The vaccinia virus N1L protein is an intracellular homodimer that promotes virulence

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The vaccinia virus (VV) N1L gene encodes a protein of 14 kDa that was identified previously in the concentrated supernatant of virus-infected cells. Here we show that the protein is present predominantly (> 90%) within cells rather than in the culture supernatant and it exists as a non-glycosylated, non-covalent homodimer. The N1L protein present in the culture supernatant was uncleaved at the N terminus and was released from cells more slowly than the VV A41L gene product, a secreted glycoprotein that has a conventional signal peptide. Bioinformatic analyses predict that the N1L protein is largely alpha-helical and show that it is conserved in many VV strains, in other orthopoxviruses and in members of other chordopoxvirus genera. However, database searches found no non-poxvirus proteins with significant amino acid similarity to N1L. A deletion mutant lacking the N1L gene replicated normally in cell culture, but was attenuated in intranasal and intradermal murine models compared to wild-type and revertant controls. The conservation of the N1L protein and the attenuated phenotype of the deletion mutant indicate an important role in the virus life-cycle.

Introduction

Vaccinia virus (VV) is the prototypic member of the Poxviridae, a group of complex DNA viruses that replicate in the cell cytoplasm (Moss, 2001). The genomes of VV strains Copenhagen (Goebel et al., 1990), modified virus Ankara (Antoine et al., 1998), and Tian Tan (accession no. AF095689) have been sequenced and contain approximately 200 genes. Genes located in the centre of the virus genome are conserved between orthopoxviruses and generally are essential for virus replication. In contrast, genes located near the termini are more variable, are often dispensable for growth in tissue culture and affect host range or virulence (Perkus et al., 1991). VV and other poxviruses have developed numerous strategies to modulate host responses to infection (Smith et al., 1997; Alcamí & Koszinowski, 2000; Moss & Shisler, 2001) and several of these immunomodulatory proteins share amino acid sequence similarity with host cell proteins, suggesting that they were acquired originally from the host.

The terminal regions of the VV genome are variable as exemplified by the isolation of spontaneous mutants with rearrangements or deletions in these regions (Wittek et al., 1978; Panicali et al., 1981). One such VV strain, Western Reserve (WR) mutant (6/2), contains a large deletion near the left terminus (Moss et al., 1981); it exhibits normal growth properties in tissue culture, but is attenuated in vivo (Buller et al., 1985). Nucleotide sequence analysis of this region of the parental virus genome (HindIII C and N fragments) and comparison to virus 6/2 revealed that the 6/2 genome had undergone a transposition event resulting in the loss of 17 open reading frames (ORFs) (Kotwal & Moss, 1988). Among the ORFs missing from the 6/2 mutant is N1L, which was predicted to encode one of several proteins of approximately 12 kDa in the supernatants.

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Accession numbers for the vaccinia virus N1L genes are: VV strain Western Reserve, AF451287; strain Wyeth, AF455803; strain Lister, AF455804; strain King Institute, AF455805; strain USSR, AF455806.
of VV-infected cells (Kotwal et al., 1989). The contribution of the N1L protein to the attenuated phenotype of the 6/2 mutant was analysed by construction of a VV-recombinant lacking a functional N1L gene. This virus was attenuated in intracranial and intraperitoneal mouse infection models (Kotwal et al., 1989), although a revertant virus was not reported.

Here the VV N1L protein has been characterized more extensively and its role in the virus life-cycle has been studied by the construction of deletion and revertant viruses. The N1L protein synthesized by VV WR-infected cells is a non-glycosylated, non-covalent homodimer that is present predominantly within infected cells. The deletion mutant was attenuated compared to wild-type and revertant controls in two murine models of infection.

Methods

**Cells and viruses.** Cell lines were grown as described (Alcamí & Smith, 1995). VV strain WR was grown and titrated in BS-C-1 cells (Mackett et al., 1985).

