Vaccinia virus kelch protein A55 is a 64 kDa intracellular factor that affects virus-induced cytopathic effect and the outcome of infection in a murine intradermal model

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The vaccinia virus (VACV) protein A55 is a BTB/kelch protein with a broad-complex, tramtrack and bric-a-brac (BTB) domain in the N-terminal region and five kelch repeats in the C-terminal half. The BTB/kelch subgroup of the kelch superfamily of proteins has been associated with a wide variety of functions including regulation of the cytoskeleton. VACV contains three genes predicted to encode BTB/kelch proteins: A55R, F3L and C2L. The A55R gene product has been identified as an intracellular protein of 64 kDa that is expressed late in infection. A VACV strain lacking 93-6% of the A55R open reading frame (vΔA55) was constructed and found to have an unaltered growth rate in vivo but a different plaque morphology and cytopathic effect, as well as reduced development of VACV-induced Ca\(^{2+}\)-independent cell/extracellular matrix adhesion. In a murine intradermal model of VACV infection, a virus lacking the A55R gene induced larger lesions than wild-type and revertant control viruses.

INTRODUCTION

Vaccinia virus (VACV) is a member of the genus Orthopoxvirus of the Poxviridae. The VACV genome encodes approximately 200 open reading frames (ORFs) (Goebel et al., 1990), including numerous genes encoding proteins that modulate the host's antiviral immune response (reviewed by Seet et al., 2003). Immunomodulators include secreted proteins that bind interleukin-1β (Alcamí & Smith, 1992; Spriggs et al., 1992), interferon (IFN)-α and γ (Upton et al., 1992; Alcamí & Smith, 1995) and complement factors C3b and C4b (Kotwal & Moss, 1988a; McKenzie et al., 1992), and intracellular immunomodulators that inhibit apoptosis (Dobbelstein & Shenk, 1996; Kettle et al., 1997; Wasilenko et al., 2003), IFN-induced antiviral proteins (Beattie et al., 1991; Chang et al., 1992) or signalling from toll-like receptors (Bowie et al., 2000; Harte et al., 2003; Stack et al., 2005). The VACV kelch protein C2 is another intracellular protein that was shown to influence the host response to VACV infection although its mechanism of action was not defined (Pires de Miranda et al., 2003).

Kelch proteins are a diverse and widely distributed family of proteins found in yeast, Caenorhabditis elegans, Drosophila melanogaster, mammals and poxviruses (Adams et al., 2000). Over 60 kelch proteins have now been identified, although the majority are still to be characterized. The first member to be described was the Drosophila kelch protein, from which the family takes its name (Xue & Cooley, 1993). The kelch motif is 44–57 aa long and generally occurs as 4–7 repeats, forming a tertiary β-propeller structure (Ito et al., 1994; Li et al., 2004). The largest subgroup of the kelch superfamily are the BTB/kelch proteins (Prag & Adams, 2003), which have a BTB (broad-complex, tramtrack and bric-a-brac) domain in the N-terminal region of the protein and kelch repeats in the C-terminal half. The BTB domain is involved in protein–protein interactions (Bardwell & Treisman, 1994) and often serves to dimerize the BTB/kelch protein, while the kelch domain mediates a variety of binding interactions with other molecules. For example, the kelch domain of the Drosophila kelch protein is necessary for association of the protein with actin filaments, while the BTB domain is required for the dimerization of two actin bound kelch proteins, resulting in the cross-linking of these filaments (Robinson & Cooley, 1997).

A recurring theme linking the majority of BTB/kelch proteins so far investigated is their involvement in the remodeling and stabilization of the cytoskeleton. The Drosophila kelch protein and the related BTB/kelch protein hDKIR are both necessary for the formation of ring-like actin structures (Xue & Cooley, 1993; Mai et al., 2004), the yeast BTB/kelch protein tea1p is an integral part of a protein complex that regulates formin distribution and actin cable assembly, thus influencing cell polarization (Feierbach et al., 2004), and the human BTB/kelch protein KBTBD2 interacts with the cytoskeleton–cell membrane linker ezrin in a phosphospecific manner (Heiska & Carpen, 2005). The BTB/kelch proteins Mayven and ENC-1 interact directly with actin...
(Hernandez et al., 1997; Jiang et al., 2005) and the Keap 1 protein requires an intact actin cytoskeleton to function (Kang et al., 2004).

