Vaccinia virus semaphorin A39R is a 50–55 kDa secreted glycoprotein that affects the outcome of infection in a murine intradermal model

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Introduction

Viruses have evolved several strategies to manipulate the host immune system (Alcamì & Koszinowski, 2000; Tortorella et al., 2000). In particular, vaccinia virus (VV) and other members of the Poxviridae encode numerous immunomodulatory proteins, some of which are secreted from infected cells (Smith et al., 1997; Smith, 2000). Amino acid similarity between several of these secreted proteins and the extracellular region of cellular proteins has helped elucidate their function and suggested that these genes were acquired from the host and modified during evolution (Bugert & Darai, 2000).

The semaphorins are a family of secreted and membrane-bound proteins each containing a ‘sema’ domain of approximately 500 amino acids (Van Vactor & Lorenz, 1999; Nakamura et al., 2000; Tamagnone & Comoglio, 2000). More than 20 semaphorins have been identified and these are classified into groups according to the presence of additional protein domains and the species of origin (Semaphorin Nomenclature Committee, 1999). Most semaphorins are involved in the neurone guidance during development, but immunological semaphorins have also been identified. For example, CD100/SEMA4D is involved in the activation of B and T lymphocytes (Hall et al., 1996; Kumanogoh et al., 2000; Shi et al., 2000), and a soluble version inhibits the migration of immunological cell types, a property shared by the SEMA3A protein (Delaire et al., 2001). SEMA7A (CDw108) is also

Vaccinia virus (VV) protein A39R has amino acid similarity to the extracellular domain of a glycosylphosphatidylinositol-linked cell surface semaphorin (SEMA7A/CDw108) that has an immunological expression profile and binding properties, thereby implicating A39R as an immunomodulator. Previously, a closely related A39R protein expressed by ectromelia virus was shown to induce cytokine production and up-regulate ICAM-1 expression in mouse monocytes in vitro. In this study, we show that the A39R gene of VV strain Copenhagen (COP) encodes a 50–55 kDa secreted glycoprotein and is expressed late during infection. The A39R protein was secreted by eight of 15 strains of VV, but not by strain Western Reserve (WR). To analyse the VV A39R function, several recombinant viruses were made, including an A39R deletion mutant of VV COP and a WR mutant containing the A39R sequence from COP. Loss of the gene from COP did not affect virus growth in vitro, or VV virulence in a mouse intranasal model, and had only a slight effect on lesion size in an intradermal model. In contrast, expression of COP A39R by VV WR was associated with an increase in the severity and persistence of skin lesions after intradermal infection of mice. Finally, a histological examination of mouse skin infected with recombinant viruses suggested that A39R has direct or indirect pro-inflammatory properties.

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The nucleotide sequence of the buffalopox virus A39R gene was assigned accession no. AJ309297.
Here, the VV A39R protein was characterized and its role in VV virulence was assessed. We report that COP A39R is a glycoprotein that is secreted late during infection. In addition, we show that A39R-like proteins are secreted by several orthopoxviruses, but that the truncated WR A39R protein is retained inside infected cells. In vivo analyses demonstrate that A39R expression affects the outcome of dermal infection by VV. Preliminary histological analyses with A39R recombinant viruses suggest a pro-inflammatory role for A39R.

**Methods**

- **Cells and viruses.** All cell lines were obtained from the Cell Bank of the Sir William Dunn School of Pathology, University of Oxford, UK and were grown as described by Alcamí & Smith (1995). The sources of VV strains and other orthopoxviruses were described previously (Alcamí & Smith, 1995). Working stocks of recombinant VVs derived from strains WR and COP were prepared by sedimentation of cytoplasmic extracts of infected cells through a cushion of 36% (wt/vol) sucrose by centrifugation at 24,000 g for 80 min at 4 °C.