**Construction of VV N1L deletion and revertant viruses.** A virus lacking 88% of the N1L ORF (nucleotides -78 to +276 relative to the N1L initiation codon) was constructed by transient dominant selection (Falletter & Moss, 1990) using the E. coli guanine xanthine phosphoribosyltransferase (gpt) gene as the selectable marker (Boyle & Coupair, 1988). The entire N1L ORF and left and right flanking regions from VV strain WR were amplified by a PCR using VV WR genome DNA as template, and as primers oligonucleotides (5’ CATATGAGGACTCATTATTAGATATATC and 5’ CTCTGGAATTCTTTATTACATATTAC) that contained Ndel and EcoRl sites (underlined). Alternatively, the N1L ORF with a C-terminal (6)His tag was amplified by PCR using the same 5’ primer and as the 3’ primer an oligonucleotide (5’ CTCATTGAGGACTTATTTTTCACTAGTA) containing a Xhol site (underlined) and lacking a stop codon. The resultant DNA fragments were digested with the appropriate enzymes and cloned into Ndel- and EcoRl- or Ndel- and Xhol-restricted pET24a (Novagen) to generate pETN1L and pETN1Lhis, respectively. Recombinant proteins were expressed from E. coli using the pET system (Novagen) according to the manufacturer’s specifications and were called EN1L and EN1L-his, respectively. EN1L-his was purified by Ni2+-affinity chromatography [HiTrap chelating HP column, AmershamPharmacia Biotech (APB)] and gel filtration using a calibrated Superdex 75 HR column (APB). EN1L protein was purified by ion-exchange chromatography using a ResourceQ column (APB), gel filtration (as described above) and a final polishing step using a MonoQ HR column (APB). Buffers all contained 1 mM dithiothreitol.

**Production of polyclonal antiserum and purification of IgG.** A polyclonal antiserum (α-N1L) was generated by immunizing New Zealand White rabbits with the purified AN1L-his protein according to standard protocols (Harlow & Lane, 1988). The IgG fraction was isolated from the antiserum by precipitation with 65% (w/v) (NH4)2SO4 and affinity purification on a HiTrap protein A-Sephrose column (APB) as instructed by the manufacturer.

**Immunoblotting.** BS-C-1 cells were mock-infected or were infected with the indicated viruses at 10 p.f.u. per cell in the presence, where specified, of 40 µg/ml β-d-arabinofuranoside (araC), 1 µg/ml tunicamycin or 1 µM monensin (all Sigma). At 24 h post-infection (p.i.) cell extracts were prepared as described previously (Ng et al., 2001). Supernatants were centrifuged (3000 g; 10 min) to remove cellular debris before concentration in a centrifugal filter device (Amicon). Where indicated, supernatants were ultraconcentrated (14 000 g for 2 h) to pellet extracellular virus. Cell extracts and supernatants were analysed by SDS–PAGE (15% gel), transferred to nitrocellulose and probed with rabbit α-N1L IgG or mouse mAb AB1.1 against the VV D8L protein (Parkinson & Smith, 1994) (each diluted 1:1000). Bound IgG was detected by incubation with the appropriate horseradish peroxidase-conjugated IgG (Sigma) (diluted 1:2000) followed by the enhanced chemiluminescence (ECL) detection system (APB).

**Pulse–chase and immunoprecipitation.** BS-C-1 cells were infected at 10 p.f.u. per cell with VV WR. At 4 h p.i. the culture medium was removed, the cells were washed in methionine- and cysteine-free medium and the cells were incubated in the same medium for 20 min. Cells were pulsed with 100 µCi [35S]methionine and [35S]cysteine (1000 Ci/mmol)/106 cells (APB) for 20 min, the cells were washed with DMEM containing 2 mM unlabelled methionine and cysteine and then incubated in the same. At the indicated times, the supernatants were collected and filtered (0.2 µm) to remove cellular debris. Cells were washed with ice-cold PBS before being scraped into lysis buffer (10 ml/106 cells) and harvested. For immunoprecipitations, cell extracts and supernatants were pre-absorbed against protein A-agarose (Pierce) and then incubated with either α-N1L or α-A41L. Immune complexes were captured with protein A–agarose, washed, eluted in Laemmli buffer,
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Fig. 1. (a) Amino acid sequence comparisons of the N1L proteins from variola virus Bangladesh–1975 (VAR, accession no. L22579), VV strain Copenhagen (VV, M35027), cowpox virus strain GRI-90 (CPV, Y11842) and myxoma virus (MYX, AF170726). The alignment was constructed using CLUSTALW (Thompson et al., 1994) and formatted using GeneDoc (Nicholas & Nicholas, 1997). Identical amino acids are shown shaded in black; identities across three of the sequences are highlighted in grey. The single Cys residue is marked with a dot. Bars above the sequence indicate regions predicted to be alpha helical. (b) Hydrophobicity profile of the N1L protein from VV Copenhagen. The hydrophobicity of the protein sequence was analysed with the program ProtScale (Kyte & Doolittle, 1982).

