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Interleukin-18 and glycosaminoglycan binding by a protein encoded by *Variola virus*

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Poxvirus interleukin (IL)-18 binding proteins (IL-18BPs) are soluble decoys that inhibit the activity of IL-18. The aim of this study was to demonstrate IL-18 binding activity of the *Variola virus* protein D7L. D7L effectively inhibited the biological activity of IL-18 in a bioassay. We compared the affinity and kinetics of D7L and the *Ectromelia virus* IL-18BP, p13, for human and murine IL-18 using surface plasmon resonance and no differences were detected, indicating that the differences in amino acid sequence did not affect binding or species specificity. Both proteins had higher affinity for murine than human IL-18. This was similar to human IL-18BP and the *Molluscum contagiosum virus* IL-18BP, which also demonstrated higher affinity for human IL-18. The host range of *Variola virus* is limited to humans and thus the affinity of D7L for IL-18 does not correlate with its host range. Furthermore, we demonstrated that D7L is capable of interacting with glycosaminoglycans (GAGs) via the C terminus, while p13 is not. Importantly, D7L interacted with both GAG and IL-18 simultaneously, indicating that the binding sites were distinct.

INTRODUCTION

Poxviruses are a family of large DNA viruses that express numerous host response modifier genes to generate more favourable conditions for virus replication and spread by altering the cellular environment or inhibiting the immune response. Throughout their evolutionary history, poxviruses have acquired host proteins involved in immune regulation, allowing them to modulate and escape the host response (Chen *et al.*, 2000; McFadden & Murphy, 2000). The poxvirus interleukin (IL)-18 binding proteins (IL-18BPs) are secreted proteins that bind to, and inhibit the activity of, IL-18 (Xiang & Moss, 1999b; Smith *et al.*, 2000; Born *et al.*, 2000; Calderara *et al.*, 2001). IL-18BPs have been identified through sequence similarity or activity in multiple orthopoxviruses, as well as the human pathogen, *Molluscum contagiosum virus* (MOCV) (Xiang & Moss, 1999a, b). IL-18BPs block interaction of IL-18 with the cellular receptor and can thus modulate the cytokine response following immunological insults such as infection or LPS treatment (Aizawa *et al.*, 1999; Novick *et al.*, 1999). Many viral cytokine binding proteins, such as the interferon (IFN)-γ BP (Upton *et al.*, 1992; Alcami & Smith, 1995, 1996) and the secreted tumour necrosis factor (TNF)-α receptors (Upton *et al.*, 1991; Smith *et al.*, 1996) are soluble forms of the receptor lacking the transmembrane domain. However, the IL-18BP family does not have significant sequence similarity to the IL-18 receptor. Furthermore, deletion of the *Ectromelia virus* (ECTV) IL-18BP, p13, results in an enhancement of IFN-γ release, NK cell activity and reduced infectivity in the spleen and liver following interperitoneal inoculation (Born *et al.*, 2000). Similarly, intranasal inoculation of mice using a *Vaccinia virus* (VACV) IL-18BP knockout results in attenuated virulence and increased IFN-γ levels, NK cell and T cell activity (Symons *et al.*, 2002; Reading & Smith, 2003).

IL-18 plays an important role both in the innate and the specific immune response, through its potent induction of IFN-γ (Okamura *et al.*, 1995; Micallef *et al.*, 1996), activation of NK cell activity (Lauwerys *et al.*, 1999), induction of pro-inflammatory cytokines and chemokines (Puren *et al.*, 1998) and promotion of a Th-1 response (Takeda *et al.*, 1998; Dinarello, 1999a, b; Akira, 2000). IL-18 has been shown to be an important mediator in the host response to many viral and bacterial infections. Treatment of mice with exogenous IL-18 confers protection in mouse models of herpes simplex virus (Fujioka *et al.*, 1999) and VACV infections (Tanaka-Kataoka *et al.*, 1999). The protective effect is likely the result of a combination of IFN-γ-, NK cell- and T cell-mediated events. Mice infected with a recombinant VACV expressing IL-18 demonstrated more rapid virus clearance due to an enhanced response involving innate and specific immune components (Gherardi *et al.*, 2003).

Variola virus (VARV), the causative agent of smallpox, was eradicated through an intensive worldwide vaccination programme; however, the possibility of clandestine stocks in rogue nations or terrorist groups make it a potential bioweapons threat. This has led to renewed interest in understanding the pathogenesis of, and immune response to, VARV. Humans remain the only identified natural host for VARV, and while ECTV, *Cowpox virus* (CPXV) or VACV

infections in mice have been used to develop a greater understanding of orthopoxvirus pathogenesis and immunity, these do not typically cause disease in humans. Thus, VARV is uniquely adapted to infect, replicate and cause disease in humans, and its immunomodulatory genes may be uniquely adapted to their cognate human ligands. In this study, we have demonstrated that VARV encodes a functional IL-18BP, D7L. The affinity of D7L for human and murine IL-18 was compared with that of the ECTV IL-18BP, p13. Furthermore, we have demonstrated that D7L is capable of interacting with glycosaminoglycans (GAGs) via residues in the C terminus, while p13 lacks this function. Importantly, D7L can interact with both GAG and IL-18 simultaneously, indicating that the binding sites are distinct.

