Truncation of gene F5L partially masks rescue of vaccinia virus strain MVA growth on mammalian cells by restricting plaque size

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Summary

Modified Vaccinia virus Ankara (MVA) is a candidate vaccine vector that is severely attenuated due to mutations acquired during several hundred rounds of serial passage in vitro. A previous study used marker rescue to produce a set of MVA recombinants with improved replication on mammalian cells. Here we extend the characterisation of these rescued MVA strains and identify vaccinia virus (VACV) gene F5L as a determinant of plaque morphology, but not replication in vitro. F5 joins a growing group of VACV proteins that influence plaque formation more strongly than virus replication and which are disrupted in MVA. These defective genes in MVA confound the interpretation of marker rescue experiments designed to map mutations responsible for the attenuation of this important VACV strain.

Main text

Modified Vaccinia virus Ankara (MVA) is a leading candidate vector for recombinant poxvirus vaccines (Gomez et al., 2008). MVA was the result of several hundred rounds of serial passage starting with the virulent strain chorioallantois vaccinia virus Ankara (CVA) in primary chicken embryonic fibroblasts (Mayr et al., 1975). In contrast to the broad host range typical of VACV, MVA fails to replicate in all but a few mammalian cell lines (Carroll & Moss, 1997, Drexler et al., 1998, Jordan et al., 2009, Okeke et al., 2006). The full genomic sequences of CVA and MVA have been published (Antoine et al., 1998, Meisinger-Henschel et al., 2007). In addition to six large deletions (termed Deletion I to VI), mutations affect coding in more than 60% of the annotated ORFs of MVA compared with CVA (Meisinger-Henschel et al., 2007, Meyer et al., 1991). However, the mutations responsible for the host range restriction of MVA in vitro and its attenuation in vivo remain unknown.

MVA does not form plaques on monolayers of most mammalian cells and this characteristic can be exploited in marker rescue experiments to map genetic lesions underlying the restricted host range. Using cosmids with genomic fragments from a replication-competent VACV strain, referred to as 'Ankara', Wyatt et al (1998) made a set of rescued MVAs that replicate on mammalian cells. These rescued MVAs were selected on the basis of increased plaque size on BS-C-1 cells and the
work broadly mapped the location of the mammalian replication defect of MVA to several regions
at the left end of the genome (Wyatt et al., 1998). One known host range gene, namely SPI-1,
which resides in deletion I, was repaired in some of the rescued MVAs (Shisler et al., 1999). While
SPI-1 may contribute to the host range defect, other work has shown that the major deletions
(even in combination) cannot account for the replication defect of MVA on mammalian cells in
general (Dimier et al., 2011, Meisinger-Henschel et al., 2010).

We began by following up apparent differences in plaque morphology across this set of rescued
MVAs (Melamed et al., 2013, Wyatt et al., 1998). BS-C-1 and HeLa cells were infected with MVA,
Ankara or the rescued MVAs and foci or plaques formed under semisolid media (0.4% w/v
carboxy-methyl cellulose) were immunostained (Staib et al., 2004) at 72 hours post infection
(h.p.i.). MVA failed to form plaques or foci on HeLa cells but small foci made up of a few tightly
packed cells were seen on BS-C-1. The rescued MVAs exhibited a range of plaque morphologies
on BS-C-1 and HeLa cells. A striking difference was seen between the plaques of v51.2 and v44.1
grown on BS-C-1: v51.2 infected cells formed tightly packed piles whereas infection with v44.1
caused the formation of obvious plaques with clearance of the monolayer at the centre (Fig 1a).
The independently rescued v51.1 and v44.2 lineages also formed piles and plaques respectively
(not shown). By contrast, and consistent with the previous report, we observed no difference in
replication rates of v51.2 and v44.1 on BS-C-1, HeLa or IEC-6 cells in multiple step growth
analyses (Fig 1b-d) (Wyatt et al., 1998).