and proteins were resolved by SDS–PAGE (15% gel) and visualized by fluorography.

Radio-iodination of the N1L protein. N1L protein was purified from the supernatants of AcN1L-infected insect cells as described above. This protein was then radio-iodinated using the Bolton–Hunter reagent as described previously for human interferon-α2 (Symons et al., 1995).

Immunofluorescence. BS-C-1 cells were grown on 13 mm glass coverslips and were infected with VV WR or vΔN1L at 1 p.f.u. per cell. For surface staining of live cells, the culture medium was removed at 18 h p.i. and was replaced with DMEM containing rabbit α-N1L IgG (diluted 1:100) or rat anti-B5R mAb 19C2 (Schmelz et al., 1994) (hybridoma culture supernatant diluted 1:100) and the incubation was continued for 1 h at 4 °C. Cells were washed three times with ice-cold PBS and then fixed and permeabilized by incubation in methanol at −20 °C for 10 min. Cells were washed thoroughly with PBS, and then incubated in PBS containing 10% FBS for 20 min at room temperature followed by rhodamine-conjugated donkey anti-rabbit IgG, or Texas red biotiniothiocyanate (TRITC)-conjugated donkey anti-rat IgG (both from Jackson Laboratories) in PBS containing 10% FBS for 30 min at 37 °C. Cells were washed three times in PBS containing 10% FBS and once in water before mounting in Mowiol as described previously (Sanderson et al., 1996).

To stain fixed and permeabilized cells, the culture medium was removed at 18 h p.i. and cells were fixed and permeabilized in methanol as described above. Primary antibody staining was performed at 37 °C for 1 h. Addition of secondary conjugated antibodies and mounting were as described above. All samples were examined with a Zeiss LSM 510 laser scanning confocal microscope and images were captured and processed using Zeiss LSM Image Browser version 3.1.0.99.

Mouse infection models. The virulence of the recombinant viruses was determined in female BALB/c mice 6–8 weeks old. Mice were anaesthetized and infected intranasally with VV in 25 µl of PBS (Gardner et al., 2001), or by intradermal injection of 10^4 p.f.u. of VV in 10 µl in the left ear pinna (Tscharke et al., 2002). For intranasal infections, mice were weighed daily and signs of illness were scored as described previously (Alcamí & Smith, 1992). Mice were euthanized if they lost more than 30% of their body weight. For intradermal infections, the sizes of lesions on infected ears were estimated daily with the aid of a micrometer. To determine virus titres, infected ears were ground with a tissue homogenizer, subjected to three cycles of freezing and thawing and two sonications and the resulting homogenate was assayed on BS-C-1 cells (Tscharke & Smith, 1999).

Results
Bioinformatic analysis of the N1L protein

The published sequence of the VV WR N1L gene predicts a protein of 117 amino acids with a size of 13.8 kDa (Kotwal
Fig. 2. Expression of the N1L protein by AcNPV and VV. (a) Metabolic labelling. Sf21 cells were mock-infected or infected with AcN1L or AcNPV at 10 p.f.u. per cell. At 24 h p.i. the cells were radiolabelled for 2 h with [35S]methionine and [35S]cysteine (70 µCi/ml; 100 µl per well) and proteins in the culture supernatant were analysed by SDS–PAGE and autoradiography. An arrow indicates the position of the N1L protein. (b) Immunoblot. Proteins in the culture supernatants of Sf21 cells that were mock-infected or infected with AcN1L, AcN1Lhis or AcNPV were separated by SDS–PAGE (15%) gel, transferred to nitrocellulose and incubated with rabbit α-N1L (Methods). (c) Immunoblot. Cells were mock-infected or infected with the indicated VVs (Methods) and at 20 h p.i. the proteins in the culture supernatant were analysed by immunoblot using either rabbit α-N1L or pre-immune serum from the same animal. For all panels the positions of molecular size markers are indicated in kDa.