METHODS

Cells and IL-18. CV-1/EBNA-1 cells were maintained in Dulbecco's modified essential medium (DMEM; BioWhittaker) containing 10 % foetal bovine serum (FBS; HyClone) and supplemented with L-glutamine and penicillin/streptomycin (Gibco). KG-1 cells were maintained in Iscoves's modified Dulbecco's medium (IMDM; BioWhittaker) containing 20 % FBS and supplemented with L-glutamine and penicillin/streptomycin. Murine (mu) IL-18 (Biosource International) was stored lyophilized until immediately before use in surface plasmon resonance experiments and stored at 4 °C with no apparent loss of activity. Human (hu) IL-18 was expressed in *E. coli* as a GST fusion protein and purified according to previously published methods (Liu *et al.*, 2000) with some modifications. The bacteria were lysed, centrifuged and the supernatant was mixed with a 50 % slurry of glutathione–agarose beads (Amersham Pharmacia) at 4 °C for 3 h. The beads were washed and resuspended in twice the bed volume of cold PBS. To cleave the protein and generate mature IL-18, Factor Xa (New England Biolabs) was added at a concentration of 1 μg (ml lysate)$^{-1}$ and incubated at 23 °C for 4 h. The cleaved product in the supernatant was harvested, assessed for purity and concentration by SDS-PAGE and silver staining and was found to be greater than 90 % pure. HuIL-18 was stored at −70 °C and thawed immediately before use.

Cloning. The ECTV p13–Fc (human IgG1) fusion gene in the expression vector pDC409 was a generous gift from Amgen. A 10·4 kbp amplicon, representing approximately 5 % of the genome of VARV (strain Bangladesh-1975), was obtained from the Centers for Disease Control, Atlanta, GA, USA. In agreement with World Health Organization regulations, all use of VARV DNA was limited to situations where accidental recombination with other poxvirus DNA could not occur. *D7L* was amplified by PCR using the following primers: BSH-D7L5′: 5′-gcgttcatagtcgacatgagaatcctatttctc-3′ and BSH-D7L-Fc: 5′-agagatctctttagctttagccaaatattc-3′. Primer BSH-D7L-Fc was used to generate an Fc-tagged fusion protein. The *D7L* PCR product was ligated into pDC409, generating pDC409-D7L-Fc. The sequence was verified on both strands.

The 5′ sequence of *D7L* was mutated using a site-directed mutagenesis kit (Genetailor; Invitrogen). Primers were designed to replace the C-terminal residues of D7L (KKNIWLK) with the sequence present in p13 (KKEYLAE): 5′-ggcgtttcaaaaaaggaatatttggctgaaagatcttgt-3′ and 5′-ctttttgaaacgccatttagcgtagt-3′. The mutagenic PCR and bacterial transformation were performed according to the manufacturer's directions. The sequence was verified on both strands.

Protein expression and purification. CV-1/EBNA-1 cells were transfected with plasmids containing the Fc fusion constructs using a DEAE–dextran method. Briefly, DNA was mixed with complete cell culture medium containing chloroquine and then complexed to DEAE–dextran. The complexes were added to a T-150 flask containing complete medium with chloroquine and incubated for 4·5 h at 37 °C, 5 % CO_2. The medium was then replaced with DMEM containing 10 % DMSO for 5 min at room temperature and finally replaced with DMEM-10. Cell culture supernatants were collected weekly for up to 4 weeks and stored at −20 °C. Expression was assessed by Western blotting. Cell supernatants were then pooled and purified by FPLC (AKTA; Amersham Pharmacia Biotech) on a 5 ml HiTrap Protein-G column. The column was equilibrated and washed with PBS, pH 7·4, and bound protein was eluted using 50 mM citrate, pH 3. The column was washed with 50 mM glycine, pH 2, to remove any remaining proteins. Fractions were collected, neutralized and assessed by silver staining (Bio-Rad). Peak fractions were pooled and dialysed against PBS, pH 7·4, and filter-sterilized using a 0·22 μm syringe filter. Purity was assessed by analysing samples by 12 % SDS-PAGE followed by silver staining. Protein concentration was determined by the Lowry method. In all experiments, full-length purified Fc fusion proteins were used.

Western blotting. Expression and purification of p13–Fc, D7L–Fc and D7L-KKEYLAE was assessed by SDS-PAGE followed by Western blotting. PVDF membranes were blocked in 5 % non-fat dried milk in PBS and incubated with rabbit anti-p13 antiserum, which is cross-reactive with D7L. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (KPL) was used as a secondary antibody and was detected using ECL-Plus (Amersham Biosciences) and exposed to film or captured by digital imaging (Alpha Innotech). Human IL-18 was detected using anti-huIL-18 rabbit polyclonal antibody (Cell Sciences) and HRP-conjugated secondary antibody as above.

IL-18 bioassay. KG-1 cells were precultured at 2×10^5 cells ml^{-1} for 4 days before use in the bioassay. Cells were centrifuged and resuspended at a concentration of 3×10^6 cells ml^{-1} in medium containing 20 ng TNF-α ml^{-1} (R&D Systems). HuIL-18 at 10 ng ml^{-1} was mixed with medium or with dilutions of D7L–Fc or p13–Fc. The IL-18/IL-18BP mixture was incubated for 1 h at 37 °C and then 100 μl cells were added and incubated for 24 h in a 96-well plate. The supernatants were harvested, centrifuged and stored at −20 °C until they were analysed for IFN-γ levels. The supernatant was assayed in an ELISA to detect IFN-γ, according to the manufacturer's recommendations (BD Pharmingen).

