Identification of residues in the ectromelia virus gamma interferon-binding protein involved in expanded species specificity

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INTRODUCTION

Ectromelia virus (ECTV), like most poxviruses, encodes multiple genes that target host responses to infection. Of the products encoded by the ECTV genome, approximately 25% target host processes that include signalling and apoptotic pathways and innate/immune responses (Chen et al., 2003). Poxviruses have many strategies within their immune-evasion repertoire. For example, poxviruses encode at least five distinct tumour necrosis factor (TNF)-binding proteins, as well as binding proteins for chemokines, interleukins and interferons (IFNs). These proteins are generally thought to bind cytokines competitively and block cell-surface receptor engagement. The poxvirus soluble immune modulators generally fall into two classes: those with and those without homology to cellular proteins. Poxvirus immunomodulators with homology to host proteins suggest that the viruses have assimilated genetic information from their host and found it advantageous to their survival (Alcami & Smith, 1996; Alcami et al., 1998; Alcami & Koszinowski, 2000; Alcami, 2003). One such assimilation appears to have included the host high-affinity receptor for IFN-γ. The virally encoded IFN-γ-binding proteins (IFN-γ-BPs) share 20–25% amino acid identity with the host IFN-γ receptor (IFN-γR1) and have been shown to block the antiviral activities of IFN-γ in vitro.

The first identification of a poxvirus protein that interacted with and inhibited the activity of IFN-γ came from work with Myxoma virus (MYXV). M-T7 (MYXV IFN-γBP) represents the major secreted product of cells infected with MYXV in vitro. Cross-linking studies with radiolabelled IFN-γ revealed that M-T7 bound to and inhibited the biological activity of rabbit IFN-γ, but not murine IFN-γ (mIFN-γ) or human IFN-γ (hIFN-γ) (Mossman et al., 1995b). Sequencing studies of this protein demonstrated significant homology to both the human and murine IFN-γR1, although neither species of ligand is recognized by M-T7. Affinity of M-T7 for rabbit IFN-γ was calculated to be approximately 1.2 × 10⁻⁸ M. This value is comparable to that calculated for the soluble human IFN-γR1 (Walter et al., 1995), but is approximately 50-fold higher than that for the...
cellular receptor complex (approx. $5.9 \times 10^{-10}$ M) (Mossman et al., 1995b). It was predicted that M-T7 functions by binding extracellular IFN-γ produced during infection and preventing its association with the IFN-γR1, thereby inhibiting downstream functions (McFadden et al., 1995). In addition to binding rabbit IFN-γ, chemical cross-linking studies have shown interaction of M-T7 with several chemokines, although binding did not occur consistently in the presence of the IFN-γ ligand. This may indicate either shared binding sites or a conformational change induced by binding of the IFN-γ ligand, which sterically prevents chemokine binding (Lalani et al., 1997). Similar cross-linking studies with other poxvirus IFN-γ-BPs did not reveal interaction with any cytokine or chemokine other than IFN-γ (Graham et al., 1997).

Although M-T7 of MYXV was the first described poxvirus IFN-γ-BP, proteins of similar function are present in viruses from the genus Orthopoxvirus as well. In contrast to M-T7, the IFN-γ-BPs from orthopoxviruses show broad species specificity with relation to ligand binding. Whereas M-T7 has only been reported to bind IFN-γ from its rabbit host, orthopoxvirus IFN-γ-BPs bind IFN-γ from several species (Alcami & Smith, 1995; Mossman et al., 1995a, b; Alcami & Smith, 1996). Of particular interest are the IFN-γ-BPs from ECTV (C4, M158 or ECTV IFN-γ-BP), Vaccinia virus (VACV; B8 or VACV IFN-γ-BP) and Variola virus (VARV; B9 or VARV IFN-γ-BP). Although the orthopoxvirus IFN-γ-BPs are related very closely to one another (> 90% identity, > 96% similarity) (Lefkowitz et al., 2005), they show differing abilities to bind and inhibit the biological actions of IFN-γ from various species (Alcami & Smith, 1995; Mossman et al., 1995a; Seregin et al., 1996). Because of the high degree of identity and similarity between these proteins, the structural and biological significance behind the varied ligand-binding specificities remains unclear.

