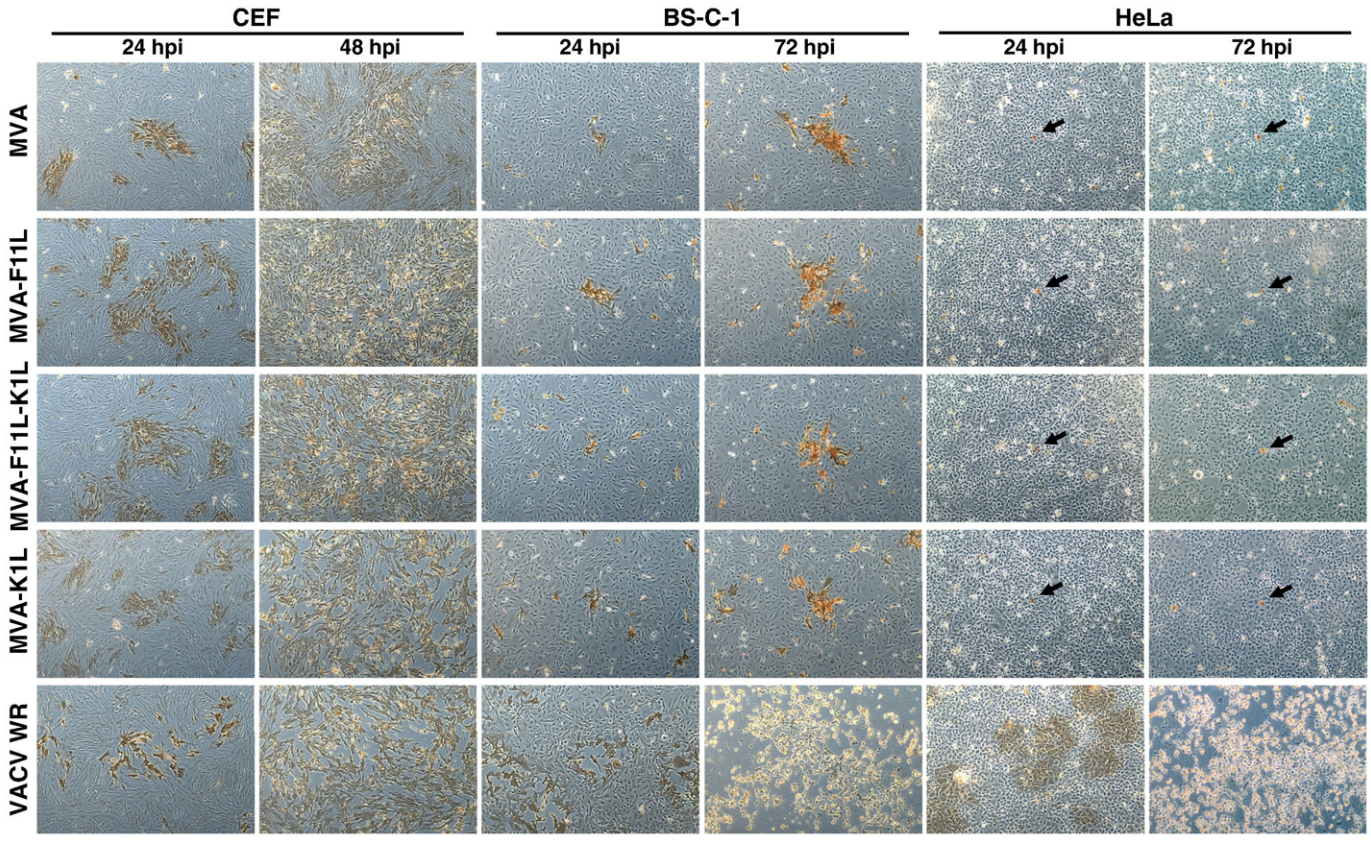
altered MVA-F11L or MVA-F11L-K1L plaque size compared to MVA. Replication of MVA in RK-13 cells was only dependent on K1L expression. In addition, insertion of F11L did not influence MVA plaque morphology. Although the lack of F11L in VACV WR infection negatively influences viral spread, addition of F11L seems to have no influence on MVA infection in the cells we analyzed.

To analyze and quantify viral growth and spread in more detail, we performed multiple-step growth analyses. We infected confluent monolayers of HeLa, NIH-3T3, BS-C-1, RK-13 and CEF cells with the indicated viruses at a MOI of 0.05 and determined viral titers 0, 12, 24, 48 and 72 hpi. Fig. 4 shows the average results of two independent experiments. There is no replication advantage for the recombinant MVA F11L viruses compared to MVA or MVAΔF11L. In HeLa and NIH-3T3 cells MVA-F11L and MVA-F11L-K1L were unable to produce progeny (Figs. 4B and D). In semi-permissive BS-C-1 cells MVA-F11L and MVA-F11L-K1L showed 10–15 times increased titers similar to those determined for MVA, MVAΔF11L or MVA-K1L by 72 hpi (Fig. 4A).