Keap 1 is the most intensively studied BTB/kelch protein. It binds to the stress protective transcription factor Nrf2, resulting in the cytoplasmic sequestration and degradation of Nrf2 via a Cul3-dependent ubiquitin ligase complex (Zhang et al., 2004). When the cell undergoes oxidative stress Nrf2 escapes this Keap 1-mediated regulation, the steady-state levels of Nrf2 increase, and the protein translocates into the nucleus where it binds to the crucial antioxidant response elements, which in turn direct the transcription of a myriad of cytoprotective genes. Deletion of the Keap 1 gene in mice causes constitutive activation of Nrf2 and post-natal lethality (Wakabayashi et al., 2003). A number of other BTB/kelch proteins also act as substrate adaptors for Cul3-dependent ubiquitin ligase complexes (reviewed by Pintard et al., 2004), including KLHL10 (Wang et al., 2006), KIAA1309, KIAA1354 and others (Furukawa et al., 2003).

A small number of BTB/kelch proteins have been linked to disease. Giant cell neuropathy is an autosomal recessive sensorimotor neuropathy characterized by neurolamin accumulation, and associates with mutations in the gene encoding the BTB/kelch protein gigaxonin (Bomont et al., 2000). Genetic alterations in the BTB/kelch protein NRP/B have been associated with glioblastomas, a brain tumour arising from astrocytes (Liang et al., 2004). In general, however, the vast majority of work on kelch proteins has concentrated on their function in vitro.

Poxviruses are the only viruses so far known to encode kelch proteins. The number of kelch genes present varies between poxvirus species: Cowpox virus contains six kelch proteins, Ectromelia virus contains four, Monkeypox virus contains one, and Goatpox virus contains six kelch proteins, leading the authors to hypothesize that these proteins are important for viral virulence (Tulman et al., 2002).

The goals of this project were to characterize the A55 kelch protein encoded by VACV and to determine if the protein affected virus growth in cell culture or virus virulence in vivo.

METHODS

**Cells.** CV-1, BS-C-1 and HeLa D98OR cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco). Infections were carried out in DMEM/2-5% FBS. Cells were incubated at 37°C in a 5% CO₂ atmosphere.

**Production of antiserum to A55.** Primers pmb1 (5′-GAATTC- ATGAAATACGCACGTGAA-3′) and pmb2 (5′-AAGCTTTCACCTAC- ACCCTATAAAACT-3′) were used to amplify the A55R gene by PCR from VACV strain Western Reserve (WR) genomic DNA, generating an EcoRI restriction site at the 5′ end and a HindIII site at the 3′ end (restriction sites are underlined). The PCR product was cloned into the pCR2.1 vector following the manufacturer’s instructions (Invitrogen) to form plasmid pPB4. The A55R ORF was then sub-cloned using the EcoRI and HindIII sites into the plasmid pET28(a) (Novagen) to create plasmid pPB6. This plasmid was sequenced to confirm the predicted protein was in-frame with an N-terminal six histidine (His) tag encoded by the vector and the absence of any PCR-induced mutations. When compared to the published sequence of VACV WR strain (NC_006998) one change was found 1032 bp downstream of the first nucleotide of A55, resulting in GAT to GAC silent mutation. This change was found in several clones obtained from independent PCRs. Rosetta Escherichia coli cells (Novagen) were transformed with plasmid pPB6 and cultured in Luria–Bertani medium at 37°C until the OD at 600 nm reached 0.6, when protein expression was induced by the addition of 1 mM IPTG at 37°C for 2 h. The cells were collected by centrifugation and lysed by Dounce homogenization in a buffer containing 50 mM Tris pH 8, 200 mM NaCl and Complete protease inhibitors (Roche). The insoluble material was pelleted by centrifugation at 15000 g for 15 min and dissolved in denaturation buffer (the above buffer also containing 6 M guanidine hydrochloride). After stirring at 4°C for 90 min the material was centrifuged at 15000 g for 15 min and the supernatant was saved. Pre-equilibrated Ni-nitrilotriacetic acid beads (Qiagen) were incubated with the supernatant for 30 min at 4°C. The unbound protein was removed and the beads were washed extensively with denaturation buffer. The beads were then washed in denaturation buffer with 10 mM imidazole, 20 mM imidazole, and finally bound protein was eluted in 0.5 M denaturation buffer with 0.5 M imidazole. The presence of His-tagged A55 in the eluate was confirmed by Coomassie blue staining and immunoblotting with a monoclonal antibody (mAb) against His (Qiagen). Samples were then stored at 4°C until use. Rabbit polyclonal antiserum was produced against the recombinant protein by Harlan Seralabs.

