The vaccinia virus kelch-like protein C2L affects calcium-independent adhesion to the extracellular matrix and inflammation in a murine intradermal model

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Chordate poxviruses encode several uncharacterized POZ-kelch proteins and three of these are present in Vaccinia virus (VV) strain Western Reserve. VV gene C2L is predicted to encode a protein of 512 amino acid residues with a POZ/BTB domain in the N-terminal region and three kelch motifs in the C-terminal half of the protein. We have identified the C2L gene product as an intracellular protein of 56 kDa and constructed and characterized a VV mutant lacking the C2L gene (vΔC2L). Compared to wild-type and revertant viruses, vΔC2L had unaltered growth in vitro, but had a different plaque morphology due to an altered cytopathic effect (CPE) of infected cells. Deleting C2L had no effect on VV-induced formation of actin tails or enhanced cell motility, but affected the development of VV-induced cellular projections and the Ca2+-independent cell/extracellular matrix adhesion late during infection. In an intranasal mouse model, C2L did not contribute to virus virulence. However, in an intradermal mouse model, infection with vΔC2L resulted in larger lesions and more cell infiltration into the infected ears during recovery from infection. Thus, in this model, C2L protein inhibits inflammation and reduces immunopathology. In summary, we found that C2L is not required for virus replication in vitro but contributes to aspects of VV-induced CPE and reduces immunopathology in vivo.

INTRODUCTION

Vaccinia virus (VV) is a member of the Poxviridae, a group of large, DNA viruses that replicate in the cytoplasm (Moss, 2001). The dsDNA genome is predicted to encode around 200 proteins (Goebel et al., 1990). Most essential genes are located in the conserved central region of the genome and encode enzymes required for virus replication and structural proteins needed for the formation of infectious virions. Non-essential genes are located mostly in the variable terminal regions of the genome (Kotwal & Moss, 1988; Perkus et al., 1991) and may influence host range and virulence.

A feature of VV infection in cell culture is the induction of cytopathic effect (CPE). This CPE is the result of numerous changes within the cell and starts with the induction of actin-containing protrusions in the plasma membrane (Locker et al., 2000) upon intracellular mature virus (IMV) binding. After virus entry, microtubules transport viral cores (Carter et al., 2003) and then early mRNAs (Mallardo et al., 2001). With the onset of early gene expression, host cell macromolecular synthesis is shut down (Buller & Palumbo, 1991). Also at early times post-infection (p.i.) cell–cell dissociation and cell rounding begin (Bablanian et al., 1978) and cell motility is enhanced (Sanderson et al., 1998). Viral DNA replication and late gene expression bring further changes to the cytoskeleton: the normal aster configuration of microtubules is lost (Sanderson et al., 1998) and actin stress fibres are disrupted. Surface microvilli (Hiller et al., 1979) and long cellular projections (Sanderson et al., 1998; Ploubidou et al., 2000) are produced as microtubules are organized into long, stable bundles. In addition, cell/extracellular matrix (ECM) adhesion changes from a Ca2+-dependent to a Ca2+-independent type, possibly mediated by integrins (Sanderson & Smith, 1998).

The VV morphogenic pathway utilizes both microtubules
and actin as it produces multiple forms of infectious progeny (for review see Smith et al., 2002). First IMV (Sanderson et al., 2000) and then intracellular enveloped virus (IEV) (Hollinshead et al., 2001; Riedorf et al., 2001; Ward & Moss, 2001) are transported within cells on microtubules. Cell-associated enveloped virus (CEV) particles are propelled away from the infected cell by polymerization of actin (Cudmore et al., 1995; van Eijl et al., 2002) and are important for cell-to-cell spread since mutants deficient in their formation form small plaques (Law et al., 2002; Rodger & Smith, 2002) (and references therein). Extracellular enveloped virus (EEV) mediates long-range dissemination in vitro and in vivo (Appleyard et al., 1971; Payne, 1980; Smith et al., 2002).

VV genes C2L, F3L and A55R are non-essential for growth in culture but otherwise remain uncharacterized (Kotwal & Moss, 1988; Perkus et al., 1991). These genes are located either at the left (C2L and F3L) or right (A55R) ends of the genome, and whilst having low overall amino acid identity with each other, they share structural motifs (Fig. 1a) and belong to the kelch superfamily of proteins.