VACV IFN-γ-BP is currently the best-studied of the orthopoxvirus IFN-γ-BPs. Cross-linking assays using IFN-γ's from several species demonstrated the ability of VACV IFN-γ-BP to associate with rabbit, rat, bovine, chicken, equine, human and murine IFN-γ, although competition assay with both ‘cold’ and radiolabelled IFN-γ revealed that binding of mIFN-γ is at a substantially reduced affinity (Alcami & Smith, 1995; Mossman et al., 1995a; Puehler et al., 1998; Symons et al., 2002). This correlated with the ability of VACV IFN-γ-BP to neutralize the bioactivity of rat, bovine, rabbit and human IFN-γ, but not mIFN-γ, in a rhabdovirus assay (Alcami & Smith, 1995). This is in contrast to the IFN-γ-BP from ECTV, a mouse pathogen, which antagonizes mIFN-γ with high affinity in both cross-linking and biological assays (Mossman et al., 1995a). We have chosen the differing ability of the VACV and ECTV IFN-γ-BPs to antagonize mIFN-γ as a model to begin to understand the expanded species specificities of the orthopoxvirus IFN-γ-BPs, a feature that makes them biochemically distinct from the cellular IFN-γR1.

**METHODOLOGY**

**Cell and virus culture.** African green monkey kidney cell lines BS-C-1 (ATCC CCL-26) and CV-1 (ATCC CCL-70) were maintained in Dulbecco’s modified Eagle’s medium containing 50 units penicillin ml⁻¹, 50 μg streptomycin ml⁻¹, 2 mM l-glutamine and 10% FetalClone II (HyClone Laboratories, Inc.) at 37 °C in a 5% CO₂ atmosphere. The Western Reserve strain of VACV and a derivative virus expressing the T7 RNA polymerase were kindly provided by Dr Bernard Moss (Fuerst et al., 1987). VACV-t7,brtr (hereafter VACV-t7) virus was constructed by deleting the VACV ifn-γ (bbr) ORF by using the gpt transient dominant-selection system (Falkner & Moss, 1990). Virus stocks were prepared in HeLa-S3 cells (ATCC CCL-2.2) and stored at −70 °C until use. Virus infectivity was measured on BS-C-1 monolayers as described previously (Chen et al., 1992).

**Plasmids and mutagenesis.** The ECTV ifn-γ-BP gene was amplified by PCR from ECTV Moscow strain DNA by using the oligonucleotides EVCA4R-5' (5'-GCGCTCATGAGGCTATAATTCATTCTCACAG-3') and EVC4R-3' (5'-GCGCTCGACATTTAGCTAAGCATGCTAAG-3'), corresponding to the 5' and 3' ends of the ifn-γ-BP gene and providing BspHl and Sall restriction sites, respectively. The VACV ifn-γ-BP gene was amplified from VACV Copenhagen DNA by using the oligonucleotides VACVB8R-5' (5'-GCGCTCATGAGGCTATAATTCATTCTCACAG-3') and VACVB8R-3' (5'-GCGCTCGACATTTAGCTAAGCATGCTAAGG-3'). The resulting fragments were cloned into NcoI and SalI-digested pTM1 vector, creating pTM1-EVCA4 and pTM1-VACVB8B (Fuerst et al., 1987). Chimeric constructs were created by digestion of the ECTV and VACV PCR products with AvalI (to separate domains I and II) and/or Ncol (to separate domains II and III), followed by coligation of the appropriate fragments into pTM1. Single-base mutations were carried out by using the Gene Tailor site-directed mutagenesis system (Invitrogen). All mutant ifn-γ-BP genes contained native IFN-γ-BP signal peptide and genotypes were confirmed by DNA sequencing.