**Recombinant virus construction.** A VAC WR strain lacking 93-6% of the A55R gene (vΔA55) was constructed using transient dominant selection (Falkner & Moss, 1990). The primers pmb3 (5′- AAGCTTGCTTCATTCCCTCACTGCAAATCT-3′) and pmb8 (5′-GACT- GACTGACTGCAATCAATTCACTGTT-3′) were used in a PCR to generate a 5′ flanking region and pmb11 (5′-AGTCATCGCTGCAATCAATTCACTGTT-3′) and pmb6 (5′-GAAACCTGGCTCT ATTACACAAACAC-3′) were used to generate a 3′ flanking region. These two regions were joined using splicing by overlap extension PCR (Horton et al., 1989). The overlapping complementary DNA
regions of pmb8 and pmb11 are shown in bold. The product was then cloned into pCR2.1 (Invitrogen) and sequenced to confirm the absence of any mutations. The fragment was then subcloned into pSJH7 to create pPB7. Plasmid pPB7 was transfected into VACV-infected cells and mycophenolic acid (MPA)-resistant viruses were isolated as described previously (Falkner & Moss, 1990). These were grown on hypoxanthine guanine phosphoribosyltransferase-negative D980R cells in the presence of 6-thioguanine (Sigma) and plaque isolates containing (vA55) or lacking (vA55) the A55R gene were identified by PCR. To construct a revertant virus a 2195 bp PCR product was produced from VACV WR genomic DNA using primers pmb3 and pmb6. This product contained full-length A55R with flanking regions and was cloned into pCR2.1 and sequenced. It was then subcloned into pSJH7 to create plasmid pPB35 that was transfected into cells infected with vA55. MPA-resistant intermediate viruses were isolated as above and resolved to obtain a revertant virus (vA55-rev) with the full-length A55R gene.

Immunoblotting. BS-C-1 cells were infected at 5 p.f.u. per cell with either deletion (vA55), wild-type (vA55) or revertant (vA55-rev) virus, or were mock-infected. Cells were harvested 20 h post-infection (p.i.), collected by centrifugation and dissolved in Laemmli buffer containing 5 % β-mercaptoethanol. The proteins present in the samples were resolved by SDS-PAGE (10 %), transferred to PVDF membranes and probed with monoclonal rabbit antiserum directed against A55 (1:500) or D8 (1:1000) (Parkinson & Smith, 1994). Proteins were visualized using Enhanced Chemiluminescence (ECL) Plus Western blotting detection reagents (Amersham Biosciences) or Alkaline Phosphatase Conjugate Substrate kit (Bio-Rad) following the manufacturer’s instructions.

Viral growth curves. One-step and multi-step growth curves were carried out as described previously (Pires de Miranda et al., 2003).