Surface plasmon resonance. Interaction of the binding proteins with IL-18 was measured by surface plasmon resonance (SPR) on a BIAcore 2000 (Biacore Inc.). All flow cells (FCs) of a CM5 chip were activated with EDC/NHS for 6 min at 5 μl min^{-1}. Anti-human IgG (Jackson Immunoresearch) was immobilized on all FCs, using a flow rate of 5 μl min^{-1}, for 7 min. The surface was blocked by injecting ethanolamine for 7 min at 5 μl min^{-1}. For kinetic studies, Fc fusion proteins were captured in FC2 or FC3, using FC1 as the reference cell, followed by injection of IL-18 at varying concentrations. Capture levels were kept low to minimize the effects of mass transfer. Cycle conditions were as follows: 7 min capture at 5 μl min^{-1}, 3 min analyte injection at 30 μl min^{-1} over all FCs, followed by 10 min dissociation time. The surface was regenerated with a 1 min injection of 5 mM HCl, pH 1·5. At least one of the dilutions was run in duplicate in each experiment and IL-18 concentrations were injected in random order. The experimental R_{max} was typically 60 % of the theoretical, indicating that a fraction of the captured protein was inactive or inaccessible for interaction with the analyte. To rule out the possibility of leaching between FCs during association and dissociation phases, experiments were repeated with protein captured on different FCs or individually on FC2. All SPR data were analysed and fitted globally using BIAevaluation 3.2 software.

For SPR analysis of interaction with heparin, a streptavidin (SA) sensor chip (Biacore Inc.) was used to capture biotinylated BSA on

FC1 and biotinylated BSA–heparin on FC2 (both from Sigma). Analyte was injected at 30 µl min^{-1} at varying concentrations for 3 min, followed by 10 min dissociation. The surface was regenerated with a 15 s injection of 1 M NaCl in 50 mM NaOH at 5 µl min^{-1}.

A GAG competition assay was performed to measure the relative affinity of D7L for different GAGs. A constant amount of D7L was pre-incubated with increasing concentrations (2·5 µg ml^{-1}, 25 µg ml^{-1}, 0·25 mg ml^{-1} and 2·5 mg ml^{-1}) of soluble heparin, heparin sulphate (HS), chondroitin sulphate B (CSB) (all three derived from porcine intestinal mucosa) or chondroitin sulphate A (CSA) (from bovine trachea; Sigma). Samples were then injected over the BSA–heparin surface and BSA alone reference surface. The maximum response was measured 20 s before the end of injection.

Heparin–Sepharose pull-down assay. Heparin–Sepharose beads (Amersham Biosciences) were washed with HEPES buffered saline and resuspended in the same buffer to generate a 50 % slurry. Beads were mixed with 130 nM protein and incubated at 4 °C for 2 h. Immediately prior to incubation an aliquot was removed for later analysis as the input fraction. The beads were recovered by centrifugation and the supernatants were collected for later analysis (unbound fraction). The bead fraction was washed three times and analysed for the presence of IL-18BP or used in a subsequent incubation with huIL-18 for 2 h at 4 °C. Beads were recovered as before and the unbound fraction collected. Samples were analysed for IL-18BP or IL-18 by Western blotting. A competition assay was performed by pre-incubating D7L–Fc with 2·5 mg soluble heparin ml^{-1} for 2 h followed by incubation with heparin–Sepharose beads as described above.

RESULTS

VARV D7L is an IL-18BP

Analysis of the genome of VARV strain Bangladesh-1975 revealed an open reading frame, *D7L*, which encoded a protein with high sequence similarity to other orthopoxvirus IL-18BPs (Fig. 1). The alignment of D7L and p13 showed that these two protein sequences differed in only 7 of the first 121 residues. The divergent residues were scattered over the length of the sequence. The C-terminal region showed the greatest divergence, with p13 including an extension that was present only in the Moscow and Hampstead strains of ECTV. The C-terminal region is most variable in this family of proteins. The other sequenced VARV major strain, India-1967, encodes a predicted IL-18BP that differs in only one position (T-65 in BSH, A-65 in IND). An alanine is also found at this position in the CPXV IL-18BP, C8L. B6L, the predicted IL-18BP of VARV minor (strain Garcia-1966), is identical to D7L after signal sequence cleavage. Consistent with its function as an immune modulator, the *D7L* promoter sequence was that of an early promoter and was identical to the p13 promoter (not shown).

To study the function of D7L, a fragment of VARV DNA was obtained from the Centers for Disease Control and the gene was amplified by PCR and cloned into the expression vector pDC409 as a C-terminal Fc fusion protein. D7L–Fc and p13–Fc were expressed in CV-1/EBNA-1 cells and purified by protein-G affinity. Analysis of the eluted protein by polyacrylamide gel electrophoresis and silver staining showed that the fusion protein was purified to greater than 90 % purity (Fig. 2a). Western blotting further demonstrated that the prominent bands between 40 and 45 kDa were D7L–Fc and p13–Fc (Fig. 2b).