C2L is predicted to encode a 59 kDa protein (Kotwal & Moss, 1988) with a POZ (poxvirus and zinc finger)/BTB (broad-complex, tramtrack and bric-à-brac) domain near zinc finger)/BTB (broad-complex, tramtrack and bric-à-brac) domain near zinc finger). This protein has structural similarity to kelch proteins (Bork & Doolittle, 1994). Kelch proteins are conserved in several orthopoxviruses, including VV strains. These proteins may have differing roles, and might affect cell function. To test this, a VV mutant lacking C2L was constructed and characterized. We report here that the C2L protein contributes to the formation of VV-induced cellular projections and the Ca^{2+}-independent adhesion of cells to the ECM and also affects the outcome of infection in a murine dermal model.

METHODS

Cells and viruses. Monkey kidney CV-1 and BSC-1 cells, human osteosarcoma TK^+143B cells and HeLa D98OR cells were obtained from the Sir William Dunn School of Pathology, University of Oxford, UK, and were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum (FBS) (DMEM/10% FBS) (Gibco BRL). Infections of cells with VV were carried out in DMEM/2.5% FBS and cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Plasmid construction. Plasmid pC2L was constructed as follows. Two DNA fragments that contained DNA from either the 5' or 3' ends of the C2L gene and flanking regions were amplified by PCR (using VV strain WR genome DNA as template). The 5' fragment was amplified with oligonucleotides 5'-AAAGATTCGATTGTGAGAGAGCC-3' (C2L2R), containing an EcoRI restriction site (underlined), and 5'-TCTCTCTGATAGTACAAATCATGCTTTG-3' (C2L2F), containing an XbaI restriction site (underlined). The 3' fragment was amplified with oligonucleotides 5'-AAATCTAGATCGCATCCATCATTGAGAGG-3' (C2L1R), containing an XbaI restriction site (underlined), and 5'-TTGAAGCTTGGTGCCTCATTCCACATG-3' (C2L1F), containing a HindIII restriction site (underlined). The 3' fragment was digested with HindIII and XbaI and ligated into HindIII- and XbaI-cut pSH7 (Hughes et al., 1991) to form pSH7A. The 5' fragment was digested with XbaI and EcoRI and ligated into XbaI- and EcoRI-cut pSH7A to form pC2L. This plasmid lacked 98% of the C2L gene corresponding to the region between nucleotides 12258 and 10404 (codons 9–510) (numbering according to Kotwal & Moss, 1988). Plasmid pC2Lrev was used to construct the C2L revertant virus and was generated as follows. A complete version of the C2L gene plus 5' and 3' flanking regions was generated by PCR, using VV strain WR genome DNA as template, and oligonucleotide primers C2L2F and C2L2R. The PCR product was digested with HindIII and ligated into HindIII- and XbaI-cut pSH7 to form pC2Lrev. The fidelity of the PCR-derived regions from all plasmids was verified by DNA sequencing. Compared to the published sequence (Kotwal & Moss, 1988), there was one nucleotide difference in the C2L 5' flanking region (G to A at position –247 relative to the first nucleotide of the C2L gene). This change is within gene C1L, but did not alter its amino acid composition (glycine-171 encoded by GGA instead of GGG). This alteration was found in several plasmids obtained from independent PCRs.

Plasmid pGS61C2LFlag was used to insert the C2L gene with the Flag epitope (N-DYKDDDDK-C) attached to the 3' end into the VV thymidine kinase (TK) locus of the VV mutant lacking C2L. The C2L gene with a C-terminal Flag sequence was obtained by PCR using VV DNA as template and oligonucleotide 5'-TTTAAAGGTTCTTACTGGCTAGCTGGTTGGAAGATG-3' (C2LFlag1), containing a BamHI restriction site (underlined) and the C2L start codon (in bold), and oligonucleotide 5'-TTTAAAGGTTCTTACTGGCTAGCTGGTTGGAAGATG-3' (C2LFlag2), containing a HindIII restriction site (underlined), the stop codon (complement of) of the C2L gene (in bold) and the sequence for the Flag epitope (in lowercase case). The PCR fragment was digested with BamHI and HindIII and ligated into BamHI- and HindIII-cut pSH7 to form pSH7C2LFlag. The PCR fragment was excised from pSH7C2LFlag using BamHI and HindIII and cloned into pGS61 (Smith et al., 1987) that had been cut with the same enzymes, to form pGS61C2LFlag.