Analysis of viral cellular projections. BS-C-1 cells were seeded thinly onto coverslips and infected at 5 p.f.u. per cell with vA55, vA55 or vA55-rev. At 18 h p.i., cells were fixed in 4 % paraformaldehyde, permeabilized with 0.05 % saponin and blocked with 5 % PBS in PBS. The actin cytoskeleton was visualized with Alexa Fluor 546 phalloidin (Molecular Probes).

Depletion of extracellular Ca²⁺. The experiments were carried out as described previously (Sanderson & Smith, 1998; Pires de Miranda et al., 2003).

Yeast transformations. The A55R ORF was subcloned from pPB6 into the EcoRI site of plasmids pGBK7 and pGADT7 to form plasmids pPB41 and 42, respectively. The C2L ORF was amplified by PCR using primers pmb69 (5′-GGATCCCATATGGAAAGCGTGATA-3′) and pmb70 (5′-GGATCCATATGGAAAGCGTGATA-3′) and cloned into pCR2.1 to form plasmid pPB37. The BamHI sites at both ends of the gene (underlined in the primer sequence) were used to subclone the C2L ORF into pGADT7 and pGBK7 to form pPB38 and pPB39, respectively. The F3L ORF was amplified by PCR using primers pmb16 (5′-GGATCCCATATGGAAAGCGTGATA-3′) and pmb17 (5′-GGATCCCATATGGAAAGCGTGATA-3′) and cloned into pCR2.1 and then into the EcoRI site of pGBK7 and pGADT7 to form pPB33 and pPB40, respectively. All plasmids were checked for the correct orientation of the insert and absence of mutations by restriction enzyme analysis and sequencing. Yeast transformations were carried out as described in the Matchmaker Gal4 two-hybrid system 3 user manual (Clontech). Growth on quadrupole knockout medium (lacking leucine, tryptophan, adenine and histidine) was scored.

Mouse intranasal and intradermal model. Groups of female BALB/c mice between 6 and 8 weeks of age were anaesthetized with inhalational isoflurane and infected intranasally with 1 × 10⁴ p.f.u. VACV in 20 μl PBS. The mice were weighed and scored daily for signs of disease as described previously (Alcami & Smith, 1992). The intradermal inoculations were carried out as described previously (Tscharke & Smith, 1999). Briefly, groups of female BALB/c mice between 8 and 10 weeks of age were anaesthetized with inhalational isoflurane and injected by subcutaneous injection of VACV in 10 μl PBS into the outer surface of the ear pinna. The lesion size was measured daily.

Statistical analysis. Student’s t-test (two tailed, unpaired) was used to test for the significance of the results in Figs 1, 3 and 6. Fisher’s exact test was used to test for the significance of the result in Fig. 2.

RESULTS

Construction and characterization of vAA55 in vitro

To investigate the role of A55 a virus lacking 93·6 % of the A55R ORF (vA55) was constructed as described in Methods. The 3′ 109 bp of A55R was retained because it contains the late promoter for A56R (Brown et al., 1991). A wild-type virus (vA55) derived from the same intermediate MPA-resistant virus was constructed in parallel. A revertant virus (vA55-rev) was constructed from vA55. The virus genomes were analysed by PCR and restriction digest using DNA extracted from virus cores (Esposito et al., 1981). These analyses confirmed the deletion of A55R in vA55 and the absence of any other detectable genomic alteration in all three viruses (data not shown).

The isolation of vAA55 proved that A55R is non-essential for VACV WR replication in vitro, a conclusion consistent with the absence of this gene from other orthopoxviruses including Variola virus (Aguado et al., 1992) and VACV strain modified vaccinia virus Ankara (Antoine et al., 1998). The growth properties of vAA55 were analysed by a one-step and multi-step growth curve. No difference was found between the rate of growth of vAA55 when compared to vA55 or vA55-rev (data not shown). However, the plaques formed on confluent BS-C-1 cells by vAA55 were different from the plaques formed by the control viruses and exhibited an indistinct border (Fig. 1a) similar to that described for the virus lacking the C2L gene (vAC2) (Pires de Miranda et al., 2003) and the VACV mutant 6/2 that lacks a large section of the left end of the genome (Kotwal & Moss, 1988b). The difference in plaque morphology was seen more clearly by phase-contrast microscopy at higher magnification of live cells (Fig. 1b). Although the plaques formed by vAA55 showed different morphology when compared with the control viruses they were of indistinguishable diameter (Fig. 1a, c).