The sequence of the N1L gene of our strain of VV WR (accession no. AF451287) differed from the published sequence by a single nucleotide change that resulted in an N23D amino acid substitution but was identical with the N1L sequence in VV strains Copenhagen and Tian. The N1L protein is highly conserved in variola major virus (VAR) Bangladesh-1975 (Massing et al., 1994) and cowpox virus (CPV) GRI-90 (Shchelkunov et al., 1998). In VV strain modified vaccinia virus Ankara (MVA) the gene is truncated so that a protein of only 113 amino acids is predicted (Antoine et al., 1998). Related genes are encoded by the leporipoxviruses Shope fibroma virus (Willer et al., 1999) and myxoma virus (Cameron et al., 1999) and by lumpy skin disease virus, a capripoxvirus (Tulman et al., 2001). In addition we sequenced the N1L gene from VV strains Wyeth (AF455803), Lister (AF455804), King Institute (AF455805) and USSR (AF455806) and found these to encode identical proteins to VV Copenhagen (data not shown). An alignment of the N1L
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Fig. 3. Analysis of the N1L protein made by VV. (a) Cells were mock-infected or infected with vN1L for 15 h in the presence of AraC, monensin or tunicamycin, or in the absence of drug (Methods). Proteins in cell extracts were resolved by SDS–PAGE, transferred to nitrocellulose and the membrane was probed with α-N1L rabbit IgG. (b) Cells were infected as in (a) in the absence of drugs and cell and supernatants were collected at 18 h p.i. Supernatants were clarified by low-speed centrifugation (2000 g, 10 min) and the clarified supernatant was then centrifuged at higher speed to pellet virus particles (14000 g, 2 h). The resulting supernatant was retained and analysed by immunoblotting before or after concentration 12-fold in a Centriprep YM-10 centrifugal filter device (Amicon). To prepare samples from cells that had comparable amounts of N1L protein to supernatants, dilutions of the cell extract was prepared. Lanes 1–4: cell extracts from 4–2 × 10^4 (1), 2–1 × 10^4 (2), 8–4 × 10^3 (3) or 4–2 × 10^3 (4) cell equivalents. Lanes 5 and 6: supernatants from 4–2 × 10^4 (5) or 5 × 10^3 (6) cell equivalents. Lane 7: pellet sample, 4–2 × 10^6 cell equivalents. Proteins in all samples were separated on duplicate SDS–polyacrylamide gels and membranes were immunoblotted with either the anti-N1L rabbit IgG or mAb AB1.1 against the VV D8L protein (Parkinson & Smith, 1994).

Use of anti-N1L antibody to detect the N1L protein

To produce protein for biochemical characterization and to raise a polyclonal antiserum, the N1L protein was expressed in recombinant AcNPV with or without a C-terminal 6(his) tag (Methods). Fig. 2(a) shows that SF21 cells infected with AcN1L express a protein of approximately 14 kDa that is not made by mock-infected cells or cells infected with AcNPV. The AcN1L-his protein (AN1L-his) was purified and used to generate a rabbit polyclonal antiserum (Methods). This antiserum detected a protein of 14 kDa in the supernatant of SF21 cells infected with AcN1L, and a slightly larger protein from cells infected with AcN1L-his, but did not detect proteins from mock-infected cells, or from cells infected with AcNPV. AN1L-his was purified from the supernatant of AcN1L-his-infected cells and its N-terminal sequence was determined and found to be identical to the predicted primary translation product.

The polyclonal antiserum was also used to identify the N1L protein in concentrated culture supernatants from cells infected with VV. Fig. 2(c) shows that a protein of 14 kDa was made by cells infected with vN1L and vN1L-rev but not by mock-infected cells or cells infected with v∆N1L (see below). These data confirmed the specificity of the antiserum and that the expression of N1L by the different viruses was as predicted.