Recombinant virus construction. A deletion mutant lacking 98% of the C2L gene was constructed by transient dominant selection (Falkner & Moss, 1990) using plasmid pC2L and VV strain WR as described previously (Ng et al., 2001). The deletion mutant, vAC2L, and a plaque-purified wild-type virus were obtained from the same
Fig. 1. (a) Schematic representation of the VV kelch proteins C2L, F3L and A55R and of the Drosophila kelch protein (ORF1), showing the location of the POZ/BTB domain and the number of KREPs. The number and location of repeats were based strictly on the presence of the highly conserved double glycine motif, a hallmark of the classic KREP (Adams et al., 2000). The fourth KREP of C2L and F3L proteins each lack one glycine residue from the double glycine motif and are, therefore, considered imperfect kelch repeats. The protein length, in amino acid residues (aa), and the percentage of amino acid identity to VV strain WR C2L are shown. (b) Immunoblot. BS-C-1 cells were mock-infected or infected with 10 p.f.u. per cell of the indicated viruses, and incubated for 14-5 h. Cell extracts were resolved by SDS-PAGE (10% gels) under reducing conditions, transferred to nitrocellulose and reacted with the anti-Flag M2 mAb (Sigma) and bound Ig was detected as described in Methods. Parallel blots were probed with mAb AB1.1 (anti-D8L) (Parkinson & Smith, 1994). The positions of molecular mass markers are shown in kDa. (c) Immunofluorescence. BS-C-1 cells were infected with indicated viruses at 5 p.f.u. per cell and fixed and processed for indirect immunofluorescence at 14 h p.i. Goat anti-DDDDK tag antibody was used to detect C2LFlag as described in Methods. Bound antibody was detected by FITC-donkey anti-goat antibody (diluted 1:100). Scale bar, 10 μm.
intermediate virus in parallel. A revertant virus (vC2L.rev) in which the C2L locus was restored to wild-type was constructed by transfecting plasmid pC2Lrev into cells infected with vC2L.

A C2LFlag gene was inserted into the TK locus of the vAC2L using plasmid pGS61C2LFlag as described previously (Smith, 1995). A recombinant virus (vC2LFlag) was distinguished from spontaneous TK– isolates by screening infected cell extracts by immunoblotting with an anti-Flag mAb (see below).

**Immunoblotting.** Samples for immunoblotting were prepared and treated as described previously (Parkinson & Smith, 1994).

**Virus growth curves.** For one-step growth curves, BS-C-1 cells were infected with 10 p.f.u. per cell for 90 min, washed with PBS to remove unbound virus and incubated in DMEM/2·5 % FBS. The culture supernatant was removed at 24 h p.i., centrifuged at 800 g at 4°C for 10 min to pellet detached cells and the supernatant was retained as the EEV fraction. Cells were harvested in DMEM/2·5 % FBS, added to the pelleted cells from above, frozen and thawed three times, and sonicated to obtain the cell-associated virus. For multiplet growth curves, BS-C-1 cells were infected at 0·01 p.f.u. per cell as above. At the indicated times, cells were scraped into the medium, frozen and thawed three times and sonicated to obtain total virus. Virus titres were determined by plaque assay on duplicate BS-C-1 cell monolayers.

**Immunocytochemistry.** Cells growing on glass coverslips (Cover glass, BDH) were infected with 1 or 5 p.f.u. per cell for 1 h on ice. After adsorption, non-adherent viruses were washed away and cells were incubated in DMEM/2·5 % FBS at 37°C. At the indicated times p.i., cells were washed with PBS and with BRB80 buffer (80 mM PIPES pH 6·8, 1 mM MgCl2, 1 mM EGTA) before being fixed in 4 % paraformaldehyde (PFA) in BRB80 buffer for 20 min at room temperature. Cells were fixed for 15 min at room temperature with 20 mM glycine in PBS, permeabilized with 0·1 % Triton X-100 in PBS and blocked with PBS/10 % FBS. For indirect immunofluorescence mouse mAbs anti-z-tubulin (B-5-1-2, Sigma), anti-vinculin (hVIN-1, Sigma) and antipaxillin (349, Transduction Laboratories) were diluted (1: 100, 1: 100 and 1: 500, respectively) in PBS/10 % FBS and added to cells for 1 h at room temperature. Primary mAbs were detected with a donkey anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Jackson Laboratories, dilution 1/50) for 30 min at room temperature. For detection of C2LFlag, goat anti-DDDDK tag antibody (Jackson Laboratories, dilution 1/100) and added to fixed and permeabilized cells for 1 h at room temperature. Bound antibodies were detected by adding donkey anti-goat FITC-conjugated antibody (Jackson Laboratories, dilution 1/100) for 30 min at room temperature. Live staining was done as described previously (Bartlett et al., 2002). For simultaneous staining of filamentous actin, tetramethylrhodamine B isothiocyanate (TRITC)–phalloidin (Sigma, diluted 1: 100) was added with the secondary antibody. Images were acquired with a Zeiss confocal laser-scanning microscope (LSM 510) and processed with Adobe Photoshop software.