The cytopathic effect (CPE) caused by vAA55 was investigated more closely using subconfluent BS-C-1 cells. Infected cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize virus factories and phalloidin to stain virus-induced actin tails. There was no difference detected in the rate of viral factory formation or the number of
actin tails formed when vΔA55-infected cells were compared with controls (data not shown). However, the formation of cellular projections (Sanderson et al., 1998) was altered. At 18 h p.i. only 43 % of cells infected with vΔA55 formed projections, compared with 62 % of cells infected with vA55 and 60 % of cells infected with vΔA55-rev (Fig. 2). A comparable difference was detected at 16, 20 and 22 h p.i. (data not shown).

The effect of the A55R gene on VACV-induced Ca\(^{2+}\)-independent cell/extracellular matrix adhesion was investigated. Monolayers of BS-C-1 cells were either mock-infected or infected with vΔA55 or control viruses at 5 p.f.u. per cell before being depleted of Ca\(^{2+}\) 18 h p.i. (Sanderson & Smith, 1998). The number of cells that had rounded up were counted at 0, 3, 10 and 20 min after Ca\(^{2+}\) depletion. As reported previously (Sanderson & Smith, 1998), 79 % of the mock-infected cells were rounded up after 10 min of Ca\(^{2+}\) depletion, compared with only 21 % of wild-type or revertant virus-infected cells. In contrast, 43 % of cells infected with vΔA55 had rounded up 10 min after Ca\(^{2+}\) depletion. A similar disparity was seen 20 min after Ca\(^{2+}\) depletion (Fig. 3). The difference between

![Fig. 1. Plaque phenotype of recombinant viruses. (a) BS-C-1 monolayers were infected with viruses as shown and overlaid with DMEM/2.5 % FBS/1.5 % carboxymethylcellulose. After 3 days, cells were stained with 0.1 % crystal violet in 15 % ethanol and representative plaques were photographed. (b) BS-C-1 monolayers were infected with the indicated viruses and incubated in DMEM/2.5 % FBS. At 24 h p.i., the edges of plaques were examined by phase-contrast microscopy. In each panel the centre of the plaque is towards the right. (c) The mean ± SEM plaque diameter (mm) at 24, 48 and 72 h p.i. is shown. The sizes of 10 plaques were measured at each time point.](image1)

![Fig. 2. Formation of VACV-induced cellular projections. Subconfluent BS-C-1 cells on coverslips were infected at 5 p.f.u. per cell with indicated viruses. Cells were fixed 18 h p.i., blocked and stained with phalloidin as described in Methods. The slides were examined blind, and the percentage of cells with projections was recorded. vΔA55 versus vA55 P < 0.01, vΔA55 versus vΔA55-rev P < 0.01 (Fisher’s exact test). Data are representative of three separate experiments.](image2)
vA55-infected cells and control virus-infected cells was statistically significant 10 and 20 min after Ca\(^{2+}\) depletion (\(P<0.001\)).

**Identification of the A55 protein**

The complete A55R ORF was cloned into the pET28a vector (Novagen), expressed in E. coli cells, and the A55 protein was purified from inclusion bodies as described in Methods. The purified protein was used to raise a rabbit polyclonal antibody against A55 and the antibody was used to detect A55 in VACV-infected cells. The antiserum recognized a 64 kDa protein in cells infected with vA55 and v\(\Delta\)A55-rev but not in cells infected with v\(\Delta\)A55 or mock-infected cells (Fig. 4a). In contrast, mAb AB1.1 directed against the D8 protein (Parkinson & Smith, 1994) detected the 35 kDa D8 protein in cells infected with all three viruses (Fig. 4b).