The three cosmids used to produce the rescued MVAs (namely c51, c44 and c47) cover the left
end of the VACV genome, but recombination sites have not been defined (Fig 2a). Three of the
major deletions of MVA (deletions I, V and II) lie within the region shared by c51 and c44 (Meyer et
al., 1991). A simple PCR-based analysis of the rescued MVAs revealed that deletion I, but not V or
II, was repaired both in v51.2 and v44.1 (not shown), consistent with reported PCR detection of the
SPI-1 (deletion I) but not K1L (deletion II) host range genes (Wyatt et al., 1998). By contrast, all
three deletions were repaired in v44/47.1, v44/47.2, v51.1 and v44.2. The relatively small repairs in
v44.1 and v51.2 made these an attractive pair to study further. As an aside, examination of
sequences surrounding the deletions suggest that Ankara is not closely related to CVA and MVA, confirming a recent report (Melamed et al., 2013). Cosmids c44 and c51 overlap substantially and both include most of the HindIII C fragment and the small HindIII N, M and K fragments. However, compared with c51, c44 extends further rightwards and into the start of the HindIII E fragment (Wyatt et al., 1998). This suggested that gene/s in the HindIII F region, unique to c44, were responsible for the larger plaques made by v44.1. To test this, six genes: K6L, F1L, F5L, F11L, F12L and F13L, distributed across this region were sequenced for v44.1, v51.2 and Ankara and compared with those published for MVA. As expected, sequences from v51.2 matched those of MVA for all six genes but in v44.1, K6L, F1L, F5L, and F11L matched Ankara and so were repaired in this virus (region shown in Fig. 2c).

To map the gene/s responsible for the plaque phenotype we carried out a set of marker rescue experiments. Firstly, K6L-F4L and F5L-F11L from Ankara were cloned into plasmids to bisect the region of interest. BHK-21 cells infected with v51.2 (m.o.i.=0.05) were transfected with 1 µg of linearized plasmid using Lipofectamine 2000 (Invitrogen). At 48 h.p.i., virus was harvested and used to infect BS-C-1. A single large plaque was isolated after recombination between the v51.2 genome and the F5L-F11L plasmid. This virus (v51.2/F5L-F11L) was plaque purified and found to contain repaired versions of two truncated genes, F5L and F11L. F5L is transcribed early and predicted to encode a 36.5kDa major membrane protein (Yang et al., 2010, poxvirus.org). The MVA homologue lacks 104 aa of the c-terminus, including a putative transmembrane domain. F11L is required for efficient release of virus particles from infected cells, normal plaque size in vitro and virus spread in vivo (Cordeiro et al., 2009, Morales et al., 2008). Next we tested whether repair of F5L or F11L alone in v51.2 might produce larger plaques. The transfer plasmids for these experiments included a GFP/bsd marker under the control of the VACV strong synthetic promoter downstream of the gene to be repaired (Wong et al., 2011). This allowed visual (eGFP) and drug (blasticidin) selection of recombinant viruses in addition to possible increases in plaque size. A complication of adding GFP/bsd downstream of F5L and F11L is that the promoters of adjacent genes (F4L and F10L, respectively) are separated from their ORF. For this reason, these promoter sequences were repeated after the GFP/bsd marker cassette (Fig. 2d). These direct repeats also
make the marker unstable in the absence of drug selection. The \textit{F5L} and \textit{F11L} rescue plasmids were linearised and transfected into BHK-21 cells infected with v51.2 (m.o.i.=0.05). Viruses with plaques larger than v51.2 were isolated after transfection with \textit{F5L} (v51.2/F5LGb) and \textit{F11L} (v51.2/F11LGb) and after 3-4 rounds of plaque purification on BS-C-1, the fidelity of repairs were verified by sequencing. In the case of v51.2/F5LGb, further passage allowed the isolation of a virus that had lost the \textit{GFP/bsd} marker, but retained the repair of \textit{F5L} (v51.2/F5L).

Having isolated these viruses we compared plaque phenotypes and sizes (Fig 3a, b). Repair of \textit{F5L} alone (v51.2/F5LGb and v51.2/F5L) had a strong effect on plaques: they were larger and there was significant monolayer clearance in the centre. The repair of \textit{F11L} also increased plaque size but did not lead to clearance of cells from their centres. Further, the effect of \textit{F5L} and \textit{F11L} was additive because plaques made by v51.2/F5L-F11L were larger than those of viruses with repairs of \textit{F5L} and \textit{F11L} alone. Next we tested virus growth and found that neither \textit{F5L} (with or without \textit{GFP/bsd}), or \textit{F11L} altered the replication of v51.2 in single or multiple step growth curves (Fig 3d, e). Finally, the use of the \textit{GFP/bsd} marker allowed us to isolate an MVA with \textit{F5L} repaired (MVA/F5LGb). Repair of \textit{F5L} did not improve MVA replication or change plaque size on BS-C-1 cells (Fig 3c, f).