IL-18 was initially identified as a potent inducer of IFN-γ. D7L was assessed for its ability to inhibit this biological function of huIL-18 in a bioassay using KG-1 cells. D7L was incubated with IL-18 to allow formation of a complex, then added to KG-1 cells in the presence of the co-stimulator, TNF-α. KG-1 cells respond to IL-18 by secreting IFN-γ and binding protein activity is seen as a decrease in IFN-γ levels. D7L effectively inhibited the release of IFN-γ, as measured

Fig. 1. Primary sequence alignment of selected viral and mammalian members of the IL-18BP family. Sequences were obtained from GenBank (accession nos: p13, NP_671531; D7L, AAA60752; muIL-18BP, AAD41052; huIL-18BP, BAA76374; MC54L, NC_001731) and aligned using CLUSTAL W. Dark grey areas surround identical residues, light grey areas are conserved residues and unboxed regions are non-conserved. Numbers indicate the residue number at the left end of each sequence. Circles above the sequences indicate residues that differ between p13 and D7L. The black bar indicates the furin cleavage site in MC54L that separates the IL-18 binding and GAG binding domains.

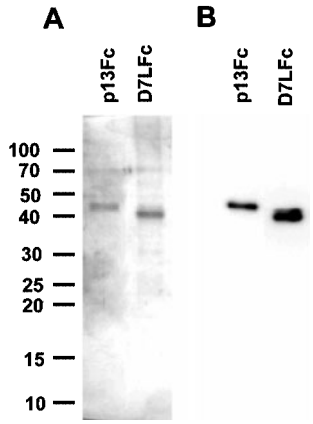

Fig. 2. Expression and purification of p13–Fc and D7L–Fc. Supernatants from CV-1/EBNA-1 cells transfected with pDC409-p13-Fc or pDC409-D7L-Fc were collected and purified by protein-G affinity by FPLC. Purified protein was analysed by 12 % SDS-PAGE and silver staining (A) and Western blotting (B). Western blots were performed by transferring the gels to PVDF membrane and probing with anti-p13 rabbit antiserum, which is cross-reactive with D7L. Marker proteins with molecular masses in kDa are indicated on the left.

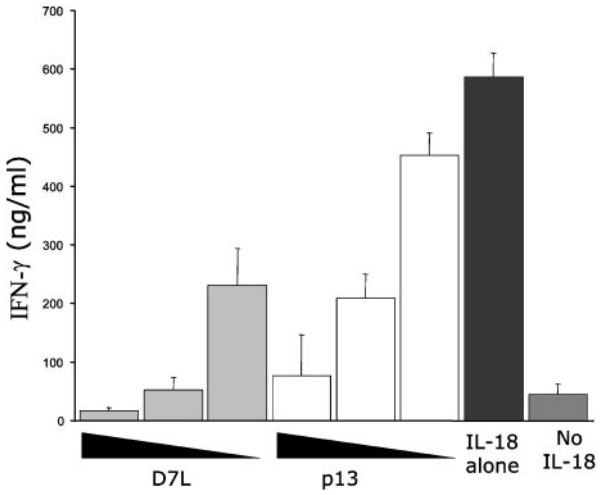

Fig. 3. D7L and p13 inhibit the biological activity of IL-18. Decreasing concentrations of p13–Fc and D7L–Fc were pre-incubated with 10 ng huIL-18 ml^{-1} for 1 h at 37 °C, then mixed with KG-1 cells and 10 ng TNF-α ml^{-1}. Twenty-four hous later, culture supernatants were collected and analysed by ELISA for IFN-γ levels. Control wells received IL-18 but no IL-18BP, or no IL-18.

by ELISA, in a dose-dependent manner (Fig. 3). Comparable results were seen with p13.

D7L and p13 interact with both huIL-18 and muIL-18

Since D7L and p13 are encoded by viruses with different maintenance hosts, we wished to determine whether these two proteins demonstrated species specificity with respect to ligand binding. To compare the binding affinities and kinetics of D7L and p13 for huIL-18 and muIL-18, SPR was used. Anti-human IgG antibody was immobilized on a BIAcore CM5 chip to capture the Fc-tagged binding proteins. For each cycle, the binding protein was captured followed by an injection of huIL-18 or muIL-18 of different concentrations. The sensograms were globally fitted to a one-to-one binding model (Fig. 4). The affinities of p13 and D7L for muIL-18 were similar and in the subnanomolar range (Table 1). The affinities of p13 and D7L for huIL-18 were also similar, but were significantly lower than for muIL-18. The difference in affinities between huIL-18 and muIL-18 was due solely to a higher association rate with muIL-18 since the dissociation rates do not differ.