The A55 protein was not detected by immunoblot in the supernatant of infected cells, suggesting that only low levels, if any, of A55 protein were released from cells (data not shown). The A55 protein was also not detected in highly purified intracellular mature virus particles, suggesting that it is not incorporated into virions (data not shown).

The time of expression of A55 during the virus life cycle was investigated. Cells were infected in the presence or absence of cytosine arabinoside (AraC), and collected at different times p.i. Proteins from cell extracts were analysed by immunoblotting. The A55 protein was detected only in the absence of AraC 24 h p.i. (Fig. 4c), indicating that it is expressed late in the virus life cycle.

**A55 homodimerizes but does not interact with F3 or C2**

To determine if the A55 protein is able to self associate, the full-length A55R gene was cloned into the pGADT7 and pGBKt7 yeast two-hybrid vectors (Clontech). The growth of yeast colonies on the high stringency quadruple knockout plates indicated that there was an interaction between A55 in the two constructs, and therefore A55 interacts with itself. The remaining two VACV kelch genes C2L and F3L were cloned into the pGBKt7 and pGADT7 vectors and further transformations carried out to determine if A55 interacted with these proteins, however, no interaction was detected (Fig. 5).

**A55 affects the outcome of infection in the murine intradermal model**

The virulence of v\(\Delta\)A55 was examined in the murine intradermal model of infection. When injected into the pinnae of the ear of C57BL/6 mice, the resultant lesion in mice inoculated with v\(\Delta\)A55 was statistically larger on days 7–14 when compared with the lesion formed by mice inoculated with either of the two control viruses.
DISCUSSION

In this paper, the VACV WR A55R gene has been characterized and shown to encode an intracellular 64 kDa protein that is expressed late in virus infection. The gene was not essential for virus replication, although it influenced both the CPE induced by the virus in vitro and the outcome of infection in vivo. A deletion mutant virus, lacking 93.6% of the A55R ORF, produced an altered plaque phenotype on BS-C-1 cells, fewer cellular projections from virus-infected cells, and affected the Ca^{2+}-independent cell/extracellular matrix adhesion of infected cells. Furthermore, vΔA55 induced larger lesions in the intradermal mouse model of VACV infection when compared with wild-type and revertant virus controls.

The reduction in the number of vΔA55-infected cells producing projections may be the cause of the altered plaque appearance. The virus-induced cellular projections are a typical feature of VACV CPE. They are produced by extending lamellipodia late during infection, and can be up to 160 μm in length (Sanderson et al., 1998). The underlying mechanism behind the formation of the projections is not known, although they are dependent on the expression of late VACV genes (Sanderson et al., 1998). Interestingly, the only other gene directly associated with the formation of the virus-induced cellular projections encodes another VACV BTB/kelch protein – C2 (Pires de Miranda et al., 2003). A number of BTB/kelch proteins have been associated with the formation of projections from other cell types. Mayven, a human BTB/kelch protein, induces process elongation when overexpressed in oligodendrocyte precursor cells (Jiang et al., 2005) and overexpression of Krp1, a rat BTB/kelch protein, leads to the formation of dramatically elongated pseudopodia in transformed rat fibroblasts (Spence et al., 2000). These data suggest that a conserved function of at least some of the BTB/kelch proteins is the modulation of cytoskeletal mechanisms that are involved in the formation of cellular protrusions. There is evidence to support this theory from other kelch protein studies. For example, mutations in the yeast BTB/kelch protein tea1p are known to result in abnormal cell shapes and growth from only one cell tip (Mata & Nurse, 1997). An explanation of this phenotype was presented in a recent paper that described how tea1p travels out to the cell periphery on microtubules, where it forms part of a multiprotein complex to regulate formin distribution and actin cable assembly at the tips of the cell (Feierbach et al., 2004). A recently described BTB/kelch protein KBTBD2 interacts with ezrin, a cell membrane–cytoskeletal linker protein with crucial signalling functions, which is usually localized to the periphery of the cell (Heiska & Carpen, 2005). Further localization work and detection of interacting partners to A55 is needed to determine if it fulfils an analogous role in VACV-infected cells, such as regulating and coordinating cytoskeletal changes at the tips of the virally induced cellular projections. Attempts to determine the localization of A55 in infected cells using the antiserum described in this paper