Wyatt et al (1998) concluded that multiple genes must be involved in the host range defect of MVA because non-overlapping cosmids improved replication and additive effects on plaque size were observed when multiple regions were repaired. However, their data are also consistent with a model where more than one gene can rescue replication, but multiple genes contribute to plaque size. We believe this latter model is a better explanation for the profound variation in plaque size but narrow range of virus titres obtained on BS-C-1 for the rescued MVAs as previously reported (Wyatt et al., 1998). It is also supported by the recent finding that v51.1, with a smaller plaque, replicates to higher titres on Vero cells than v44/47.1 (Melamed et al., 2013). From the literature, three VACV proteins that increase plaque size without enhancing replication are inactive or missing in MVA, namely C2, F11 and O1. C2 is a kelch protein that is required for the usual distinct borders of plaques made by VACV strain WR, but is lost from MVA, due to major deletion V (Pires
de Miranda et al., 2003). As noted above, F11 plays roles in virus-induced cell motility (Valderrama et al., 2006) and in normal plaque size (Cordeiro et al., 2009, Morales et al., 2008). O1 is required for sustained activation of the RAF/MEK/ERK pathway and is truncated in MVA. Deletion of O1L decreases the plaque size of CVA (Schweneker et al., 2012). Despite their association with altered plaques, none of these genes has a strong influence on growth of VACV in vitro and for O1L and F11L this has been shown for MVA (Antoine et al., 1998, Morales et al., 2008, Pires de Miranda et al., 2003, Schweneker et al., 2012). F5L is now the fourth VACV gene function missing from MVA that is required for normal plaques, but not replication.

We determined the status (repaired or not) of each of these four genes and plaque phenotypes across the full set of rescued MVAs allowing some further observations (Fig. 3g). 1) Of all the rescued MVAs, v44/47.1 has the largest plaques but their size remains smaller than Ankara. This suggests that genes outside the region mapped by Wyatt et al (1998) affect plaque size or replication. 2) The similarity (no significant difference in size) between v51.2/F5L-F11L and v44.2 suggests that the individual contribution of C2L to plaque size is minor. 3) Repair of F5L and F11L increased plaque size, but F5L was required for the clearance of cells from the middle of plaques. Repairing both genes gave an additive increase in plaque size and together these suggest that F5 and F11 act independently. 4) Plaques from v44/47.1 were larger again than v51.2/F5L-F11L suggesting that a gene in the region covered by c47 also has a strong influence on plaques. The most likely candidate here is O1L, consistent with results obtained when this gene was deleted from CVA (Schweneker et al., 2012). 5) Restoration of F5 and F11 to v51.2 gave larger plaques than v44.1. F5L and F11L are intact in v44.1, but the repairs in this virus do not extend as far to the left of the genome as in v51.2. It seems likely that this region also contains genes that affect replication or plaque formation (Fig 2a, b).

In summary, we have identified the truncation of F5 as a determinant of plaque morphology but not in vitro replication in MVA. Further, the existence of F5L and several other genes required for normal plaque formation complicate the interpretation of work done to map attenuating mutations of MVA, which has assumed plaque size is an accurate surrogate for replication. We also show
here that the relatively small single repair in v51.2 alone produces a substantial improvement in
replication on three mammalian cell lines. Together these lead us to conclude that the range of key
genomic changes associated with the replication defect of MVA in mammalian cells has been
previously overestimated.

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Figure legends

**Fig 1:** Rescued MVAs show a range of plaque size and morphology on BS-C-1 and HeLa cells. (a) Representative plaques formed by the viruses shown on BS-C-1 or HeLa cells under semisolid media. Cells were immunostained at 72 h.p.i. (100× final magnification, scale bars 100µm). (b-d) Multiple step growth analysis (m.o.i.=0.01) in (b) HeLa, (c) BS-C-1, or (d) IEC-6. Cells were incubated for 1h with virus then washed and fresh media added. 0 h.p.i. samples were harvested immediately after addition of fresh media. Cell associated virus collected at 24, 48 and 72h.p.i. was titrated and divided by virus titre present after absorption. Data points represent mean ± SEM of three independent wells.