Growth properties of N1L deletion and revertant viruses

Previous work demonstrated that the N1L gene is not essential for virus replication in tissue culture and that a mutant virus with the N1L gene disrupted was attenuated in vivo (Kotwal et al., 1989). However, in the absence of a revertant virus it was not proven that the loss of N1L contributed to this attenuated phenotype. Therefore, we constructed wild-type, deletion and revertant viruses (Methods) and studied their properties in cell culture and in animal models. The genomic structure of these viruses was analysed by PCR and Southern blotting (see supplementary data 1 at JGV Online: http://
After DNA was extracted from purified virus and digested with HindIII, the HindIII N and C fragments (approximately 1–6 kb and >12 kb, respectively) were present with vN1L and vN1L-rev. In contrast, with vΔN1L the HindIII N fragment was not detected and the C fragment increased in size slightly due to the loss of the HindIII site between the N and C fragments (Supplementary data Fig. 1a). Similarly, after digestion with either EcoRI or BglII, bands of equivalent size were detected with vN1L and vN1L-rev but these were reduced in size by approximately 350 bp for vΔN1L. These observations confirmed the absence of the N1L gene from vΔN1L, consistent with the lack of N1L protein expression by this virus (Fig. 2c).

The growth properties of vN1L, vΔN1L and vN1L-rev were examined in cell culture and found to be indistinguishable. The rate of increase in virus infectivity after infection and the final titre obtained were the same for each virus (Supplementary data 2a). Moreover, there were no differences in the amount of intracellular or extracellular virus produced and the plaques formed by each virus were of equal size (Supplementary data 2b). These data confirm that the N1L gene is dispensable for virus growth in cell culture.

The N1L protein is predominantly intracellular

To examine the kinetics of N1L protein expression, cells were infected with VV WR in the presence or absence of AraC, an inhibitor of DNA replication and late gene expression. Immunoblotting detected the 14 kDa N1L protein in cell extracts in the presence of AraC indicating early expression (Fig. 3a). This was consistent with transcriptional data that identified early and late RNA start sites upstream of the N1L initiation codon (Kotwal & Moss, 1988). Addition of monensin or tunicamycin did not affect the size of the N1L protein indicating this protein was not post-translationally modified by attachment of O- or N-linked carbohydrate (Fig. 3a).

Preliminary experiments indicated that the majority of the N1L protein was present in cell extracts rather than culture supernatants at 24 h p.i. (data not shown). To investigate the...
Fig. 5. Analysis of the location of the N1L protein in infected cells by immunofluorescence. Cells were infected with the indicated viruses and processed for immunofluorescence (Methods). In (a)–(c) cells were fixed and permeabilized before staining with the indicated Ab. In (d)–(i) live cells were incubated at 4 °C with the indicated Ab prior to fixation and permeabilization. Bound Abs were detected with secondary conjugates and examined by confocal microscopy (Methods). Phase-contrast images of panels (d) and (g) are shown in panels (e) and (h). Scale bars, 20 µm.

relative distribution of the N1L protein, cell extracts and supernatants were prepared from VV WR-infected cells and analysed by immunoblotting. To obtain an N1L signal from the cell extract that was equivalent to that present in the culture supernatant, a series of dilutions of the cell extract was prepared and the supernatant was analysed before and after concentration 12-fold. These analyses showed that > 90% of the N1L protein was associated with cells and, at the exposure shown, N1L was detected in the supernatant only after the sample had been concentrated (Fig. 3b). Kotwal et al. (1988) had used supernatants concentrated between 20- and 50-fold to detect the N1L protein, but did not analyse cell extracts. To compare the release of N1L into the supernatant with other VV proteins, the samples were also immunoblotted with mAb AB 1.1, which recognizes the D8L protein (Parkinson & Smith, 1994) that is present in cytoplasmic factories, on the surface of IMV particles and in virions released from cells (Fig. 3b). As for N1L, the majority (> 90%) of D8L was retained in the cell fraction, and the released D8L protein was detected only after concentration of the supernatant. If the supernatant was centrifuged to collect virus particles, the great majority of the D8L protein was detected in the pellet fraction. In contrast, N1L remained in the soluble fraction after such treatment. Collectively, these data indicated that N1L was predominantly a cell-associated protein but that some (< 10%) was released into the culture medium as soluble protein.