GAG binding by D7L

Despite no apparent differences between p13 and D7L in terms of IL-18 binding, we turned our attention to the C terminus of these proteins, which demonstrated the most significant sequence divergence. Analysis of the D7L sequence revealed a potential GAG binding region, S-K-K-N-I-W-L-K, closely fitting a common motif X-B-B-B-X-X-B-X, where B is a basic residue and X is any hydropathic residue (Cardin & Weintraub, 1989). Although the double lysine is present in p13, the third lysine is absent and acidic residues are present. Since many chemokines, cytokines and binding proteins, including the MOCV IL-18BP, have been shown to bind GAGs, we used SPR to test heparin binding by D7L and p13. The binding proteins were injected over a sensor chip with immobilized heparinated BSA. D7L interacted with the heparin surface, but p13 did not demonstrate a detectable interaction (Fig. 5a). Furthermore, untagged p13 expressed in *E. coli* and refolded, which demonstrates IL-18 binding activity, also did not interact with heparin, indicating that the Fc tag was not obstructing heparin binding (data not shown). Affinity and kinetics of D7L binding were determined by injecting varying concentrations of D7L over the heparin surface (Fig. 5b). D7L bound heparin with an affinity of $55{\cdot}7 \pm 17{\cdot}5$ nM ($k_a = 5{\cdot}95 \times 10^3 \pm 2{\cdot}76 \times 10^3$ M^{-1} s^{-1}, $k_d = 3{\cdot}07 \times 10^{-4} \pm 5{\cdot}02 \times 10^{-5}$ s^{-1}). To test whether the C-terminal motif is responsible for the observed interaction, a mutant of D7L was constructed in which the C-terminal sequence was converted to that found in p13 (KKNIWLK to KKEYLAE). No interaction of D7L-KKEYLAE with heparin was detectable by SPR (Fig. 5a), identifying this region as necessary for heparin binding. There was no difference between wild-type and D7L-KKEYLAE binding to IL-18 (data not shown). We were unable to generate a p13 construct with the D7L C-terminal sequence to determine whether this is the only determinant that is needed for heparin binding.

Interaction with heparin was tested further using heparin–Sepharose beads. Beads were incubated with D7L, p13, D7L-KKEYLAE or no protein and the beads with bound

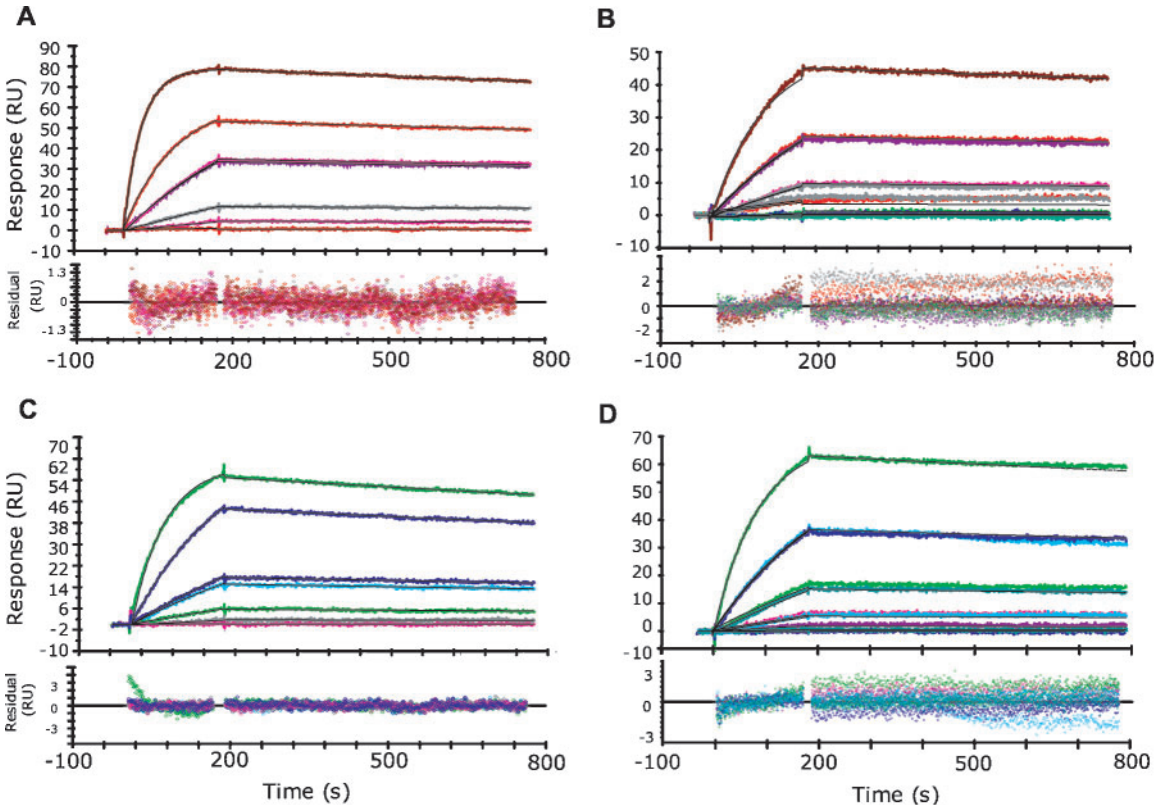

Fig. 4. Surface plasmon resonance analysis of p13 and D7L binding to huIL-18 and muIL-18. SPR sensograms showing binding kinetics of p13 and muIL-18 (A), D7L and muIL-18 (B), p13 and huIL-18 (C) and D7L and huIL-18 (D). A CM5 chip with immobilized anti-huIgG was used to capture Fc-tagged p13 or D7L. Murine IL-18 was injected at concentrations of 30, 10, 3·3, 1·11, 0·37, 0·12 and 0·013 nM and human IL-18 at concentrations of 278, 93, 31, 10·3, 3·4, 1·14 and 0·12 nM. To correct for bulk refractive index changes, responses in FC1 were subtracted from FC2. Top panels, binding sensograms in colour, with the fitted curves for a 1 : 1 binding model superimposed in black. Bottom panel, the residual plot showing the deviations of the experimental from the fitted curves. Gaps were regions not used in data analysis due to excessive noise from movement of the autosampler needle.