**Fig 2:** MVA genome and repairs in v51.2 and v44.1 (a) Map of the MVA genome showing the location of the six major deletions, indicated by grey boxes and the approximate location of cosmids used to make the rescued MVAs (blue bars). HindIII fragments of Copenhagen are marked for reference. (b) Approximate locations of identified repairs in v51.2 and v44.1 are indicated in relation to the rescuing cosmid (blue bar). Repairs surrounding deletion I in v51.2 and v44.1 are indicated by the green boxes and a second repair, unique to v44.1, by the red box. (c) Mapping of the repair unique to v44.1: ORFs shown in black are truncated in MVA compared with CVA. Genes shown in white are identical between CVA and MVA. Genes shown in grey contain small mutations in MVA (4 aa deletions in F1L and F3L, single aa substitution in F8L). Genes labelled in red were sequenced in v44.1 and v51.2 to identify the extent of the repair. For these four genes v44.1 matches Ankara not v51.2 or MVA.(d) Structure of F5L region in v51.2/F5LGb. F5L is followed by GFP/bsd driven by a strong synthetic promoter (not shown) and flanked by repeated sequences (rpt) to preserve the promoter for F4L. The structure of F11L in v51.2/F11LGb was similar.

**Fig 3:** Restoration of F5L or F11L to v51.2 alters plaque morphology but not replication. (a-b) Plaques formed on BS-C-1 cells under semisolid media by the recombinant viruses shown were immunostained 72 h.p.i. (a) Representative plaques, original magnification 100× (scale bar
(b) Areas of individual plaques are plotted with the average shown by the solid line. (***) v51.2 significantly different to all other viruses (p<0.001), *v51.2/F5L-F11L is significantly different to all other viruses (p<0.05 for v51.2/F5L, all others p<0.001) One-way ANOVA (n=50) and Tukey pairwise test. (c) Fluorescent foci of MVA/F5LGb and v51.2/F5LGb formed on BS-C-1 under semisolid media at 72h.p.i. (100× final magnification). (d-e) Replication analysis in BS-C-1. Data are mean ± SEM of three independent wells (d) Multiple step growth analysis (m.o.i.=0.01). (e) Single step growth analysis (m.o.i.=5). (f) Multiple step growth analysis (m.o.i.=0.01, BS-C-1) of MVA and two independent rescues of F5L in MVA (MVA/F5LGb #1 and MVA/F5LGb #2). Data expressed as fold increase (mean ± SEM of three independent wells). (g) Disposition of genes associated with plaque phenotype in rescued MVAs.
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(a) v51.2  v51.2/F5L-F11L  v51.2/F5LGb  v51.2/F5L  v51.2/F11LGb

(b) 

(c) MVA/F5LGb  v51.2/F5LGb

(d) BS-C-1  m.o.i.=0.01

(e) BS-C-1  m.o.i.=5

(f) BS-C-1  m.o.i.=0.01

(g) C2L  F5L  F11L  O1L  left end of genome

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Medium plaque
Similar morphology to v44.1, larger
Small pile of cells
Small pile of cells, similar to v51.1
Similar morphology to v44.1, larger
Medium plaque, similar to v44.1
Larger pile of cells (no clearance)
Large plaque, but smaller than Ankara
Medium plaque, decreased clearance
Fig 3: Restoration of F5L or F11L to v51.2 alters plaque morphology but not replication. (a-b) Plaques formed on BS-C-1 cells under semisolid media by the recombinant viruses shown were immunostained 72 h.p.i. (a) Representative plaques, original magnification 100x (scale bar 100µm). (b) Areas of individual plaques are plotted with the average shown by the solid line. (***) v51.2 significantly different to all other viruses (p<0.001), *v51.2/F5L-F11L is significantly different to all other viruses (p<0.05 for v51.2/F5L, all others p<0.001) One-way ANOVA (n=50) and Tukey pairwise test. (c) Fluorescent foci of MVA/F5Lgb and v51.2/F5Lgb formed on BS-C-1 under semisolid media at 72h.p.i. (100x final magnification). (d-e) Replication analysis in BS-C-1. Data are mean ± SEM of three independent wells. (d) Multiple step growth analysis (m.o.i.=0.01). (e) Single step growth analysis (m.o.i.=0.01, BS-C-1) of MVA and two independent rescues of F5L in MVA (MVA/F5Lgb #1 and MVA/F5Lgb #2). Data expressed as fold increase (mean ± SEM of three independent wells). (g) Disposition of genes associated with plaque phenotype in rescued MVAs.