**Quantification of cellular projections.** BS-C-1 cells were seeded onto glass coverslips to give well-isolated cells, and were infected with 5 p.f.u. per cell. Cells were processed for immunocytochemistry as described above and stained with TRITC–phalloidin. For each coverslip, 200 cells were analysed by fluorescent microscopy and scored positive for VV-induced cell projections when either two or more projections were observed, or a single ramified projection was observed.

**Depletion of extracellular Ca2+ and quantification of Ca2+-independent adhesion.** Depletion of extracellular Ca2+ was performed as described (Sanderson & Smith, 1998). Confluent monolayers of BS-C-1 cells were mock-infected or infected with 3 p.f.u. per cell and incubated for 18 h at 37°C. After three washes with PBS, cells were incubated with PBS/1 mM EGTA at room temperature. The morphology of cells was recorded by phase-contrast microscopy before and after depletion of extracellular Ca2+. Three random areas of the monolayer (~150 cells per field) were photographed under a 10× objective and the number of round or adherent cells was scored from projected images.

**Mouse intradermal model.** Groups of 8- to 10-week-old female BALB/c mice were injected intradermally in their left ear pinnae with 10^3 p.f.u. in 10 μl of PBS as described (Tscharke & Smith, 1999). The diameter of the lesions was estimated daily to the nearest 0·5 mm using a micrometer. The titres of infectious virus in the ears was determined by plaque assay using extracts from the ears prepared by grinding in a glass homogenizer, followed by three freeze–thaw cycles and sonication.

**Analysis of cell populations in infected ears by fluorescence activated flow cytometry (FC).** Groups of 6- to 8-week-old female C57BL/6 mice were injected intradermally in both ears with 10^3 p.f.u. At 14 days p.i. mice were sacrificed and both ears were removed, washed in 70 % ethanol and dried. The ventral and dorsal leaflets of each ear were separated and placed internal side down onto RTPM 1640 medium (Gibco) containing 50 IU of penicillin and streptomycin (Gibco), 10 % FBS and 2·5 mM HEPES, pH 7·4 in a plastic bacterial plate. Samples were incubated at 37°C for 8 h to allow cells to migrate from the ear. Non-adherent cells were collected and adherent cells were washed with PBS and removed from the plate by incubation in PBS (Ca2+ and Mg2+-free) with 2 mg glucose ml^-1 for 20 min at 37°C. Adherent and non-adherent cells were pooled, washed once with RPMI/10 % FBS, once with Tris/NaCl buffer (0·14 M NaCl in 17 mM Tris, pH 7·2) and twice with FC buffer (0·1 % BSA, 0·1 % NaN3 in PBS). The number of viable cells was determined by trypan blue exclusion.

About 10^6 cells (corresponding to pooled cells from six ears per infection group) in FC buffer were incubated for 10 min on ice with 10 % rat serum and 0·5 μg rat anti-mouse CD16/CD36 (BD Pharmingen) to block Fc receptors on cells. Cells were then double or triple stained with rat mAbs conjugated to FITC, phycoerythrin (PE) or tricolor (TC), or incubated with the appropriate isotype controls, for 30 min on ice. Cells were washed with FC buffer and with PBS before being fixed in PBS/2 % PFA for 30 min at room temperature. Fixed cells were collected, resuspended in PBS and analysed in a fluorescence activated cell sorter (FACScalibur, Becton Dickinson) using CellQuest software.

The dermal cells were identified by characteristic size (forward scatter) and granularity (side scatter) combined with two-colour analysis. CD4+ and CD3+ T lymphocytes were identified by their small size, low granularity and bright CD3 (PE anti-CD3, Caltag Laboratories) and CD4 (FITC-anti-CD4, Caltag Laboratories) or CD8 (FITC-anti-CD8, Sigma) staining, respectively. Neutrophils were identified by their small size, high granularity and bright Ly6-G (PE anti-Ly6-G, Caltag Laboratories) staining. Macrophages and dendritic cells were identified by their large size and granularity, and bright F4/80 (PE-anti-F4/80, Serotech) or DEC205 (FITC-anti-DEC205, Serotech) staining, respectively.