- **Plasmid construction.** Deletion versions of the A39R gene of VV COP and WR were assembled using splicing by overlap extension (SOE)–PCR with Pfu DNA polymerase (Stratagene) and virus DNA from the appropriate VV strain as template. Two DNA fragments were amplified that contained sequence from either the 5’ or 3’ ends of the A39R ORF and flanking regions. The 5’ fragment was generated using oligonucleotides A39R-1 (CCCCCAAGCTTAAAGATTAAGTATGAGCAAGCAGCTAGGCTGATGCGCGACTATTTTC) and A39R-4 (GGGGGATTATCATCTGAGTCTTCCCTC), which contained complementary sequence to the 5’ fragment (annealing regions are shown in bold). The 3’ fragment was amplified using oligonucleotides A39R-3 (AGGATGCGGATAAGGATTATCATCTGAGTCTTCCCTC), which contained complementary sequence to the 5’ fragment, and A39R-4 (GGGGGATTATCATCTGAGTCTTCCCTC) containing an EcoRI restriction site (underlined). The 5’ and 3’ fragments were joined by PCR using oligonucleotides A39R-1 and A39R-4 to form a deletion version of the A39R ORF that lacked 73% of the A39R gene (from strain COP), corresponding to the region between nucleotides 146918 and 147798 (numbering according to Goebel et al., 1990). Plasmids pCOP A39R-rev and pWR A39R-rev were generated by ligating the deletion fragments from VV strains COP or WR into pSJH7 (Hughes et al., 1991). Plasmids pCOP A39R-rev and pWR A39R-rev were constructed by generating a wild-type copy of the COP or WR A39R gene using PCR with oligonucleotides A39R-1 and A39R-4 and virus genomic DNA as template, and then cloning the products into pSJH7. The VV DNA inserts in all plasmids were confirmed by DNA sequencing.

- **Construction of A39R recombinant viruses.** VV recombinants were generated by transient dominant selection using the E coil gene as a selectable marker as described (Smith, 1993) with either pCOP AA39R and VV COP, or pWR AA39R and VV WR. Mycophenolic acid-resistant intermediate viruses were rescued into deletion (vCOP AA39R and vWR AA39R) or wild-type (vCOP A39R-wt and vWR A39R-wt) viruses on the hypoxanthine-guanine phosphoribosyl transferase (hprt)-negative HeLa cell line D98OR in the presence of 6-thioguanine. Revertant intermediate viruses were resolved into deletion (vCOP AA39R-rev and pSJH7) and then cloning the products into pSJH7. The VV DNA inserts in all plasmids were confirmed by DNA sequencing.

The A39R gene was identified in VV strain Copenhagen (COP) (Goebel et al., 1990) and the product of the VV Lister A39R gene was identified as a 55 kDa secreted protein (Comeau et al., 1998). In VV strain Western Reserve (WR) a 13 bp deletion introduces a frameshift and a premature stop codon into the A39R gene (Smith et al., 1991). Thus A39R from WR represents a truncated version of the VV semaphorin with a predicted molecular mass of 33-6 kDa (Fig. 1).

The ectromelia virus A39R-like protein up-regulates expression of ICAM-1, IL-6 and IL-8 in primary monocytes (Comeau et al., 1998). In addition, it was stated (data not shown) that ectromelia A39R was monomeric, caused a rapid aggregation of mouse dendritic cells, and in monocytes induced TNF-α production but inhibited chemoattractant-induced migration (Spriggs, 1999). The ectromelia A39R protein was also used to identify a cell surface receptor with a wide tissue expression that was named plexin-C1 (Comeau et al., 1998).

![A39R genes from VV strains COP and WR](image-url)
A WR virus containing the full-length COP A39R gene (vWR COPA39R) was generated using vWR ΔA39R and pCOP A39R-rev. As a control, the full-length A39R gene was deleted from this virus by infecting cells with vWR COPA39R and transfecting with pCOP ΔA39R, forming virus vWR ΔA39R-rev.

The virus genomes were analysed by Southern blotting and PCR using DNA extracted from virus cores (Esposito et al., 1981). These analyses confirmed that the genomic structure of each virus was as expected and that no other detectable alterations had occurred (data not shown).