To examine the release of the N1L protein further, the kinetics of N1L protein release from cells were investigated by
Fig. 6. The N1L protein made by VV is a homodimer. (a) Concentrated supernatants from BS-C-1 cells infected with VV WR and N1L purified from E. coli were resolved by SDS–PAGE in the presence or absence of 2-ME. Proteins were transferred to nitrocellulose and immunoblotted with α-N1L IgG. Alternatively, N1L protein expressed by AcN1L was purified, labelled with...
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Fig. 7. The N1L protein contributes to virulence in a murine intranasal model.

(a) Groups of five mice were infected intranasally with \(10^4\) (a, c) or \(10^5\) (b, d) p.f.u. of vN1L, vΔN1L or vN1L-rev and the weights (a, b) of the animals and signs of illness (c, d) were recorded daily. The mean weight of each group of animals on each day is expressed as a percentage of the mean weight of the same group of animals on day zero. Signs of illness were assigned an arbitrary scale of 1 to 4 as defined previously (Alcamí & Smith, 1992). Mean values (±SEM) for each group of animals are shown.

pulse–chase analysis and were compared with the secretion of the VV A41L protein, an immunomodulatory glycoprotein secreted into the culture supernatant (Ng et al., 2001). VV-infected cells were pulsed with \([^{35}\text{S}]\)methionine and \([^{35}\text{S}]\)cysteine and chased for the indicated periods of time. Cells and supernatants were harvested and proteins were immunoprecipitated with either \textit{x-N1L} IgG or anti-A41L polyclonal serum, resolved by SDS–PAGE and visualized by fluorography (Fig. 4). The A41L protein was exported efficiently from infected cells and the majority of radioactive protein was in the supernatant within 60 min of pulse. In contrast, the N1L protein appeared to be released from cells slowly and at all times examined the great majority remained in the cell extract. Therefore, it is unlikely that N1L is a true secreted protein, but rather its presence in supernatants is most likely a result of leakage from infected cells.

The distribution of N1L protein was also examined in baculovirus-infected Sf21 cells (data not shown). Cells were infected with AcNPV, AcN1L or AcA41L, which expresses the VV strain WR A41L secretory glycoprotein (Ng et al., 2001). At 24, 48 or 72 h p.i. the cells and supernatants were collected and analysed by immunoblotting with either \textit{x-N1L} or anti-A41L Ab. At 24 h p.i. N1L was an abundant protein in cells but was absent from the supernatant, but the N1L protein appeared in the supernatant by 48 and 72 h p.i. In contrast, the A41L protein was distributed equally between cells and the supernatant by 24 h p.i. and continued to accumulate in the supernatant with time. The presence of the N1L protein in the supernatant at only very late times after infection is consistent with non-specific release due to cell lysis late after infection. Consonant with these observations, recombinant N1L protein made by \textit{Pichia pastoris} was retained within yeast cells and was not secreted (data not shown).

The location of the N1L protein was also examined by immunofluorescence (Fig. 5). BS-C-1 cells were infected with vN1L or vΔN1L for 16 h and were stained with \textit{x-N1L} IgG and mAb 19C2 directed against the B5R protein that is present on the cell surface and cell-associated enveloped virus and extracellular enveloped virus particles. Bound Abs were detected with appropriate secondary Abs and visualized by confocal microscopy (Methods). The N1L protein was detected predominantly in the cytoplasm of WR-infected cells that had been permeabilized prior to addition of Ab (Fig. 5a), but only background fluorescence was observed if the cells were stained prior to permeabilization (Fig. 5d), or if the cells were infected with vΔN1L (Fig. 5b, g). Phase contrast images confirmed the