**Statistical analysis.** Student’s t-test (two tailed, unpaired) was used to test for the significance of the results.
RESULTS

Identification of the C2L protein

To identify the C2L protein a short epitope recognized by a mAb was attached to the C terminus. This resultant gene, C2LFlag, was placed downstream of the VV 7.5K promoter and inserted into the VV TK locus of a deletion mutant lacking the C2L gene (vΔC2L, see Methods). A recombinant virus was isolated and called vC2LFlag. Expression of the C2L protein was examined by immunoblotting extracts from mock-infected cells, or cells infected with vC2L-wt, vΔC2L or vC2LFlag using an anti-Flag mAb (Fig. 1b). In cells infected with vC2LFlag a protein of 56 kDa was detected that was absent from the other samples. This protein was not detected in the supernatant of infected cells and inhibitors of glycosylation (monensin and tunicamycin) did not affect its size (data not shown). As a control to determine if cells had been infected equally with each virus, parallel blots were re-probed with a mAb against the VV structural protein D8L. This showed equivalent amounts of virus antigen in each infected sample.

The location of the C2L protein was analysed by immunofluorescence (Fig. 1c). Cells were infected with vC2L-wt, vΔC2L, or vC2LFlag or mock-infected and were processed for immunofluorescence. Cells infected with vC2LFlag showed a diffuse cytoplasmic fluorescence that was absent from cells infected with vC2L-wt or vΔC2L or from mock-infected cells. If the anti-flag mAb was added to live cells infected with vC2LFlag no fluorescence was detected indicating that the C2L protein was intracellular (data not shown).

The C2L gene does not affect VV replication in vitro

To examine the function of C2L, a deletion mutant virus lacking 98% of the C2L gene (vΔC2L), a plaque-purified wild-type virus (vC2L-wt) and a revertant virus (vC2L-rev) were constructed (see Methods). The virus genomes were analysed by restriction digestion, PCR and Southern blotting, using DNA extracted from virus cores (Esposito et al., 1981). These analyses confirmed the deletion of C2L in vΔC2L and the absence of gross genomic alterations or changes in the regions surrounding C2L in the recombinant viruses (data not shown).

The isolation of vΔC2L confirmed previous observations (Kotwal & Moss, 1988) that the C2L gene is non-essential for virus replication in vitro. Nevertheless, it was possible that there were differences in virus replication and therefore the growth properties of vAC2L were analysed. After infection of BS-C-1 cells at 10 p.f.u. per cell for 24 h, the virus yields of the vC2L-wt, vAC2L and vC2L-rev in the cell or supernatant [see supplementary data (a) at JGV Online: http://vir.sgmjournals.org/] were indistinguishable. Similarly, there were no differences in the virus yields after infection of BS-C-1 cells at 0-01 p.f.u. per cell [supplementary data (b)].

Plaque phenotype

Although VV replication in vitro was unaffected by loss of C2L, it was evident that the plaques formed by vAC2L in BS-C-1 cells had a different morphology to those formed by vC2L-wt and vC2L-rev (Fig. 2). The plaques formed by vAC2L in BS-C-1 cells (Fig. 2b) were similar to those of VV strain WR mutant 6/2 (Fig. 2d), which has a large deletion in the left end of the genome including C2L (Kotwal & Moss, 1988). Despite the fact that plaques from vAC2L appeared smaller than those from the control viruses, measurements of the diameter of the plaques from the outer edge of virus-induced CPE showed there were no differences (Fig. 2a–c). The different plaque morphology of vAC2L compared to controls is seen more clearly under higher magnification of the edge (Fig. 2e, f, g) or centre (Fig. 2h, i, j) of plaques.

VV contributes to aspects of VV-induced CPE

The plaque phenotype of vAC2L prompted further investigation of the role of C2L in VV-induced morphological changes in the host cells. A kinetic analysis of infected cells up to 12 h p.i. showed that the formation of viral factories, dispersal of virus to the cell periphery, formation of actin tails in BS-C-1 cells and reduction of actin stress fibres were all unaffected by the loss of C2L (data not shown). Additionally, there was no difference between the motility of cells infected with the different viruses in an in vitro wound healing assay described previously (Sanderson et al., 1998) (data not shown). However, analysis of cellular projection formation in infected cells revealed differences (Fig. 3). Cells infected with vAC2L formed projections by the bundling of microtubules with a similar structure to those induced in cells infected by vC2L-wt or vC2L-rev (Fig. 3a–d). However, the proportion of vAC2L-infected cells forming projections was reduced consistently (Fig. 3e). At 12 and 16 h p.i. projection formation was reduced by 60% compared to controls and by 40% at 20 and 24 h p.i. Therefore, C2L contributes to the formation of cellular projections, although it is not essential for this phenotype.