Production and purification of polyclonal antisera. The COP A39R ORF (amino acids 28–403) was cloned after PCR amplification into pET 16b (Novagen) such that translated products carried a 10-His tag at the N terminus. The primers used were A39R-5 (GGGGG-CATATGGTCACTACTATTAGAAGACGAC) and A39R-6 (GGGGG-GGATCCCTCGATTAAAGATTACATTTTAAAG), which contained NdeI and BamHI restriction sites respectively (underlined). The protein was expressed in E. coli and purified from inclusion bodies under denaturing conditions (using 6 M guanidine hydrochloride; Sigma) with His-bind resin (Novagen) according to the manufacturer’s instructions.

A polyclonal antisera (α-A39R) was obtained by immunizing a New Zealand White rabbit with the His-tagged COP A39R protein according to standard protocols (Harlow & Lane, 1988). The IgG fraction was purified using Protein G Sepharose 4 Fast Flow gel (Pharmacia Biotech) according to the manufacturer’s instructions. To improve the specificity of the antibody, the purified IgG was adsorbed at 4 °C for 1 h against a 10% packed cell volume of TK + A39R (data not shown).

Immunoblotting. BS-C-1 or TK-143B cells were infected at 10 p.f.u. per cell or mock-infected in MEM containing 10% FBS and, where indicated, 40 µg/ml β-d-arabinofuranoside (AraC), 1 µg/ml tunicamycin or 1 µM monensin (all Sigma). At 24 h post-infection (p.i.) (except where specified) cells were harvested, washed in PBS and cellular material was dissolved in Laemmli buffer (Laemmli, 1970) containing β-mercaptoethanol, heated at 95 °C for 5 min and sonicated to shear DNA. Proteins in the culture supernatant were precipitated in 10% (vol/vol) trichloroacetic acid overnight at 4 °C, recovered by centrifugation (15 000 r.p.m., 30 min, 4 °C), washed in methanol, separated by SDS–PAGE (10% gel), transferred to nitrocellulose (Sambrook et al., 1989) and incubated with α-A39R (diluted 1:1000) or α-A41L (diluted 1:2500) (Ng et al., 2001). Bound IgG was detected by incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) (diluted 1:2000) followed by the enhanced chemiluminescence detection system (Amersham). Typically, virus proteins were detected using extracts from 1.5 x 10⁶ cells and the supernatant from 1.8 x 10⁶ cells.

Virulence assays in mice. For intranasal infections, groups of five female BALB/c mice (6 weeks old) were inoculated under general anaesthesia with viruses in 20 l PBS. Each day, mice were weighed individually and monitored for signs of illness, and those suffering a severe infection or having lost 30% of their original body weight were sacrificed. For the intradermal infection model, groups of seven female BALB/c mice (8–10 weeks old) were anaesthetized and injected intradermally into left ear pinnae with 10⁶ p.f.u. of viruses diluted in 10 µl of MEM. The diameter of lesions was estimated daily to the nearest 0.5 mm using a micrometer. Infectious virus was determined by plaque assay using extracts from ears prepared by grinding in a glass tissue homogenizer, followed by three freeze–thaw cycles and sonication for 1 min.

Histological analysis of VV-infected mouse ears. Mice were infected intradermally as described above and at the indicated days p.i., two mice were sacrificed and the infected ears were removed. Cryosections (10 µm) of infected ears were incubated with antibodies specific for CD3e (goat polyclonal, diluted 1:100; Santa Cruz Biotechnology), F4/80 and class II major histocompatibility complex (rat monoclonals, used at 1:5 dilution and obtained from Sir William Dunn School of Pathology, University of Oxford, UK) or the VV B5R protein (a concentrated stock of rat monoclonal 19C2 used at a 1:100 dilution; Schmelz et al., 1994). Bound antibodies were detected with an appropriate biotinylated secondary antibody, AB enzyme reagent (Santa Cruz Biotechnology) and a peroxidase substrate (diaminobenzidine; Park Scientific). Digital images of histological sections were obtained using a Zeiss Axioplan microscope under bright field illumination and SPOT software, version 2.2.2 (Diagnostic Instruments). The width of infected ears was measured from digital images using SPOT. Measurements were made from the widest points of five sections taken at intervals in infected regions, and the three highest values were used to calculate a mean maximum width for each mouse at each time-point. To determine the proportions of positive-staining cells, histological sections were analysed using Meta Morph software, version 4.5 (Universal Imaging Corporation). By altering the RGB colour threshold settings, the software could measure selectively the proportion of positive (dark brown) coloration within digital images of infected ears. Typically, three areas of infiltrate of 25 000–50 000 µm² were analysed at each time-point to obtain a mean positive value for each mouse.