There were no significant differences in growth capacity between MVA-F11L-K1L and MVA-K1L in RK-13 cells, both of which produced about 1000-fold more progeny than the non-replicating MVA, MVA-F11L or MVAΔF11L (Fig. 4C). Interestingly, at 72 hpi VACV WR produced about 10-times more viruses in RK-13 cells than MVA-K1L or MVA-F11L-K1L. As anticipated, in CEF cells we detected no difference in viral titers between MVA and the recombinant viruses (Fig. 4E). These results clearly show that reinsertion of functional F11L alone or in combination with VACV K1L cannot change the general growth restriction of MVA in mammalian cells.

F11L expression does not alter virion morphogenesis

To determine whether MVA with a functional F11L is capable of undergoing additional processing steps in the virion maturation process, we infected confluent HeLa cell monolayers and analyzed particle morphogenesis by electron microscopy at 24 hpi. In MVA



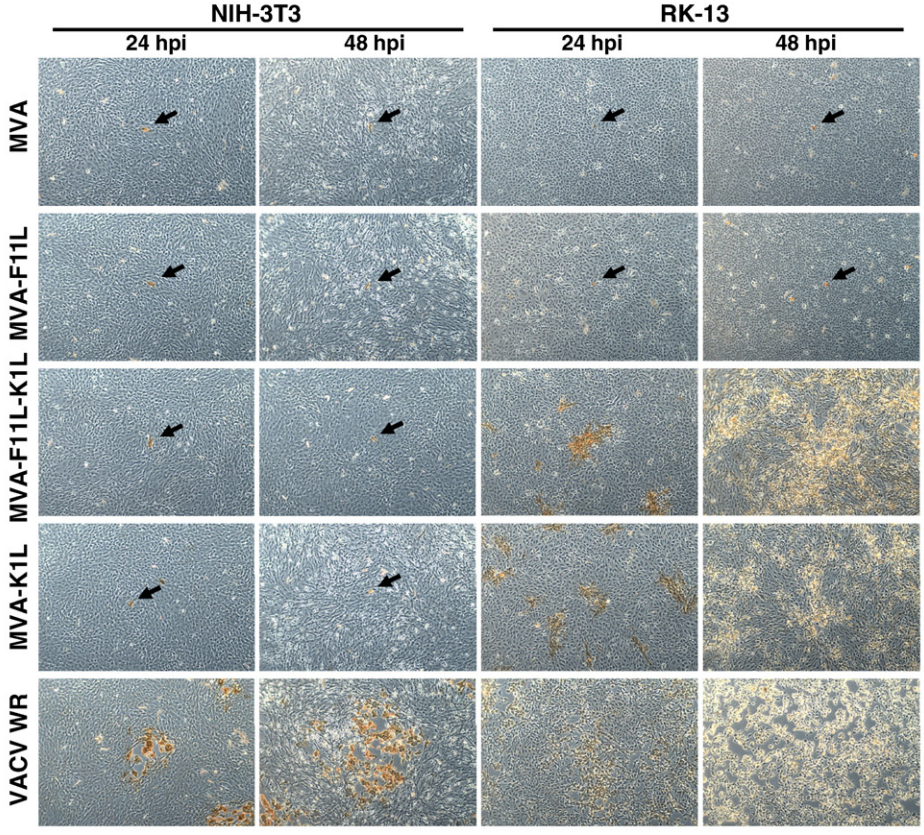


Fig. 3. Analysis of viral spread. Confluent monolayers of BS-C-1, HeLa, NIH-3T3, RK-13 and CEF cells were infected with the indicated viruses at a MOI of 0.05. After the indicated time points, cells were fixed and immunostained with an anti-vaccinia serum. Arrows indicate single infected cells.