Next, we investigated whether C2L was required for the VV-induced Ca2+-independent cell–ECM adhesion, a phenotype that is evident after the onset of VV-induced cell motility, but occurs prior to the formation of VV-induced cellular projections (Sanderson & Smith, 1998). BS-C-1 monolayers were compared for their ability to adhere to the ECM after depletion of extracellular Ca2+. In uninfected cells, 80% or 100% of cells rounded up due to cell–cell and cell–ECM detachment within 10 or 20 min, respectively, after extracellular Ca2+ depletion (Fig. 4a, e, i). In contrast, in vC2L-wt- or vC2L-rev-infected monolayers, most cells remained adherent with only approximately 15% of cells rounding up 20 min after Ca2+ depletion. This is similar to previous results (Sanderson & Smith, 1998). However, cells infected with vAC2L showed a phenotype intermediate between mock-infected cells and cells infected with the control viruses. Although this was already evident 10 min after Ca2+ depletion (Fig. 4a–h), it became more obvious by...
20 min when 40% of vΔC2L-infected cells had rounded up (Fig. 4i). Therefore, the C2L protein also contributes to the VV-induced Ca\(^{2+}\)-independent adhesion to the ECM, although it is not the only component necessary.

Given that C2L contributes to the Ca\(^{2+}\)-independent adhesion phenotype, it was possible that the C2L protein may alter the distribution or composition of focal adhesions.

Therefore, the distribution of paxillin and vinculin, two proteins present in cellular focal adhesions, was investigated. In mock-infected cells vinculin (Fig. 5a, d, g) and paxillin (data not shown) were concentrated in focal adhesions (arrowhead-like) at the end of actin stress fibres and focal complexes (dot-like) at the edges of lamellipodia or filopodia. In infected cells, vinculin concentrated in small dot-like areas at the edges of lamellipodia and filopodia,
particularly in the growth cone and along condensed regions of the virus-induced projections (Fig. 5e, f). Similar data were obtained for paxillin (data not shown). Both paxillin and vinculin were also concentrated at the ends of fine stress fibres, in the cell body or projecting into the leading edge of a developing projection. Overall, there was no

Fig. 3. Formation of VV-induced cellular projections. (a–d) BS-C-1 cells were mock-infected (a) or infected with 1 p.f.u. per cell of vC2L-wt (b), vΔC2L (c) or vC2L-wt (d). At 14 h p.i. cells were fixed and processed for immunofluorescence and stained with anti-α-tubulin mAb, as described in Methods. Scale bar, 20 μm. (e) Quantification and kinetic analysis of the formation of cellular projections. Well-isolated BS-C-1 cells were infected with 5 p.f.u. per cell and at the indicated times were fixed, stained with TRITC-phalloidin and quantified for the formation of projections as described in Methods. In each experiment, 200 cells were analysed per time point. Data show the mean percentage of cells presenting projections (from three independent experiments) ± SD. As a control for efficiency of infection, the formation of viral factories containing virus DNA was examined by DAPI staining, which confirmed that all cells were infected (data not shown). The horizontal bar represents a comparison of means of vΔC2L versus vC2L-wt and vC2L-rev groups, across 20–24 h p.i. (P<0.04).
difference in the distribution of paxillin or vinculin in cells infected by vC2L-wt (b, e, h) and vΔC2L (c, f, i) indicating that loss of C2L does not affect the distribution of focal contacts in infected cells.

**Virulence of VV lacking C2L**

The virulence of vΔC2L was assessed in murine intranasal and intradermal models of infection. In the intranasal model, no difference in the weight loss or mortalities of the animals infected with the different viruses was observed (data not shown). However, in the intradermal model, although all mice developed lesions by day 5 p.i. and these enlarged at the same rate, it was clear from day 8 p.i. that lesions caused by vAC2L were more pronounced and took longer to heal than those caused by vC2L-wt and vC2L-rev (Fig. 6a). This difference was statistically significant on days 11 to 18 p.i. (*P* < 0.001) (Fig. 6a). These results were confirmed in a repeat experiment, in which lesions caused by vAC2L remained longer at their peak size and were significantly (*P* < 0.02) larger, across days 13–24 p.i., than lesions caused by the control viruses (Fig. 6b). Despite this difference, virus yields obtained from the infected ears were the same for all groups, with titres reaching a peak of between 1 and 2·5 × 10⁶ at day 5 p.i. before declining (Fig. 6c). The higher titre obtained for the vC2L-rev infection group at day 11 p.i. (Fig. 6c) was due to a 10⁴-fold higher titre from a single mouse, and reflects a higher variability of virus titres at these later time-points, because they depend on whether the scab, where most of the infectious virus persists, has fallen off (Tscharke & Smith, 1999; Tscharke *et al*., 2002).