Results

To assist in the characterization of the A39R ORF, deletion mutant and revertant viruses were constructed (see Methods). More than 70% of the A39R ORF was deleted from VV strain COP, and because of the differences between the A39R genes of VV strains COP and WR (Fig. 1), a similar portion of the WR A39R region was also deleted. Additional recombinants were constructed to study the influence of the full-length A39R in a WR background. These were created by inserting the COP A39R coding sequence into vWR ΔA39R (vWR COPA39R) and then re-deleting this sequence to obtain a revertant (vWR ΔA39R-rev).

Growth properties of mutant viruses

To determine if the A39R gene affected virus growth in cell culture the plaque phenotypes of representative viruses were analysed and found to be indistinguishable (see JGV Online for supplementary data, http://vir.sgmjournals.org). The production of intracellular and extracellular virus by these groups of viruses were also analysed after low and high m.o.i. and found to be similar (data not shown; see JGV Online for supplementary data, http://vir.sgmjournals.org). These data demonstrated that the A39R gene was non-essential for replication in vitro.

Characterization of the A39R protein

To detect the A39R protein, a rabbit polyclonal antiserum (α-A39R) was raised against a His-tagged version of the COP
Fig. 2. Immunoblot analysis of the A39R protein. TK143B (a) or B-SC-1 (b) cells were infected with the indicated viruses at 10 p.f.u. per cell and at 24 h p.i. proteins in the supernatant or cells were analysed by immunoblotting using antibodies against A39R or A41L (Ng et al., 2001). The positions of molecular size markers are indicated in kDa. (a) Expression of A39R by recombinant viruses. (b) A39R is secreted late during the infection cycle. Cells were infected with vCOP A39R-wt in the presence or absence of AraC and supernatant proteins were analysed at the times indicated p.i. (c) A39R contains both N- and O-linked carbohydrate. Infections with vCOP A39R-wt were performed in the presence or absence of tunicamycin or monensin and both cell-associated (cellular) and secreted proteins were analysed by immunoblotting. A39R protein expressed in E. coli (see Methods). Immunoblotting with this antibody detected a single band of 50–55 kDa in the supernatants of cells infected by vCOP A39R-wt, vCOP A39R-rev and vWR COPA39R (Fig. 2a). No band was detected in the supernatants from vCOP ΔA39R, vWR A39R-wt, vWR ΔA39R, vWR A39R-rev or vWR ΔA39R-rev. As a control, blots were stripped and re-probed with an antibody specific for A41L, a VV secretory protein (Ng et al., 2001). The detection of A41L in each sample confirmed that all cells had been infected. Although the deletion viruses were not expected to express A39R, these data demonstrated that WR-infected cells did not secrete the truncated version of A39R. The experiment also confirmed the secretion of the COP A39R protein from a WR background (vWR COPA39R).

After a long exposure, α-A39R detected a minor protein of approximately 34 kDa in cells infected with vWR A39R-wt and vWR A39R-rev, but not with other viruses (data not shown). This corresponded to the predicted size of the truncated WR A39R protein and suggests that this version of A39R is retained inside cells following infection. Blots were also probed with an antibody specific for VV B5R (a 42 kDa glycoprotein) (Engelstad et al., 1992), which verified that virus-encoded proteins were present at similar levels in cells infected by each recombinant.