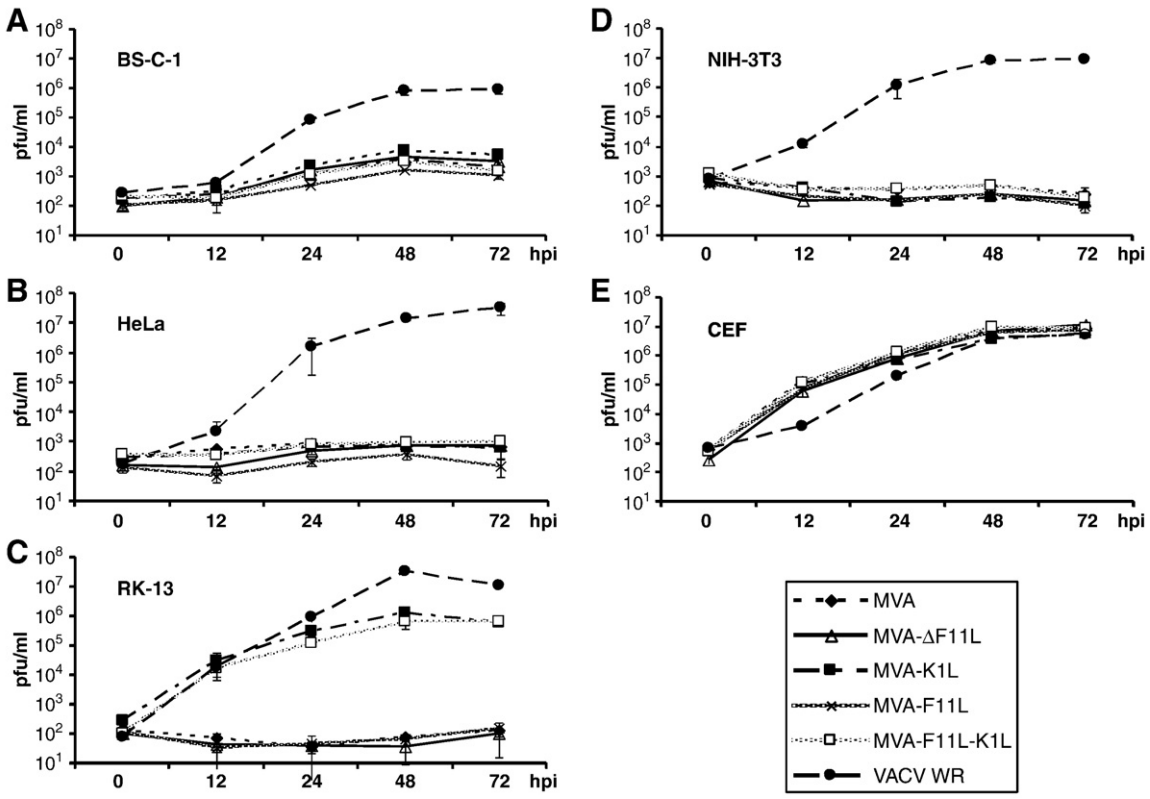


Fig. 4. Multiple step-growth analysis. Monolayers of BS-C-1 (A), HeLa (B), RK-13 (C), NIH-3T3 (D) and CEF (E) cells were infected with the indicated viruses at a MOI of 0.05. Zero, 12, 24, 48 and 72 hpi cells and supernatants were harvested and virus titers were determined by titration on CEF. The average titers of two independent experiments are plotted at each time point.

infected HeLa cells we found accumulated spherical immature virions as previously described (Sutter and Moss, 1992) (Fig. 5A). In MVA-F11L infected cells, these accumulated particles showed no obvious difference in virion morphology compared to MVA infected cells (Fig. 5B). Fully matured viral particles were only detected in VACV WR-infected HeLa cells (Fig. 5C). We conclude that expression of a functional F11L in recombinant MVA infected HeLa cells is not sufficient to alter the phenotype of virion morphogenesis normally observed with MVA wild-type infection.

Discussion

The host-range phenotype of MVA in HeLa and many other cells of mammalian origin is characterized by a block in virion morphogenesis resulting in the accumulation of immature virions (Sutter and Moss, 1992). A similar phenotype was observed after F11L gene knock-down in VACV WR-infected HeLa cells, provoking a defect in virion morphogenesis and decreased viral growth (Valderrama et al., 2006). This implicated a function of F11 in virion morphogenesis, which was further supported by its co-localization with viral factories (Valderrama et al., 2006). Since MVA presumably does not encode a functional F11 protein, we hypothesized that reconstituting the F11L gene in MVA might beneficially modulate MVA infection in mammalian cells. In addition, we analyzed whether simultaneous co-expression of the VACV host-range gene K1L could contribute to a potential host-range function of F11.

Reintroduction of F11L gene sequences into MVA resulted in F11 protein expression, cell motility and rearrangement of the actin cytoskeleton in infected cells. Migration of BS-C-1 cells was induced after infection with F11L-positive viruses (VACV WR, MVA-F11L and MVA-F11L-K1L). Although the number of migrating cells was significantly higher in MVA-F11L compared to MVA infected cells, it clearly did not reach the cell numbers found after VACV WR infection (Fig. 2B). A rescue of actin remodeling by F11L expression in MVA infection was first described by Valderrama et al. (2006) and further characterized by others (Morales et al., 2008). Our observed F11-dependent VACV-induced remodeling of the actin cytoskeleton (Fig. 2C) resembled MVA infected PtK-2 cells transiently expressing F11L (Morales et al., 2008).