protein were collected by centrifugation. Unbound protein was collected from the supernatant fraction. Following incubation with the binding proteins, the same beads were incubated with huIL-18 to determine whether bead-bound protein was capable of simultaneously interacting with IL-18. Samples were analysed by Western blot to detect the binding proteins or IL-18. Only D7L was detectable in the bead fraction, indicating that only D7L was capable of interaction with heparin (Fig. 6a, left panel). Furthermore, IL-18 was detectable only in beads pre-incubated with D7L (Fig. 6a, right panel). IL-18 did not directly interact with the heparin–Sepharose beads. To demonstrate that the interaction of D7L with the heparin–Sepharose beads was specific, D7L was incubated with soluble heparin prior to

Table 1. Summary of BIAcore studies using captured D7L–Fc and p13–Fc with human or murine IL-18 analyte

		K_D (nM)	k_a (M^{-1} s^{-1}) ($\times 10^5$)	k_d (s^{-1}) ($\times 10^{-4}$)
Murine IL-18	p13	0·16±0·05	9·54±2·52	1·42±0·21
	D7L	0·44±0·27	4·47±2·55	1·43±0·61
Human IL-18	p13	5·14±1·41	0·53±0·17	2·43±0·34
	D7L	2·62±0·67	0·38±0·10	2·02±0·90

Data were obtained as described in Fig. 4. Each combination was tested at least three times and data were analysed using BIAevaluation 3.2 software.

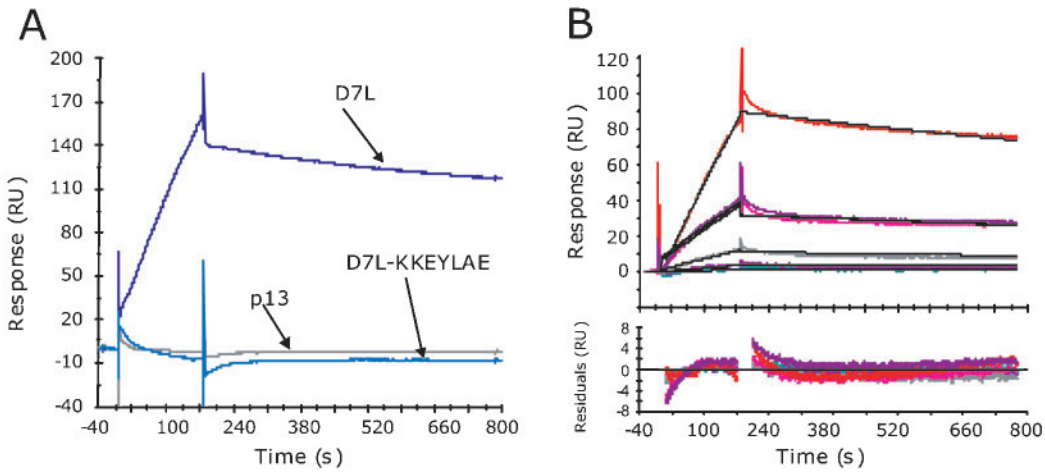

Fig. 5. D7L but not p13 or the mutant D7L-KKEYLAE binds to immobilized heparin. A BIAcore SA chip was used to capture biotinylated BSA on the reference flow cell (FC1) and biotinylated BSA–heparin on FC2. (A) D7L, p13 and D7L-KKEYLAE were injected for 3 min at the same concentration (130 nM) and allowed to dissociate for 10 min. Binding was measured as the response difference in RU after contribution of bulk shift was eliminated by subtraction of the reference cell from FC2. (B) D7L was injected at five different concentrations (130, 43·3, 14·4, 4·8 and 1·6 nM, with a replicate of 43·3 nM) for 3 min followed by 10 min dissociation time. A blank injection and the signal in FC1 were subtracted and sensograms were globally fitted to a 1 : 1 binding model. A residual plot is shown beneath the sensogram, which shows the deviation of the experimental data from the fitted curves. The same concentration series was repeated in three independent experiments.

incubation with the beads. Soluble heparin effectively competed with the immobilized heparin (Fig. 6b).

The structure of GAGs is highly variable and tissue dependent. Heparin is commonly used as a generic GAG, although it is the least common component of the extracellular matrix. To demonstrate that D7L can interact with other GAGs, a competition assay was performed using SPR. D7L was incubated with varying concentrations of soluble heparin, HS, CSA, CSB or no GAG, then injected over a BIAcore chip coated with immobilized heparin. Competition by the soluble GAG was detected as a drop in the maximum binding compared with D7L alone. All soluble GAGs competed with immobilized heparin in a concentration-dependent manner, but with varying efficacy (Fig. 7). Heparin was the most effective competitor, while CSB and HS were intermediate and CSA was the least effective. This suggested that the affinity of D7L was highest for the best competitor, heparin.