(Fig. 6). A repeat experiment gave the same result. No clear difference was detected in the virulence of vΔA55 in the intranasal model of infection (data not shown).
have so far been unsuccessful because the antibody does not work well for immunofluorescence.

The loss of the A55R gene also affects the VACV-induced Ca\(^{2+}\)-independent cell/extracellular matrix adhesion. Uninfected cells normally round up and detach from the culture flask under conditions of Ca\(^{2+}\) depletion, however VACV-infected cells remain attached (Sanderson & Smith, 1998). Cells infected with vAA55 showed an intermediate phenotype, with 43% of the cells rounding up and detaching after 10 min of Ca\(^{2+}\) depletion, compared with 21% of wild-type virus-infected cells or 79% of mock-infected cells. Again, vAC2 showed a very similar increased sensitivity to Ca\(^{2+}\) depletion (Pires de Miranda et al., 2003). In fact, the vAA55 and vAC2 appear phenotypically indistinguishable in vitro, at least for the parameters tested, suggesting their functions may be linked, possibly by a physical interaction. Consequently, potential interactions between A55 and the other two VACV BTB/kelch proteins, C2 and F3, were investigated using yeast two-hybrid transformations. It was found that A55 interacted with itself, but there was no evidence that it interacted with either C2 or F3. This result was consistent with previously reported work on BTB/kelch proteins that have been widely reported to homodimerize (Robinson & Cooley, 1997), but only one report describes an interaction between two different BTB/kelch proteins (Mai et al., 2004). A comprehensive genome-wide yeast two-hybrid analysis to identify VACV protein–protein interactions has been reported (McCraith et al., 2000). The authors found 37 protein–protein interactions from approximately 70,000 combinations including 13 self-interacting proteins, but they did not report an interaction between A55 and itself or any other VACV protein. However, the paper concludes that the 37 interactions it reported could represent only a fraction of those that truly occur.

Most of the work on BTB/kelch proteins so far has been performed in vitro. Deletion of the BTB/kelch protein A55 from VACV was an excellent opportunity to examine the role of this protein family in vivo. The virus lacking A55R induced a larger lesion size compared with wild-type and revertant viruses in the intradermal model (Fig. 6), but no clear difference in an intranasal model under the conditions tested (data not shown). It is not unusual for the deletion of a gene to produce a phenotype in only one of the two models of infection (Tscharke et al., 2002).

Interestingly, the vAA55 and vAC2 mutant viruses that are so similar in vitro produced a slightly different phenotype in the intradermal model of VACV infection. The virus lacking C2 caused a delay in the healing lesions in the pinnae of mice when compared with control viruses (Pires de Miranda et al., 2003), but the sizes of the lesions were similar. In contrast, the lesion caused by vAA55 was significantly larger (Fig. 6) than the control viruses. The time course of lesion resolution in mice inoculated with vAA55 was not assessed because the lesion size by day 14 necessitated the curtailing of the experiment.

The identical phenotype exhibited by vAA55 and vAC2 in vitro suggests that, while they do not interact directly, the two proteins have roles in the same process, such as regulation of the virally induced cellular processes. The fact that deletion of either gene gave the same phenotype in vitro, shows that the functions of the two proteins are distinct, for if one could substitute for the other, no phenotype would be expected if either was deleted. The increased size of lesions in mice infected intradermally with the deletion mutant suggests that, like the C2 protein, A55 influences the inflammatory response to infection. How each protein functions to do this will require additional study and the identification of binding partners in infected cells.

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