F11 induces cell motility by directly binding to the small GTPase RhoA, inhibiting its downstream signaling (Valderrama et al., 2006). However, Arakawa et al. (2007) speculated that two additional RhoA-family proteins, Rac or Cdc42, involved in regulating the actin and microtubule cytoskeleton, might be targeted by other viral proteins encoded by VACV WR, and this could have an impact on virus-induced cell motility. Although our MVA F11L recombinants did express the same amounts of F11 as VACV WR, the fact that cell motility was not induced to VACV WR levels (Figs. 2A and B) suggests that F11 is not the only VACV protein involved in promoting cell migration. MVA lacks many other genes found in VACV WR, any of which could additionally influence cell migration.

One gene absent in MVA that we also analyzed here is K1L. The K1 protein interacts with cellular ACAP2, a GTPase activating protein (GAP) for Arf6 (Bradley and Terajima, 2005). Although the ACAP2 interaction was not suggested as being linked to the host-range function of K1 (Meng and Xiang, 2006), interestingly, Arf6 is involved in actin remodeling and cell migration (Donaldson, 2003). Logically, this would make K1 a good candidate for being involved in VACV infection-induced cell migration. Our data, however, clearly indicate that K1 is not the missing factor, since wound healing assays revealed no differences between MVA-F11L and MVA-F11L-K1L infected cell migration (Fig. 2B).

The central question in our study was whether restoring the F11L gene in MVA would change its replicative capacity, hence possibly overcoming MVA's host-range restriction in mammalian cells. However, our multiple-step growth analyses in mammalian cells

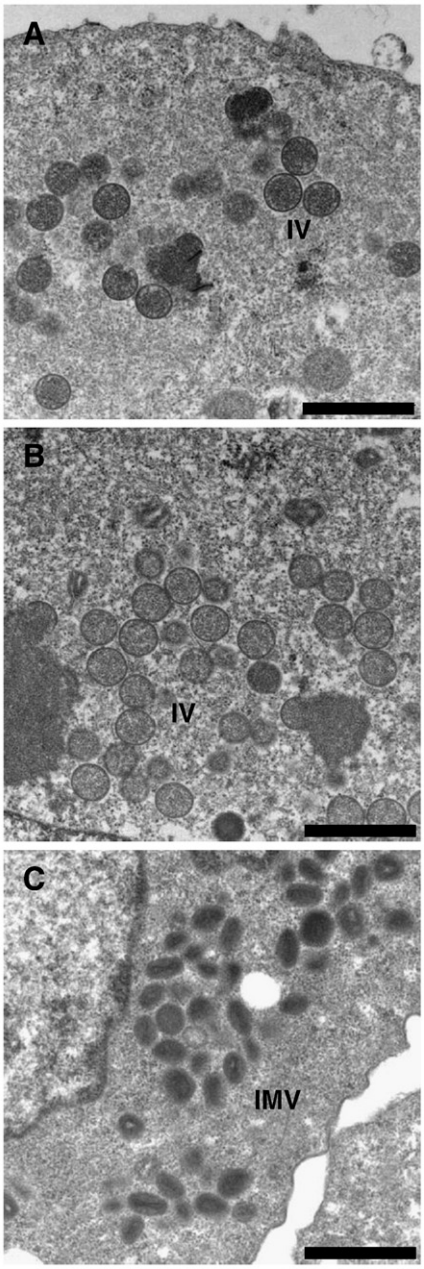


Fig. 5. Analysis of virion morphogenesis. Confluent HeLa cell monolayers were infected with MVA (A), MVA-F11L (B) and VACV WR (C) at a MOI of 5. Cells were fixed 24 hpi and epon embedded slides were prepared. Bars: 1000 nm. Immature (IV) and intracellular mature virions (IMV) are indicated, the latter only visible in the VACV WR positive control.

failed to detect any F11-specific modulation of virus growth in the cell types investigated. Moreover, the permissiveness of the cells played no role. This was further supported by electron microscopy of virions in infected HeLa cells demonstrating that F11L gene expression in MVA did not alter the characteristic phenotype of incomplete virion morphogenesis under non-permissive conditions (Sutter and Moss, 1992). In contrast, the expected host-range rescue upon K1L insertion was easily confirmed, since both recombinant viruses expressing K1L (MVA-K1L and MVA-F11L-K1L) productively replicated in RK-13 cells.

Morales et al. (2008) showed that a VACV WR F11L-deletion mutant (VACVΔF11L) induced a smaller plaque size on BSC40 cell monolayers than plaques induced by VACV WR. Therefore, we analyzed plaque formation of the recombinant MVA on CEF (permissive for MVA), BS-C-1 (semi-permissive for MVA) as well as RK-13, HeLa and NIH-3T3 cells (all non-permissive for MVA). However, reconstituting F11L in MVA had no influence on viral spread of recombinant MVA in the tested cell lines (Fig. 3). This finding may agree with our data from the high MOI wound healing assays (Fig. 2), since it may be difficult to find a difference in plaque size between F11L positive MVA and MVA wild-type, considering motility was only partially restored by F11L expression. Alternatively, cell motility may not be the major factor in virus transmission. The induction of actin tails was also shown to promote viral spread (Ward and Moss, 2001) and though the ability of MVA to form actin tails was found to be conserved in CEF (Meiser et al., 2003) other data suggested deficient formation of actin tails upon infection of BSC-40 cells (Gallego-Gomez et al., 2003). Moreover, even upon high MOI infection the overall growth kinetics of MVA are reduced in BS-C-1 cells in comparison to VACV WR (Carroll and Moss, 1997) (our data not shown). Thus, we believe that rather the kinetics of virus replication/virion formation are the limiting factors in MVA spread at least in BS-C-1 cells.