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125I, and analysed by SDS–PAGE in the presence or absence of 2-ME before visualization by autoradiography. (b) N1L protein made by VV or \textit{E. coli} as in (a) was separated by PAGE (10% gel) in the absence of SDS, with or without 2-ME, and analysed by immunoblotting with rabbit \textit{x-N1L} IgG. N1L protein expressed by \textit{E. coli} and AcN1L was analysed in parallel together with the 35 kDa chemokine-binding protein from VV Lister (Alcamí et al., 1998); both were detected by Coomassie blue staining. (c) N1L protein made by VV as in (a) was mixed with protein size markers (APB) ribonuclease A (15-7 kDa), chymotrypsin A (20 kDa), ovalbumin (48-6 kDa) and albumin (63-7 kDa) and analysed by gel filtration. Proteins present in fractions 7–17 were analysed by SDS–PAGE and immunoblotting with \textit{x-N1L} IgG. The positions of molecular size markers in kDa are indicated on the gels shown in (a) and (c).
The presence of cells in the fields shown in panels (d) and (g) (Fig. 5e, h, respectively). In contrast, the B5R protein was detected in cells infected with either virus and was detected in cells that had or had not been permeabilized (Fig. 5c, f, i). These data show that N1L is an intracellular protein and is not present on the cell surface.

**The VV N1L protein is a non-covalent homodimer**

To determine if the VV N1L protein was monomeric or oligomeric, the protein was analysed by PAGE with or without 2-mercaptoethanol (2-ME) and SDS (Fig. 6a, b). Recombinant N1L proteins from *E. coli* or Sf21 cells were analysed in parallel. The N1L protein made by VV migrated as a monomer (~ 14 kDa) in the presence of SDS with or without reducing agent, whereas much of the N1L protein from *E. coli* and AcN1L migrated as a dimer in the absence of 2-ME (Fig. 6a). Under native conditions (without SDS) and in the presence of 2-ME, N1L from VV and *E. coli* each had an electrophoretic mobility similar to the monomeric 35 kDa chemokine-binding protein from VV strain Lister (Alcamí et al., 1998) (Fig. 6b). On these native gels, the 14 kDa form of N1L from VV or *E. coli* was detected only if SDS was included in the sample loading buffer (data not shown). However, N1L protein from bacteria formed larger oligomers in the absence of 2-ME. These data suggested that the N1L protein made by VV might be a dimer or trimer and this was investigated further by gel filtration (Fig. 6c). The concentrated supernatant from VV-infected cells (Fig. 6c) or from VV-infected cell extracts (data not shown) was fractionated by gel filtration and analysed by SDS–PAGE and immunoblotting. The N1L protein eluted in fractions 11, 12 and 13 with the majority of protein in fraction 12 and more in fraction 13 than 11. The sizes of proteins eluting in fractions 11, 12 and 13 were calculated by comparison with protein molecular size markers to be 34 ± 2, 28 ± 0 and 23 ± 7 kDa, respectively. The predicted size of the N1L complex was therefore slightly less than 28 ± 0 kDa, indicating N1L exits as a dimer under physiological conditions.

**Deletion of N1L reduces the virulence of vaccinia virus**

The virulence of the deletion mutant vΔN1L was examined in two murine models in comparison with wild-type and revertant controls. In the intranasal model, infection with vΔN1L at doses of 10⁴ and 10⁵ p.f.u. induced fewer signs of illness and induced less weight loss than controls (Fig. 7a). These differences were significant for both weights (P < 0.02) and signs of illness (P < 0.02) at 10⁴ p.f.u. and for signs of illness at 10⁵ p.f.u. (P < 0.05) on days 12 to 17 inclusive (Fig. 8a). In addition, the titre of infectious virus present in infected ears was reduced after infection with vΔN1L (Fig. 8b). Therefore, in both models of infection the deletion of N1L caused significant virus attenuation.