DISCUSSION

Since its eradication, studies on VARV have been limited due to the absence of an animal model and restricted access to virus, viral DNA and related reagents, as well as reduced interest in what is essentially an extinct pathogen. However, the threat of bioterrorism has reinvigorated research in VARV. This study is only the third specifically addressing the biological activity of a VARV immunomodulatory protein (Smith *et al.*, 1997; Rosengard *et al.*, 2002). Using surface plasmon resonance and inhibition of IL-18 activity, we have demonstrated that the VARV gene *D7L* encodes a functional IL-18BP. D7L differs from p13 in only 7 of the N-terminal 121 residues, scattered throughout the length of the protein, but the C terminus is truncated and shows the most divergent sequence. SPR studies demonstrated that these differences in primary sequence do not result in significant changes in activity with respect to IL-18 binding. Both proteins effectively bind both huIL-18 and muIL-18 and are capable of inhibiting the biological activity of IL-18.

Orthopoxvirus immunomodulatory proteins frequently display broad species specificity (Mossman *et al.*, 1995; Alcami & Smith, 1995, 1996; Symons *et al.*, 1995; Smith & Alcami, 2000). Orthopoxviruses may have had multiple maintenance hosts throughout their evolution, resulting in diversification of ligand binding capability. Similarly, the IL-18BPs can bind multiple species of IL-18. Interestingly, both D7L and p13 demonstrated higher affinity for muIL-18 than huIL-18. The affinity for muIL-18 is comparable with that of the high-affinity (0·4 nM) IL-18 receptor (Yoshimoto *et al.*, 1998; Debets *et al.*, 2000) found on the cell surface, suggesting that p13 and D7L could effectively compete against the receptor for muIL-18. The affinity for huIL-18 is significantly lower and is in the range of the low-affinity (11–31 nM) cellular receptor (Yoshimoto *et al.*, 1998). Inspection of the association and dissociation rates of p13 and D7L reveals that the difference in binding affinities for the mouse and human ligands is due to a faster association rate for murine IL-18. Therefore, the binding protein and muIL-18 complex is formed more rapidly; however, once formed the complex with either ligand is

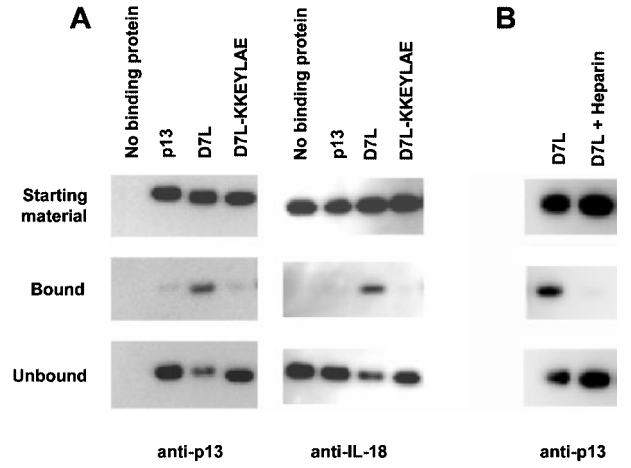

Fig. 6. D7L binds heparin and huIL-18 simultaneously. (A) D7L, p13, the heparin-binding site mutant D7L-KKEYLAE or no protein were incubated for with heparin–Sepharose beads. The beads were separated from unbound material by centrifugation and washed extensively. The same beads were then incubated with huIL-18 for 2 h, pelleted by centrifugation and unbound material was collected. Beads were washed and samples were analysed by SDS-PAGE and probed by Western blotting against p13 (left), then stripped and reprobed with antibody against IL-18 (right). Starting material, initial amount of p13 or IL-18 prior to incubation with beads; Bound, bead fraction recovered following incubation and centrifugation; Unbound, supernatant fraction containing unbound material following incubation with beads and centrifugation. (B) D7L was pre-incubated with or without 2·5 mg soluble heparin ml^{-1}, then incubated with heparin–Sepharose beads. Samples were centrifuged to separate bound and unbound fractions and analysed by Western blotting using anti-p13.

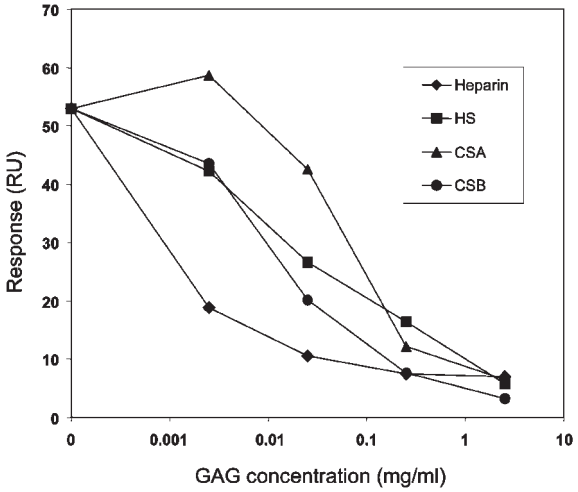

Fig. 7. Soluble GAGs can compete with immobilized heparin for D7L binding. D7L was pre-incubated with increasing concentrations of soluble heparin, heparin sulphate (HS), chondroitin sulphate A (CSA), chondroitin sulphate B (CSB) or no competitor, then injected over a BIAcore sensor chip with an immobilized heparin surface as described in Fig. 5. The maximum binding of each sample is shown.

equally stable. It is important to note that huIL-18BP and MC54L also display an approximately fivefold higher affinity for muIL-18 than huIL-18 (Xiang & Moss, 1999b) indicating that the species preference is common among the IL-18BP family. The higher affinity for muIL-18 in these proteins is also primarily due to a faster association rate. The higher-affinity binding may be due to an intrinsic property of muIL-18 that allows better binding.