Although not seen in our experiments, F11 function in VACV WR was reported to contribute to VACV spread *in vitro* and *in vivo*, and deletion of F11L in VACV was suggested as a potential approach to improve the safety of VACV-based vaccines (Cordeiro et al., 2009). Importantly, we show here that F11L alone cannot influence the growth capacity of MVA in non-permissive cells. Restoring (an)other VACV host-range gene(s) in addition to K1L is probably required to relieve this restriction. Thus, it seems very likely that F11L gene expression does not influence the *in vivo* attenuation of MVA. In contrast, it is tempting to speculate that restoring F11L and possibly the migration of MVA infected cells under non-permissive (*in vivo*) conditions might influence vaccine immunogenicity.

Furthermore, by deleting the residual F11L sequence in MVA we prove that this sequence is dispensable, since MVAΔF11L and MVA wild-type behaved alike. We suggest it as an alternative site for inserting exogenous sequences into the MVA genome, as was also already shown for the F11L orthologue in fowlpoxvirus (Boulanger et al., 2002).

In conclusion, the question posed by Valderrama et al. (2006) as to whether F11 could contribute to overcoming the host-range restriction of MVA in mammalian cells now clearly has a negatively answer, and co-expression of the host-range gene K1L did not change the outcome.

Materials and methods

Cells, viruses and antibodies

HeLa and BHK-21 cells were grown in Roswell Park Memorial Institute medium (RPMI), NIH-3T3 and BS-C-1 cells in Dulbecco's modified eagle medium (DMEM), and primary chicken embryo fibroblasts (CEF) and RK-13 cells in Eagle's basal medium (EMEM) at 37 °C in 10% CO_2. CEF were freshly prepared and propagated for one passage. All other cell lines were obtained from ATCC (CCL-2, CRL-1658, CCL-10, CCL-37 and CCL-26). All media were supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin/streptomycin and EMEM additionally with non-essential amino acids (Invitrogen, Karlsruhe, Germany). Modified vaccinia virus Ankara II new (MVA) has been described previously (Staib et al., 2003). Vaccinia virus strain Western Reserve (VACV WR) was kindly provided by Bernard Moss, National Institutes of Health, Bethesda, Md.

Polyclonal antibodies against F11 and K1 were raised by immunizing rabbits with two peptides (aa 11–23 RSKMLKRGSRKSSSIl and aa 105–120 LDAVLDRDGNFRPADC for F11, 84–98 SGMDDSQFDDKGNTAL and aa 243–258 HVEYKSDSYTKDLDIV for K1) derived from the VACV WR sequence in case of F11 and VACV Copenhagen sequence in case of K1 protein (Eurogentec, Cologne, Germany). Monoclonal antibody against β-Actin was purchased from Sigma-Aldrich (Taufkirchen, Germany). Secondary antibodies against mouse and rabbit IgG, coupled to

IRDye680 and IRDye800 were purchased from LI-COR Biosciences (Bad Homburg, Germany).

Construction of vector plasmids

All plasmid constructions were carried out using standard cloning procedures (Sambrook et al., 1989). Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany).

The K1L gene was amplified by PCR using VACV WR DNA as a template. The following primers were used: 5′-AATTCTGCAGATCTCCTTAATATGGGTACG-3′ and 5′-AATTGGATCCAATGTTAACAAAAATGTGGG-3′. The PstI and BamHI restriction sites (underlined) were used to insert the amplified K1L gene into a transfer vector to generate pLW9-E-K1L, which carries 500 bp up- and downstream sequences flanking deletion III of the MVA genome for recombination.

To delete most of the residual F11L sequences of MVA, the flanking sequences were amplified by PCR using the primers 5′-AAAAGCGGCCGCGTGAATAATTTCATTACCAAAG-3′ and 5′-AAAAGGGCCCTACTGTGAACTCCGCCGTAG-3′ for the downstream region, including NotI and ApaI restriction sites, and the primers 5′-AAAAGAGCTCCAATGGAATGCAAAACCCCATAG-3′ and 5′-AAAAACGCGTCTACTAACGACCATATTCCAG-3′ with SacI and MluI restriction sites (underlined) for the upstream region. Both amplified DNA fragments were inserted into the pΔK1L plasmid (Staib et al., 2000) to obtain the F11L deletion plasmid pΔF11L.