The presence of a potential late transcription initiation motif (TAAAATG) overlapping the A39R putative start codon and early transcription termination signals (TTTTTTNT) 16 bp before this ATG and approximately 400 bp before the stop codon suggested that A39R might be transcribed late during infection. To examine this experimentally, cells were infected with vCOP A39R-wt in the presence or absence of AraC, an inhibitor of DNA synthesis and, therefore, intermediate and late viral gene expression. Immunoblotting detected the A39R protein in supernatants from 8 h p.i. and levels continued to increase until 22 h (Fig. 2b). AraC inhibited A39R expression. In contrast, A41L was detected in the same blots after 4 h and was not inhibited by AraC. In infected cells A39R was detectable by 6 h p.i. (data not shown). The time of initial synthesis, the increase in amounts of protein in the supernatant over time and the inhibition of expression by AraC indicated that A39R is expressed late during infection.

The COP A39R contains three potential sites for N-linked glycosylation (NXS/T, where X represents any amino acid). To determine if the A39R protein is modified by glycosylation, cells were infected with vCOP A39R-wt in the presence or absence of either tunicamycin (an inhibitor of N-linked glycosylation) or monensin (which disrupts the trans-Golgi apparatus and affects lysosome and acidic endosome function, thereby inhibiting O-linked glycosylation). Cells and supernatants were analysed at 24 h p.i. by immunoblotting with antibodies to detect A39R or A41L. In the presence of monensin, the size of the major form of A39R in supernatant over time and the inhibition of expression by AraC indicated that A39R is expressed late during infection.

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Vaccinia virus semaphorin A39R

A41L protein was also decreased in size by both glycosylation inhibitors, but its secretion was not inhibited by tunicamycin, as reported previously (Ng et al., 2001). In Fig. 2(c), some of the A39R protein was associated with the cellular fraction (even in the absence of drug treatment), but further experiments showed this was not the case for untreated B-SC-1 or RK13 cells (data not shown). These results show that A39R contains N-linked carbohydrate that is essential for its secretion. The reduction in size of the A39R protein could be due to direct inhibition of O-linked glycosylation or indirect effects on the maturation of N-glycans.

Supernatants from cells infected with vCOP A39R-wt and vCOP ΔA39R were also analysed by immunoblotting after electrophoresis under reducing and non-reducing conditions. In reducing conditions, A39R ran slower than under non-reducing conditions (data not shown). This suggests that disulphide bridges exist between some of the eight cysteines that are present within this protein.

**Distribution of the A39R protein in orthopoxviruses**

Sequence information indicates that the A39R gene is broken into three segments in all sequenced variola virus strains (Aguado et al., 1992; Shchelkunov et al., 1993, 2000; Massung et al., 1994) as well as camelpox virus (C. Gubser & C. L. Smith, unpublished data) and VV strain MVA (Antoine et al., 1998). The A39R gene of VV strain Tian Tan is truncated in an identical manner to strain WR (Jin et al., 1998). Consequently, secretion of an A39R protein would not be expected by cells infected with any of these viruses. A previous study found that the A39R protein of VV strain Lister is secreted (Comeau et al., 1998). To determine if the A39R protein was secreted by other orthopoxviruses, supernatants were collected at 24 h p.i. and analysed by immunoblotting. A secreted A39R protein was expressed by VV strains COP, Evans, King Institute, Lister, Patwadangar, USSR, buffalopox and rabbitpox, and two strains of cowpox virus (Brighton red and elephantpox) (Fig. 3). The buffalopox virus A39R protein was a doublet, with both bands of a higher molecular mass than other A39R proteins. No protein was detected in supernatants with the other VV strains, including WR and Tian Tan, as...
predicted from DNA sequence data. As a control, blots were stripped and re-probed using an A41L-specific antibody and A41L was detected for each virus.