The results suggested that the persistence of lesions caused by vΔC2L might be caused by enhanced immunopathology. To investigate this, the cell infiltrate in the ears of C57BL/6 mice infected with 10⁴ p.f.u. was quantified by flow cytometry. C57BL/6 mice were used here because they develop larger lesions that have a larger number of infiltrating cells than BALB/c mice (Tscharke *et al*., 2002), thus facilitating a quantitative analysis. As noted previously, VV-induced lesions in C57BL/6 mice were larger than those observed in BALB/c mice (compare lesion diameters in Fig. 6a, b with those in Fig. 7a). Although the maximum lesion sizes from the different infection groups were indistinguishable in C57BL/6 mice after inoculation with 10⁴ p.f.u., the lesions caused by vAC2L remained at their peak size longer and were significantly (*P* < 0.01) larger at days 13 and 14 p.i. than lesions caused by the control viruses. Quantification of cell populations in infected ears by flow cytometry showed that, for all infection groups, neutrophils were the main component of the cell infiltrate, followed

**Fig. 4.** Ca²⁺-independent adhesion to the ECM. (a–h) Monolayers of BS-C-1 cells were mock-infected (a, e) or infected with 3 p.f.u. per cell of vC2L-wt (b, f), vAC2L (c, g) and vC2L-rev (d, h) for 18 h. Photographs of cell monolayers under phase contrast microscopy were taken immediately before (a–d) or 10 min after (e–h) depletion of extracellular Ca²⁺ as described in Methods. (i) Quantification and kinetic analysis of Ca²⁺-independent cell–ECM adhesion. Cell morphology was recorded as above 18 h.p.i. before or 3, 10 and 20 min after Ca²⁺ depletion. In each experiment, three random areas of the monolayer with approximately 150 cells per field were photographed. The number of round or spread cells was scored from projected images. Data points are the mean of three independent experiments ± SD.
Fig. 5. Distribution of focal contacts in VV-infected cells. BS-C-1 cells were mock-infected (a, d) or infected with 1 p.f.u. per cell of vC2L-wt (b, e, h) or vΔC2L (c, f, i) and processed for immunofluorescence (see Methods) at 18 h p.i. Cells were stained with anti-vinculin (d–f) and filamentous actin was visualized with TRITC–phalloidin (a–c). (g–i) Merged images of panels (a) and (b), (c) and (d), and (e) and (f), respectively. Similar results on the distribution of focal contacts were obtained with cells infected with vC2L-rev (not shown). Insets show higher magnification of cellular projections, indicated in the main panel by the white arrows. Scale bars, 10 μm.
by T-cells, macrophages and dendritic cells (Fig. 7b). Ears infected with vΔC2L had a 2- to 2.4-fold increase in the total number of cells when compared with ears infected with the control viruses. This was due to an increase in the total number of neutrophils (2-2.9-fold), CD8+ T-cells (1.7- to 2.1-fold), macrophages (2.2- to 2.6-fold) and dendritic cells (2.6- to 3-fold) (Fig. 7b). The total number of CD4+ T-cells was the same for all infection groups. The relative proportions of the identified cell populations were the same for all viruses except for the population of CD4+ T-cells, which was proportionally reduced in the vΔC2L group compared to controls.

Lastly, the immune response following infection with the different viruses was investigated by measurement of the antibody response against VV on day 29 p.i. and the cytotoxic T lymphocyte activity of cells recovered from VV-infected ears (against VV-infected targets) at day 14 p.i. No differences were found following loss of C2L (data not shown).

**DISCUSSION**

In this report the role of VV gene C2L was analysed by comparison of vΔC2L with wild-type and revertant viruses. C2L was not required for virus replication or spread in cell culture. However, infection with vΔC2L produced plaques with a different morphology, even though their size was unaltered. vΔC2L-infected cells presented a reduction in the formation of VV-induced cellular projections and in the VV-induced Ca2+-independent cell–ECM adhesion phenotype. The reduction in the formation of projections in vΔC2L-infected cells may explain the difference in plaque morphology.