Functional F11L was amplified by PCR using VACV WR DNA as a template and the primers 5′-ATAAGAATGCGGCCGCATACGTTAGTAGCTCTTATAAAG-3′ and 5′-TTGGCGCGCCGGCAACACCCATTTATTGTAC-3′ containing NotI and AscI restriction sites (underlined) for insertion into a MVA deletion III transfer vector carrying K1L surrounded by repetitive sequences, to form pGSV23-F11L. Genomic DNA was isolated from infected cells using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out using Taq polymerase (Roche, Germany) according to the manufacturer's instructions and was verified by sequencing.

Generation of recombinant viruses

Confluent BHK-21 cells were grown in 6-well tissue culture plates and infected with MVA at a multiplicity of infection (MOI) of 0.05. 1.5 hours post infection (hpi) either pLW9-E-K1L or pΔF11L were transfected with lipofectamine 2000 (Invitrogen, Karlsruhe, Germany) using the procedure recommended by the manufacturer. 48 h later, cells and supernatant were harvested, freeze-thawed three times and homogenized in a cup sonicator (Sonopuls HD 200; Bandelin, Berlin, Germany). The subsequent procedure has been described previously (Staib et al., 2000).

MVA-F11L and MVA-F11L-K1L were generated by infecting BHK-21 cells with MVAΔF11L and transfection of pGSV23-F11L as described above. The K1L gene was either lost by repeated plaque purification of recombinant MVA-F11L-K1L on CEF due to its flanking repetitive sequences, or preserved by passaging on RK-13 cells. Viral DNA from cloned MVA isolates was routinely analyzed by PCR as described previously (Staib et al., 2000). The presence of the original F11L locus was analyzed using the primers 5′-AAAAGGGCCCTACTGTGAACTCCGCCGTAG-3′ and 5′-AAAAACGCGTCTACTAACGACCATATTCCAG-3′, which were already used for cloning the flanks of pΔF11L. To prove whether the inserted full-length F11L is present in MVA-F11L and MVA-F11L-K1L we used the primers 5′-TGAATTGTTTCTTTTAGAAGTG-3′ and 5′-AGGAGATACTATCGTCAGTA-3′ which anneal to regions of the F11L sequence that are deleted in the residual F11L locus of MVA (Fig. 1A). The presence of K1L was analyzed using the primers 5′-ACGTGCGTCTAGTATGTAC-3′ and 5′-TCTCAATGTCTTTATTATCTATA-3′.

Western blot analysis

Confluent monolayers of HeLa cells were infected at a MOI of 10 with the appropriate viruses. At 5 hpi cell lysates were prepared as described previously (Ludwig et al., 2006) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electroblotted onto a PVDF membrane (Bio-Rad, Munich, Germany). After blocking, membranes were incubated with primary antibodies at 4 °C overnight. After washing, the blots were incubated with secondary antibodies for one hour at room temperature. Detection of fluorescence was performed with the Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany).

Wound healing assay

Confluent monolayers of BS-C-1 cells in 6-well cell culture dishes were starved for 2 h and afterwards infected with VACV at a MOI of 10. After virus adhesion for one hour, several wounds were scratched into the cell monolayer with a transparent tip. Cells were washed three times with PBS before adding cell culture medium without serum. Wounds were photographed (DX40 camera, Kappa, Gleichen, Germany) directly and 24 h later at marked positions with phase contrast at a magnification of 100 (Axiovert 40 CLT, Carl Zeiss, Göttingen, Germany).

Fluorescence microscopy

HeLa cells were grown on coverslips and infected at a MOI of 20. 20 min after infection, cells were washed three times with warm, serum-free medium to ensure synchronized infection. 3 hpi cells were fixed with 3.7% paraformaldehyde in PBS for 15 min, afterwards washed twice with PBS and then permeabilized for 10 min with 0.1% Triton-X-100 in PBS at room temperature. Cells were blocked for 30 min with 5% BSA in PBS to prevent unspecific binding. The actin cytoskeleton was stained with phalloidin–rhodamine (Chemicon International, Temecula, USA) and DNA with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Steinheim, Germany) for 45 min in 2% BSA in PBS. After washing three times with PBS coverslips were mounted using mowiol. Confocal microscopy was performed using a LSM510 META (Carl Zeiss, Göttingen, Germany) at a magnification of 630. Images were processed using Zeiss LSM image browser version 3.5.

Electron microscopy

Confluent HeLa cells grown in 10 cm cell culture dishes were mock infected or infected with VACV at a MOI of 5. After one hour of viral adhesion the cell media was renewed. 24 hpi cells were fixed for 45 min with 2.5% glutaraldehyde in medium. Epon embedding and staining procedures have been described previously (Steidl et al., 2004). Scans were documented with the help of a transmission electron microscope (Type 902, Carl Zeiss, Göttingen, Germany) with a slow-scan CCD camera.