The increased molecular mass of the buffalopox virus A39R proteins might be explained by additional coding sequence or more extensive glycosylation. To investigate this, the A39R region from the buffalopox virus genome was amplified by PCR (in duplicate) and sequenced. The predicted amino acid sequence (Fig. 4) shows that the buffalopox virus A39R protein is 14 amino acids longer than that of COP due to a frameshift caused by the deletion of nucleotide 1198 (relative to the putative start codon of the buffalopox virus/COP A39R ORF) and thus utilization of a different stop codon. A potential motif for N-linked glycosylation is also present within this additional sequence. Together these features explain the higher molecular mass of this protein; the doublet observed in Fig. 3 is most likely due to variations in glycosylation.

**Virulence of A39R recombinant viruses**

Several secreted poxvirus proteins act as virulence factors by interacting with components of the immune system.
Therefore, the virulence of recombinant VVs with or without the A39R protein was assessed in murine models. The A39R protein of VV strain WR is truncated and not secreted (Figs 1 and 4), therefore we were most interested in the COP A39R deletion virus (vCOP ΔA39R), the WR in which A39R was replaced with that of COP (vWR COPA39R) and the relevant plaque-purified parent and revertant controls.

Viruses were studied initially using a murine intranasal model of infection; however, no differences in either weight loss or signs of illness were noted among sets of COP or WR viruses (data not shown). Viruses were then compared using a murine intradermal infection model (Tscharke & Smith, 1999). Mice were infected in their ear pinnae with 10^4 p.f.u. of the A39R recombinant viruses, and lesion sizes were measured daily (Fig. 5). Apart from a slightly quicker resolution of lesions in vCOP ΔA39R, no difference was observed in terms of lesion size resulting from infection with vCOP A39R-wt, vCOP ΔA39R or vCOP A39R-rev (Fig. 5a). However, a difference was seen in the lesions induced by vWR COPA39R compared to vWR A39R-wt, vWR ΔA39R and vWR ΔA39R-rev. The vWR COPA39R-infected mice developed larger lesions, and the difference between the pooled mean values of the lesions sizes was statistically significant between days 6 and 14 p.i. compared to those of vWR A39R-wt, vWR ΔA39R and vWR ΔA39R-rev (P < 0.02, Mann–Whitney test) (Fig. 5b). In a repeat experiment, the maximum size of lesions was not different between the three groups, but the mean lesion size of mice infected with vWR COPA39R remained at its maximum value for longer than the other groups (Fig. 5c). Furthermore, the lesions of control groups were healed fully by day 13, compared to day 17 for vWR COPA39R-infected mice (Fig. 5c). The similar maximum lesion sizes observed in the second intradermal infection experiment with WR A39R recombinant viruses were most likely due to the use of older mice, a variable that has been shown to affect lesion development in other experiments (D. C. Tscharke & G. L. Smith, unpublished observations). Both experiments showed the lesions obtained with vWR COPA39R were more pronounced than those obtained with controls.

The formation of lesions in the mouse intradermal infection model is likely to be a combination of direct viral cytopathology and immunopathology. To investigate the basis for the difference in lesions sizes, infectious virus in the ears of mice infected with vWR ΔA39R, vWR COPA39R and vWR ΔA39R-rev was quantified by plaque assay (Fig. 5d). Virus titres were similar for all infection groups on each of the days examined. These results suggested that the increased severity of lesions associated with the expression of the A39R protein may be due to immunopathology rather than increased virus replication.