Recently, the smallpox inhibitor of complement enzymes, SPICE, was shown to be significantly more potent at inactivating human complement than its homologue in VACV (Rosengard *et al.*, 2002). The VARV and VACV complement inhibitors differ by less than 5 %, suggesting that minor changes can significantly change species specificity. The current study demonstrates that VARV immune modulators are not necessarily better suited for human ligands and that adaptations overcoming human immunity may not explain the narrow host range or considerable pathogenicity of VACV. The species specificity of CPXV and ECTV IL-18BPs has been assessed and has correlated with the host range of the pathogens (Smith *et al.*, 2000; Calderara *et al.*, 2001). These studies demonstrate that, despite the fact that VARV is a human pathogen, D7L has a lower affinity for the ligand from its natural host, as does the IL-18BP of another obligate human pathogen, MOCV (Xiang & Moss, 1999b). Therefore, it is possible that higher-affinity binding of huIL-18 is not possible since huIL-18BP and IL-18BPs from two human pathogens demonstrate higher-affinity binding for muIL-18. Thus these proteins may already possess optimal binding within the limitations of the structure of the IL-18BP family.

GAGs are an abundant component of the extracellular matrix and cell surface and are composed of repeating disaccharide units, usually modified with sulphyl groups or alkyl groups, attached to a protein core. An enormous diversity of structures can be formed based on variations in the disaccharide backbone and modifications and differential tissue expression results in a complex and unique extracellular environment. GAGs participate in a variety of processes including binding of growth factors and chemokines, which contributes to protein function by helping to establish a solid-phase gradient, maintain a high local concentration or aid in association of a ligand with its receptor (Schonherr & Hausser, 2000). Viral proteins such as the *Myxoma virus* chemokine binding protein, M-T1 (Seet *et al.*, 2001), and MC54L, the MOCV IL-18BP (Xiang & Moss, 2003), can also bind GAGs. Since there are several examples of secreted immunomodulatory poxvirus proteins that bind GAGs, it is likely that this activity is important in maximizing the ability of the proteins to compete with the cellular receptor by maintaining high concentrations at the site of infection. In this study we have

demonstrated that the VARV IL-18BP can also interact with GAGs *in vitro*. D7L has an appreciable affinity for heparin (56 nM); however, p13 does not demonstrate detectable binding. For comparison, MC54L binds heparin with an affinity that is two orders of magnitude higher than D7L due to the longer, heavily charged C terminus (Xiang & Moss, 2003).

GAG binding is conferred primarily, but not exclusively, through ionic interactions with positively charged residues (Mulloy & Linhardt, 2001). We have provided evidence that the region involved in binding to GAGs is the C-terminal sequence present in D7L but not p13, and that the binding site is physically distinct from the IL-18 binding site, since both ligands can be bound simultaneously. The C terminus of D7L contains three lysines that closely fit a common heparin-binding motif, and although p13 contains two of these lysines, acidic residues are also present. Interaction with GAGs is primarily driven by ionic interactions with positively charged amino acids; thus the presence of negative charges is likely to interfere with binding.

In a competition assay, D7L bound heparin best, HS and CSB equally but less than heparin, and CSB with the lowest affinity. Although specific conclusions on the components of GAGs that may be involved in binding cannot be made, some structures are suggested based on the relative affinities for each. Binding may be dependent on the degree of sulphation, with the most highly sulphated GAG, heparin, binding best. In addition, unlike the other GAGs tested, CSA does not contain L-iduronic acid, indicating that this may also contribute to binding.

At this time, it is only possible to speculate why D7L, but not p13, binds GAGs. The maintenance hosts of ECTV and VARV differ, indicating that this function may be more important in the human host. The IL-18BP of MOCV, a human pathogen, also binds GAGs via residues in the C terminus (Xiang & Moss, 2003). However, analysis of the nucleotide sequences of *D7L* and *p13* reveals that the different C termini are the result of a single nucleotide deletion, resulting in a frame shift and later termination in p13. Furthermore, the C terminus of p13 differs among ECTV strains; p13 of ECTV Moscow (used in this study) and Hampstead are identical, while other strains, including ECTV Naval, have a C terminus identical to D7L. The CPXV IL-18BP C terminus is unique among orthopoxviruses and is likely to lack GAG binding activity, while VACV and *Monkeypox virus* encode IL-18BPs with D7L-like C termini. Thus it is difficult to conclude whether GAG binding is a gain-of-function mutation or whether some viruses have lost this function during their evolutionary history.

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