Analysis of viral growth and plaque formation

Confluent monolayers of indicated cell lines or primary CEF were grown in 6-well plates and infected with VACV at a MOI of 0.05. For multiple-step growth curves, viruses were adsorbed to the cells for 1 h at 37 °C, washed two times and cultivated at 37 °C in media with 2% serum. Zero, 12, 24, 48 and 72 hpi cells and supernatants were harvested, freeze-thawed and sonicated three times. Virus titers were determined on CEF cells following standard procedures as described previously (Hornemann et al., 2003).

For spreading analysis, immunostaining of infected cells was performed as described previously (Staib et al., 2004). Briefly, cells were infected with the indicated viruses at a MOI of 0.05. 24, 48 and 72 hpi cells were fixed with ice-cold methanol/acetone, blocked with PBS containing 3% FCS and stained with polyclonal VACV antiserum (Abgene, Epsom, United Kingdom) and an HRP-conjugated secondary antibody. Detection was performed using 0.3% dianisidine solution in PBS with 0.01% H_2O_2 as the substrate. Pictures were taken by phase contrast microscopy (Axiovert 40 CLT, Carl Zeiss, Göttingen, Germany) with 100-fold magnification.

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References

Antoine, G., Scheiflinger, F., Dorner, F., Falkner, F.G., 1998. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. Virology 244, 365–396.

Arakawa, Y., Cordeiro, J.V., Way, M., 2007. F11L-mediated inhibition of RhoA–mDia signaling stimulates microtubule dynamics during vaccinia virus infection. Cell Host Microbe 1, 213–226.

Backes, S., Sperling, K.M., Zwilling, J., Gasteiger, G., Ludwig, H., Kremmer, E., Schwantes, A., Staib, C., Sutter, G., 2010. Viral host-range factor C7 or K1 is essential for modified vaccinia virus Ankara late gene expression in human and murine cells, irrespective of their capacity to inhibit protein kinase R-mediated phosphorylation of eukaryotic translation initiation factor 2alpha. J. Gen. Virol. 91, 470–482.

Boulanger, D., Baier, R., Erfle, V., Sutter, G., 2002. Generation of recombinant fowlpox virus using the non-essential F11L orthologue as insertion site and a rapid transient selection strategy. J. Virol. Meth. 106, 141–151.

Bradley, R.R., Terajima, M., 2005. Vaccinia virus K1L protein mediates host-range function in RK-13 cells via ankyrin repeat and may interact with a cellular GTPase-activating protein. Virus Res. 114, 104–112.

Carroll, M.W., Moss, B., 1997. Host range and cytopathogenicity of the highly attenuated MVA strain of vaccinia virus: propagation and generation of recombinant viruses in a nonhuman mammalian cell line. Virology 238, 198–211.

Cordeiro, J.V., Guerra, S., Arakawa, Y., Dodding, M.P., Esteban, M., Way, M., 2009. F11-mediated inhibition of RhoA signalling enhances the spread of vaccinia virus in vitro and in vivo in an intranasal mouse model of infection. PLoS ONE 4, e8506.

Donaldson, J.G., 2003. Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. J. Biol. Chem. 278, 41573–41576.

Drexler, I., Heller, K., Wahren, B., Erfle, V., Sutter, G., 1998. Highly attenuated modified vaccinia virus Ankara replicates in baby hamster kidney cells, a potential host for virus propagation, but not in various human transformed and primary cells. J. Gen. Virol. 79 (Pt 2), 347–352.

Gallego-Gomez, J.C., Risco, C., Rodriguez, D., Cabezas, P., Guerra, S., Carrascosa, J.L., Esteban, M., 2003. Differences in virus-induced cell morphology and in virus maturation between MVA and other strains (WR, Ankara, and NYCBH) of vaccinia virus in infected human cells. J. Virol. 77, 10606–10622.

Hornemann, S., Harlin, O., Staib, C., Kisling, S., Erfle, V., Kaspers, B., Hacker, G., Sutter, G., 2003. Replication of modified vaccinia virus Ankara in primary chicken embryo fibroblasts requires expression of the interferon resistance gene E3L. J. Virol. 77, 8394–8407.

Lin, S., Broyles, S.S., 1994. Vaccinia protein kinase 2: a second essential serine/threonine protein kinase encoded by vaccinia virus. Proc. Natl. Acad. Sci. U S A 91, 7653–7657.

Ludwig, H., Suezer, Y., Waibler, Z., Kalinke, U., Schnierle, B.S., Sutter, G., 2006. Double-stranded RNA-binding protein E3 controls translation of viral intermediate RNA, marking an essential step in the life cycle of modified vaccinia virus Ankara. J. Gen. Virol. 87, 1145–1155.

Mayr, A., 1976. TC marker of the attenuated vaccinia vaccide strain "MVA" in human cell cultures and protective immunization against orthopox diseases in animals. Zentralbl. Veterinärmed. B 23, 417–430.

