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

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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 extended the characterization of these rescued MVA strains and identified 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.

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). Whilst 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 in 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 (Fig. 1, Fig. 2a,b) (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 medium (0.4%, w/v, carboxymethyl cellulose) were immunostained (Staib et al., 2004) at 72 h post-infection (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 cells. 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, Fig. 2a,b) [rescued MVAs were named based on the cosmid used for marker rescue, two separate lineages were produced from each cosmid (Wyatt et al., 1998)]. 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).

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Modified vaccinia virus Ankara (MVA) is a leading candidate vector for recombinant poxvirus vaccines (Gómez et al., 2008). MVA is 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 vaccinia virus (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 deletions I–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.

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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 deletion V or II, was repaired both in v51.2 and v44.1 (Fig. 2b), 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 suggested 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 F region 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. 5.0.05) were transfected with 1 mg linearized plasmid using Lipofectamine 2000 (Invitrogen). At 48 h p.i., virus was harvested and used to infect BS-C-1 cells. A single large plaque was isolated after recombination between the v51.2 genome and the F5L–F11L plasmid. This plaque was then titrated and divided by the virus titre present after absorption. Data points represent means ± SEM of three independent wells.

**Fig. 1.** Rescued MVAs show a range of plaque sizes 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 semi-solid medium. Cells were immunostained at 72 h p.i. (×100 final magnification; bars, 100 μm). (b–d) Multiple step growth analysis (m.o.i. = 0.01) in HeLa (b), BS-C-1 (c) and IEC-6 (d). Cells were incubated for 1 h with virus and then washed and fresh medium added. Samples were harvested immediately after addition of fresh medium (0 h p.i.). Cell-associated virus collected at 24, 48 and 72 h p.i. was titrated and divided by the virus titre present after absorption. Data points represent means ± SEM of three independent wells.
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 36.5 kDa major membrane protein (Yang et al., 2010, http://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 was that the promoters of adjacent genes (F4L and F10L, respectively) were separated from their ORF. For this reason, these promoter sequences were repeated after transfection with F5L (v51.2/F5LGb) and F11L (v51.2/F11LGb), and after three to four rounds of plaque purification on BS-C-1 cells, the fidelity of repairs was verified by sequencing. In the case of v51.2/F5LGb, further passage allowed the isolation of a virus that had lost the GFP/bsd marker but retained the repair of F5L (v51.2/F5L).

Having isolated these viruses, we compared plaque phenotypes and sizes (Fig. 3a, b). Repair of 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 F11L also increased plaque size but did not lead to clearance of cells from their centres. Furthermore, the effect of F5L and F11L was additive because plaques produced by v51.2/F5L–F11L were larger than those of viruses with repairs of F5L and F11L alone. Next, we tested virus growth and found that neither F5L (with or without GFP/bsd) nor F11L altered the replication of v51.2 in single- or multiple-step growth curves (Fig. 3d, e). Finally, the use of the GFP/bsd marker allowed us to isolate an MVA with F5L repaired (MVA/F5LGb). Repair of 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 were 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 cells for the rescued MVAs.

**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 semi-solid medium by the recombinant viruses shown were immunostained at 72 h p.i. (a) Representative plaques (x100 original magnification; bars, 100 µm). (b) Areas of individual plaques were plotted, with the mean shown by a horizontal line. Significant differences were determined by one-way ANOVA (n=50) and Tukey’s pairwise test: ***, v51.2 significantly different to all other viruses at P<0.001; *, v51.2/F5L–F11L significantly different to all other viruses at P=0.05 for v51.2/F5L and at P<0.001 for all others. (c) Fluorescent foci of MVA/F5LGb and v51.2/F5LGb formed on BS-C-1 cells under semi-solid medium at 72 h p.i. (x100 final magnification). (d, e) Replication analysis in BS-C-1 cells. Data are means ±SEM of three independent wells for multiple-step growth analysis (m.o.i.=0.01) (d) and single-step growth analysis (m.o.i.=5) (e). (f) Multiple-step growth analysis (m.o.i.=0.01, BS-C-1 cells) of MVA and two independent rescues of F5L in MVA (MVA/F5LGb #1 and MVA/F5LGb #2). Data are expressed as fold increase (means ±SEM of three independent wells). (g) Disposition of genes associated with the plaque phenotype in rescued MVAs.
as reported previously (Wyatt et al., 1998). It is also supported by the recent finding that v51.1, with a smaller plaque size, replicates to higher titres in 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 produced by VACV strain WR, but is lost from MVA, due to the 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 (Antoine et al., 2012; Morales et al., 2008; Pires de Miranda et al., 2003; Schweneker et al., 2012). F5L is now the fourth VACV gene function to be determined as 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). We found that: (i) of all the rescued MVAs, v44/47.1 had the largest plaques but their size remained smaller than those of Ankara, suggesting that genes outside the region mapped by Wyatt et al. (1998) affect plaque size or replication; (ii) 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; (iii) 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; (iv) plaques from v44/47.1 were larger again than those of 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); and (v) 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 did not extend as far to the left of the genome as in v51.2, so 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. Furthermore, the existence of F5L and several other genes required for normal plaque formation complicate the interpretation of work carried out to map attenuating mutations of MVA, which has assumed that plaque size is an accurate surrogate for replication. We also showed here that the relatively small single repair in v51.2 alone produces a substantial improvement in replication on three mammalian cell lines. Together, these data lead us to conclude that the range of key genomic changes associated with the replication defect of MVA in mammalian cells has been overestimated previously.

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References