**Discussion**

A characterization of the VV protein N1L is presented. Hitherto, the N1L protein was described as a secreted protein that contributes to virulence. However, data presented here show that the protein is predominately intracellular (< 90%) and exists as a non-covalent homodimer. Using an N1L deletion mutant and appropriate wild-type and revertant controls, the N1L protein was shown to enhance VV virulence in intradermal and intranasal models of infection.
Although the N1L protein was described originally as a secreted protein, analysis of the distribution of the N1L protein showed that it was present predominantly within infected cells, and only a small fraction (< 10%) was released into the cell culture medium (Fig. 3b). How this protein is released is unclear, but the N terminus of N1L lacks a conventional signal peptide and amino acid sequencing showed that the N1L protein released from insect cells infected with AcN1L was uncleaved at the N terminus. Moreover, pulse-chase analyses established that in comparison with glycoprotein A41L, which is expressed by VV during the same phase of infection, has a signal peptide and is secreted from infected cells, the N1L protein leaks out of cells very slowly and the majority of the protein is always present within cells (Fig. 4). The N1L protein also has a distribution comparable with the VV D8L protein that is described as intracellular and a component of released virions. In addition, immunofluorescence showed the protein was predominantly cytoplasmic and was not present on the cell surface (Fig. 5). Collectively, these observations suggest that the small fraction of the total N1L protein released into the cell supernatant might be due to lysis of some cells following virus infection; alternatively, some N1L protein might be released by an unidentified and unconventional pathway.

Biochemical analyses of N1L protein made by VV showed that the protein is a homodimer, and that this dimer can be disrupted by treatment with SDS but not reducing agents. So that the protein is a homodimer, and that this dimer can be released by an unidentified and unconventional pathway. Recombinant N1L protein, synthesized in E. coli or in insect cells infected with recombinant baculoviruses, forms additional oligomeric structures that contain disulphide bonds. As there is only a single cysteine residue in N1L, these bonds must be intermolecular. Purification of recombinant N1L was therefore performed in the presence of reducing agent. VV expresses many proteins that are non-essential for virus replication in cell culture but which contribute to virus virulence in vivo and several proteins of this group function as immunomodulators to affect the host response to infection. VV immunomodulators may be grouped according to their site of action. Those proteins secreted from the infected cell usually bind to and inhibit the function of host proteins such as interferons, cytokines, chemokines or complement, whereas intracellular proteins may interfere with interferon-induced antiviral proteins, cell signalling pathways or apoptosis. The correct location of the protein is essential to mediate these different functions. In several cases the function of VV immunomodulatory proteins has been inferred from computational analyses of amino acid sequences that revealed similarities between a VV protein and a host protein of known function. In other cases, the function of a virus protein has been deduced by functional studies. In the case of N1L, although bioinformatic analyses established that the protein is conserved in several orthopoxviruses and in other chordopoxvirus genera, no amino acid similarity with host proteins was detected. Predictions of the secondary structure of N1L showed a largely alpha-helical protein that has a similar overall topology to other small alpha-helical proteins such as IL-10 and interferon-γ. This similarity, taken together with the proposed secreted nature of N1L, led this protein to be described by some as a virokin (Chang et al., 1992) and suggested a possible mechanism by which N1L contributed to virulence. However, data presented here cause the mechanism of action of N1L to be reconsidered. Computational analyses suggest that N1L is also related structurally to intracellular alpha-helical proteins such as the N-terminal actin-cross-linking domain of fimbrin (pdb 1aa0), histone acetyltransferase bromodomain (pdb 1b91) and the N-terminal domain of transcription elongation factor TFIIS (pdb 1eo0). It remains possible that the small fraction of N1L that is released from cells mediates an important extracellular function; however, it is more likely that the site of action of N1L is intracellular where the majority of the protein is located. Determination of the mechanism of action of N1L requires additional study.

Data presented here confirm that N1L promotes VV virulence and does so in both the intradermal and intranasal models of infection in mice. This is in contrast to a variety of other VV proteins that are associated with virulence only after inoculation by one of these routes (Tscharke et al., 2002). Furthermore in each model, the attenuation caused by deletion of N1L is amongst the strongest seen for genes that do not affect growth in culture. The attenuation seen in the intradermal model was accompanied by a reduction in virus titre in the infected skin, indicating that N1L expression may affect either virus growth in vivo or influence the rate at which virus is cleared by the host response to infection.

In conclusion, the N1L protein is demonstrated to be a non-covalent homodimer that is largely intracellular and contributes to virulence in intradermal and intranasal models of infection by an unknown mechanism.

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