Previously, it was suggested that the transition to Ca2+-independent adhesion might be necessary for the formation of cellular projections because: (i) the Ca2+-independent adhesion phenotype precedes projection formation; (ii) VV-induced projections adhere to the ECM in a Ca2+-independent manner and their formation was supported by the same ECM components that support the Ca2+-independent adhesion; and (iii) both phenotypes required late gene expression (Sanderson & Smith, 1998). Therefore, the disruption of C2L could have affected the Ca2+-independent adhesion directly and thereby reducing the formation of cellular projections.

The mechanism of VV-induced Ca2+-independent cell–ECM adhesion is probably mediated by integrins (Sanderson & Smith, 1998). Integrins are heterodimeric glycoproteins formed by an α and β subunit, and constitute the major transmembrane components of the focal

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**Fig. 6.** Virulence of vΔC2L in a murine intradermal model. (a, b) Lesion diameters of BALB/c mice inoculated with 10^4 p.f.u. of the indicated viruses. (a) Data points are the mean lesion diameter ± SEM of groups of seven (8-week-old) mice. The horizontal bar represents days on which there was a statistically significant difference between vΔC2L and both vC2L-wt and vC2L-rev (P<0.001). (b) Data points are the mean lesion diameter ± SEM in groups of 18 (8- to 10 week-old) BALB/c mice up to day 2, 15 mice up to day 5, 12 mice up to day 8, 9 mice up to day 11 and 6 mice thereafter. Horizontal bar: as for (a) (P<0.02). (c) Virus titres in infected mouse ears. On the indicated days p.i., three mice per group were sacrificed and the titre of virus in the infected ears was determined by plaque assay. Bars represent mean virus titres (expressed as log_{10} p.f.u. per ear) from three mice ± SEM.
adhesions (Hynes, 1992). Their extracellular domains participate in binding to ECM components, whereas their short cytoplasmic tails interact with a multimolecular complex of proteins (Petit & Thiery, 2000; Geiger et al., 2001). Most integrins require extracellular divalent cations for function (Mould, 1996) but there are a few exceptions (Lallier & Bronner-Fraser, 1992; Sanderson & Smith, 1998). Possibly, late in VV infection the class of integrins expressed does not require extracellular Ca$^{2+}$ for function. Alternatively, since the conformation of integrins can be regulated and binding to the ECM is influenced by conformation (Mould, 1996), it is possible that during VV infection the requirement for extracellular Ca$^{2+}$ for integrin conformation, and therefore function, could be reduced. Like other POZ-KREP proteins, C2L is intracellular and therefore might interfere with integrin function by interacting directly or indirectly with the integrin cytoplasmic tail. Alternatively, C2L could interact directly or indirectly with other focal adhesion transmembrane proteins, which influence integrin binding to the ECM (Petit & Thiery, 2000).

Although loss of C2L had no effect on VV virulence in an intranasal mouse model, in an intradermal mouse model the lesions produced by vΔC2L remained at their maximum size longer and healed more slowly than lesions produced by the control viruses. This was not due to a difference in virus titres, suggesting that the larger lesions reflect increased immunopathology. The latter hypothesis was supported by the increased cellular infiltrate in the vΔC2L-infected ears. This represented a general increase in the quantity of all cell types analysed (except CD4$^+$ T-cells). Therefore, in this model C2L reduces the cell infiltrate in VV-infected ears and reduces pathology associated with VV infection, promoting healing of lesions.

The finding that an intracellular kelch-like protein reduces the general cell infiltrate in VV-infected ears is intriguing. As the larger lesions caused by vΔC2L were observed at later stages of infection, C2L may affect the complex process of healing. The C2L protein may alter the balance of cytokines and chemokines produced by infected leukocytes or dermal cells, thereby influencing the recruitment of leukocytes. C2L might also influence the migratory properties of immune cell types or epidermal cells, thereby influencing the lesion resolution, or co-operate with other intracellular VV proteins that halt the immune response to VV infection. The mechanism of C2L action in this regard is not clear, but we note that inflammation and wound healing involve integrin-mediated adhesion, providing a potential link with the in vitro findings and possible role of C2L discussed above.

In summary, VV C2L is an intracellular 56 kDa protein that contributes to the formation of VV-induced cellular projections and to the development of the Ca$^{2+}$-independent adhesion of infected cells to the ECM. In addition, C2L reduces the pathology of VV infection in an intradermal mouse model.
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