Mayr, A., Munz, E., 1964. Changes in the vaccinia virus through continuing passages in chick embryo fibroblast cultures. Zentralbl. Bakteriol. Orig. 195, 24–35.

Meiser, A., Boulanger, D., Sutter, G., Krijnse Locker, J., 2003. Comparison of virus production in chicken embryo fibroblasts infected with the WR, IHD-J and MVA strains of vaccinia virus: IHD-J is most efficient in trans-Golgi network wrapping and extracellular enveloped virus release. J. Gen. Virol. 84, 1383–1392.

Meng, X., Xiang, Y., 2006. Vaccinia virus K1L protein supports viral replication in human and rabbit cells through a cell-type-specific set of its ankyrin repeat residues that are distinct from its binding site for ACAP2. Virology 353, 220–233.

Meng, X., Jiang, C., Arsenio, J., Dick, K., Cao, J., Xiang, Y., 2009. Vaccinia virus K1L and C7L inhibit antiviral activities induced by type I interferons. J. Virol. 83, 10627–10636.

Meyer, H., Sutter, G., Mayr, A., 1991. Mapping of deletions in the genome of the highly attenuated vaccinia virus MVA and their influence on virulence. J. Gen. Virol. 72 (Pt 5), 1031–1038.

Morales, I., Carbajal, M.A., Bohn, S., Holzer, D., Kato, S.E.M., Greco, F.A.B., Moussatche, N., Locker, J.K., 2008. The vaccinia virus F11L gene product facilitates cell detachment and promotes migration. Traffic 9, 1283–1298.

Perkus, M.E., Goebel, S.J., Davis, S.W., Johnson, G.P., Limbach, K., Norton, E.K., Paoletti, E., 1990. Vaccinia virus host range genes. Virology 179, 276–286.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Lab Press, Plainview, NY.

Shisler, J.L., Jin, X.L., 2004. The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. J. Virol. 78, 3553–3560.

Staib, C., Drexler, I., Ohlmann, M., Wintersperger, S., Erfle, V., Sutter, G., 2000. Transient host range selection for genetic engineering of modified vaccinia virus Ankara. Biotechniques 28, 1137–1142.

Staib, C., Lowel, M., Erfle, V., Sutter, G., 2003. Improved host range selection for recombinant modified vaccinia virus Ankara. Biotechniques 34, 694–696.

Staib, C., Drexler, I., Sutter, G., 2004. Construction and isolation of recombinant MVA. Meth. Mol. Biol. 269, 77–100.

Steidl, S., Schule, S., Muhlebach, M.D., Stitz, J., Boller, K., Cichutek, K., Schweizer, M., 2004. Genetic engineering of onco/lentivirus hybrids results in formation of infectious but not of replication-competent viruses. J. Gen. Virol. 85, 665–678.

Stickl, H.A., 1974. Smallpox vaccination and its consequences: first experiences with the highly attenuated smallpox vaccine "MVA". Prev. Med. 3, 97–101.

Stickl, H., Hochstein-Mintzel, V., 1971. Intracutaneous smallpox vaccination with a weak pathogenic vaccinia virus ("MVA virus"). Münch. Med. Wochenschr. 113, 1149–1153.

Sutter, G., Moss, B., 1992. Nonreplicating vaccinia vector efficiently expresses recombinant genes. Proc. Natl. Acad. Sci. U S A 89, 10847–10851.

Sutter, G., Ramsey-Ewing, A., Rosales, R., Moss, B., 1994. Stable expression of the vaccinia virus K1L gene in rabbit cells complements the host range defect of a vaccinia virus mutant. J. Virol. 68, 4109–4116.

Sutter, G., Staib, C., 2003. Vaccinia vectors as candidate vaccines: the development of modified vaccinia virus Ankara for antigen delivery. Curr. Drug Targets Infect. Disord. 3, 263–271.

Valderrama, F., Cordeiro, J.V., Schleich, S., Frischknecht, F., Way, M., 2006. Vaccinia virus-induced cell motility requires F11L-mediated inhibition of RhoA signaling. Science 311, 377–381.

Ward, B.M., Moss, B., 2001. Vaccinia virus intracellular movement is associated with microtubules and independent of actin tails. J. Virol. 75, 11651–11663.

Werden, S.J., Rahman, M.M., McFadden, G., 2008. Poxvirus host range genes. Adv Virus Res. , pp. 135–171.

Willis, K.L., Patel, S., Xiang, Y., Shisler, J.L., 2009. The effect of the vaccinia K1 protein on the PKR-eIF2alpha pathway in RK13 and HeLa cells. Virology 394, 73–81.

Wyatt, L.S., Carroll, M.W., Czerny, C.P., Merchlinsky, M., Sisler, J.R., Moss, B., 1998. Marker rescue of the host range restriction defects of modified vaccinia virus Ankara. Virology 251, 334–342.