**Histological examination of infected mouse ears**

The cellular events occurring during the intradermal infection process were examined further in cryosections of infected mouse ears. The increased thickness of mouse ears infected with vWR COPA39R compared to vWR ΔA39R and vWR ΔA39R-rev. Mice were infected with 10^4 p.f.u. of the indicated viruses in ear pinnae. After 4 days, cryosections (10 µm) of ears were prepared and stained with haematoxylin and eosin.
infected ears from 4, 7 and 10 days p.i. All ear lobes infected with VV showed the marked increase in thickness noted previously in this model (Tscharke & Smith, 1999), but interestingly, ears infected with vWR COPA39R were thicker than those infected with control viruses at 4 days p.i. (Fig. 6). Quantification (see Methods, Fig. 7a) showed that ears infected with vWR COPA39R had a mean maximum width of almost 900 μm, compared to less than 700 μm for vWR ΔA39R and vWR ΔA39R-rev at 4 days p.i. This difference was not seen at day 7 and 10 p.i. These data correlated with observations made at early time-points that ears infected with vWR COPA39R appeared more red and inflamed than those infected with vWR ΔA39R or vWR ΔA39R-rev (data not shown). Upon closer observation, it appeared that the increased thickness of ears infected with vWR COPA39R was due both to increased infiltration and oedema. This finding suggests that the secretion of the A39R protein may have a pro-inflammatory effect during VV intradermal infections.

To search for differences in inflammatory infiltrate between the groups of viruses, sections were stained using antibodies against CD3ε, F4/80 and B5R, markers of T cells, monocytes/macrophages and virus-infected cells, respectively. At 4 days p.i., low numbers of T cells were scattered throughout the infiltrate in all infection groups, and on days 7 and 10, increased numbers of these cells were detected in the cellular infiltrate (data not shown). In all day 4 sections, monocytes/macrophages were found to be associated closely with sites of virus infection, but by days 7 and 10 p.i., substantial numbers of F4/80+ cells were present throughout the infiltrates (data not shown). Cells staining for CD3ε and F4/80, including sections adjacent to those positive for virus antigen (days 4 and 7 p.i.), were quantified using image analysis software (Fig. 7b, c). Similar proportions of both markers were observed in sections irrespective of the infecting virus at all time-points examined, suggesting that secretion of A39R did not affect the influx of CD3ε+ or F4/80+ cells into infected sites. Proportions of class II major histocompatibility complex-positive cells, representing Langerhans’ cells and activated macrophages in the infected ears, were also comparable (data not shown). These initial histological analyses suggest that monocytes/macrophages and T cells are unlikely to be the source of the increased thickness apparent in ear pinnae infected with vWR COPA39R compared to vWR ΔA39R or vWR ΔA39R-rev.

**Discussion**

This study presents a characterization of the A39R protein from VV strains COP and WR and studies the contributions of these proteins to virus virulence. Data presented show that VV COP A39R is a 50–55 kDa secreted glycoprotein that is expressed late during infection and affects the outcome of infection in a murine intradermal model. The WR A39R protein, in contrast, is an intracellular 34 kDa protein that was not secreted, did not affect virulence, and is assumed to be a non-functional truncated version of the COP A39R protein.

Immunoblotting showed that the A39R protein was secreted by cells infected with eight of 15 strains of VV and two strains of cowpox virus. It is notable that a secreted A39R protein is encoded by VV strains that have been isolated from identified hosts (i.e. buffalopox and rabbitpox), as well as other orthopoxviruses found in nature (both cowpox strains and ectromelia virus). Therefore, secretion of A39R may be a selective advantage during natural infections. The lack of an
intact A39R ORF in all sequenced strains of variola virus is contradictory, but there are other examples of moderately well conserved orthopoxvirus immunomodulatory genes that are fragmented in variola virus (Alcamí & Smith, 1992; Moore & Smith, 1992).

To investigate the role of the VV A39R protein in vivo, sets of COP and WR A39R recombinant viruses were compared in murine models of infection. Following inoculation via the intranasal route, which causes a systemic infection, no differences were associated with the expression of COP A39R in either the COP or the WR background. In contrast, while mice infected intradermally with the A39R deletion mutant of COP had only slightly more rapidly resolving lesions and no difference in lesion size, significantly larger or more prolonged lesions were seen when COP A39R was expressed by WR. The reason why A39R was associated with more severe lesions when expressed by WR but not by COP is unclear but may be influenced by expression of other proteins unique to either virus, or to the replicative ability of either parent virus in this model. By analogy, the VV IL-1βR (gene B15R) prevented enhanced weight loss and induction of fever in some circumstances only (Alcamí & Smith, 1992, 1996; Spriggs et al., 1992).

To determine whether the increased lesion sizes associated with A39R expression were caused by increased virus replication or immunopathology, we quantified infectious virus and examined infected ears histologically and immunohistochemically. At no time after infection could we find any influence of A39R on virus titres in infected ears. However, histological analysis at 4 day p.i. found that COP A39R expression by WR was associated with greater thickening of the ears due to increased cellular infiltration and oedema. Taken together, these results suggest that A39R secretion is associated with increased immunopathology and not with damage caused by enhanced virus replication. The observed increases in immunopathology and cellular infiltration suggest that the A39R protein has direct or indirect pro-inflammatory properties.

Work with the A39R-like protein of ectromelia virus suggested that this virus product might affect monocyte migration (Spriggs, 1999). Monocytes and macrophages can cause tissue damage mediated by enzymes and reactive oxygen species (Chensue & Ward, 1996) and T cells have also been implicated in virus-induced immunopathology. Therefore, infected ear sections were stained with antibodies to detect F4/80 and CD3e. The proportions of cells expressing these markers were the same for all viruses, indicating that increased infiltration of these cell types was not likely to be the cause of greater immunopathology associated with A39R expression. Increased activation of these cells cannot be ruled out, although equally, other inflammatory cells such as neutrophils might be involved. The ectromelia A39R protein was found to inhibit migration of monocytes and induce secretion of TNF-α, IL-6 and IL-8 from these cells (Comeau et al., 1998; Spriggs, 1999).

While we found no evidence for A39R-induced attenuation of monocyte migration in vivo, TNF-α and IL-6 are potent activators of a variety of cells, and IL-8 is chemotactic for neutrophils. Thus, increased levels of these cytokines in the presence of A39R would be consistent with our data. Furthermore, ectromelia A39R was found to bind to most primary immune cell types and immunological cell lines (Comeau et al., 1998), suggesting that multiple cell types may be affected.

VV secretes a variety of proteins that are likely to be anti-inflammatory, for example soluble receptors for IL-1β, TNF, IFN-γ and chemokines, and the secretion of an apparently pro-inflammatory molecule might seem counter-intuitive. However, there are examples of other viruses that secrete proteins to recruit subsets of immune cells that favour virus infection. The murine cytomegalovirus (CMV) m131/129 protein attracts macrophages in vitro and mutant viruses lacking the encoding gene have a decreased dissemination within mice (Fleming et al., 1999; Saederup et al., 1999). Functional chemokines are also encoded by human CMV and human herpesvirus-6 (Penfold et al., 1999; Zou et al., 1999). VV is unable to disseminate from cutaneous inoculation sites in mice, but other orthopoxviruses do disseminate from this site during infection of their natural host and a role for phagocytes in this process has been proposed (Buller & Palumbo, 1991). Possibly, the A39R protein might aid VV dissemination within or between hosts during natural infection.

Studying viral immunomodulators can also help elucidate the functions of their ligands (Alcamí & Smith, 1996). The VV A39R protein is more closely related to SEMA7A than other known host proteins, including those in the recently published human genome (data not shown) (International Human Genome Sequencing Consortium, 2001; Venter et al., 2001). A39R and SEMA7A bind to the same receptor, plexin-C1, with a similar affinity (Comeau et al., 1998; Tamagnone et al., 1999) and therefore data presented here and elsewhere (Comeau et al., 1998) suggest that cell–cell interactions between SEMA7A and plexin-C1 may be involved in the process of inflammation. Consequently, blocking this molecular interaction may be useful during chronic inflammation, and A39R or SEMA7A may be beneficial during immunization by stimulating an inflammatory response. Human immunodeficiency virus causes up-regulation of SEMA7A on the surface of infected cells (Frank et al., 1996), which also suggests a role for these molecules during viral disease.

In conclusion, the VV A39R gene of strain COP encodes a secreted glycoprotein that is non-essential for virus replication but affects the outcome of infection in a murine intradermal model.

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