**Transfection.** We have previously expressed ECTV IFN-γ-BP that retains its biological activity by using the VACV-t7 expression system with yield of approximately 0.5 μg protein per 10⁶ CV-1 cells (Fuerst et al., 1987; Alexander et al., 1992; Bai et al., 2005). In brief, CV-1 cells were grown in six-well tissue-culture plates to approximately 80% confluence and were infected with VACV-t7 at 10 p.f.u. per cell for 1 h at 37 °C. The cells were then washed in Opti-MEM (Invitrogen) and transfected with pTM1 (2 μg, vector control) or pTM1 containing wild-type (WT) or mutant ifn-γ-BP genes (2 μg) by using LipofectAMINE 2000 reagent in Opti-MEM according to the manufacturer’s protocol (Invitrogen). At 24 h post-transfection, the supernatant was collected, clarified and filtered to remove virus by using 0.1 μm centrifugal filters (Millipore). Expressed proteins were assayed and quantified via Western blot densitometry using a purified IFN-γ-BP standard, as described previously (Bai et al., 2005).

**Surface plasmon resonance (SPR).** Real-time interaction of IFN-γ-BP with mIFN-γ was measured by SPR on a Bicore 2000 (Biacore Inc.). Flow cells of a carboxymethylated dextran (CM5) sensor chip were activated using 50 mM N-hydroxysuccinimide and 200 mM N-ethyl-N’-(dimethylaminopropyl)carbodiimide for 7 min at a flow rate of 5 μl min⁻¹. mIFN-γ (R&D Systems) diluted in 10 mM sodium acetate (pH 5.0) was immobilized at a flow rate of 5 μl min⁻¹ for 5 min. The surface was treated by using 1 M ethanolamine hydrochloride at pH 8.5 for 7 min with a flow rate of 5 μl min⁻¹ to deactivate excess reactive esters and remove non-covalently bound ligand. The mIFN-γ surface was stable following repeated rounds of regeneration and remained so for several weeks. Three mIFN-γ surfaces were created at varying densities, along with a control surface (activated and blocked), on each CM5 chip. Surfaces were tested for mass-transport limitation by using the
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Results

ECTV/VACV chimeric IFN-γBPs have reduced affinity for mIFN-γ

ECTV IFN-γBP binds to mIFN-γ with high affinity and inhibits its biological activity, whereas VACV IFN-γBP does not. Each IFN-γBP can be divided into three domains: the first two (DI and DII) are based on homology to the IFN-γ1 fibronectin type III domains (Walter et al., 1995) and the third (DIII) is unique to the poxvirus IFN-γBPs (Fig. 1). To evaluate the basis of the expanded species specificity of ECTV IFN-γBP, we constructed chimeric IFN-γBPs wherein ECTV IFN-γBP domains were replaced with the corresponding VACV domains (designated by vertical lines in Fig. 1), either singly or in combination, and tested for mIFN-γ affinity by SPR. ECTV IFN-γBP was found to bind to mIFN-γ with high affinity; however, no binding could be detected by VACV IFN-γBP. Substitution of VACV domains into the ECTV IFN-γBP resulted in decreased affinity for mIFN-γ (Fig. 2). Interestingly, inclusion of VACV domains into the ECTV IFN-γBP did not diminish the ability of IFN-γBP–mIFN-γ complexes to form (k₄), but the stability of the complexes (k₃) was affected. Inclusion of VACV I alone had the greatest effect of an individual domain, with a 4.6-fold faster dissociation rate (P < 0.0001 vs ECTV WT by unpaired Student’s t-test). The dissociation rate was increased further by the inclusion of either VACV II or III along with VACV I, by 8.6- and 12.6-fold, respectively (P = 0.0031 and P = 0.0028 vs VACV I alone by unpaired Student’s t-test, respectively). The importance of DI in ligand binding is consistent with this region being homologous to the ligand-binding domain of the IFN-γ1. Interestingly, none of the three VACV domains alone or in pairs eliminated mIFN-γ binding by ECTV completely. This observation alludes to a cooperative role for several of the 17 amino acid differences between ECTV and VACV in enabling ECTV to bind mIFN-γ. Rate constants for each of the chimeric constructs can be found in Table 1.

High-affinity binding of mIFN-γ is mediated by amino acids within the receptor-homologous region

There is a high level of identity within the orthopoxvirus IFN-γBP family. ECTV and VACV IFN-γBPs differ by only 17 amino acid substitutions (excluding the signal sequence and VACV C-terminal extension). To address which amino acid differences between the ECTV and VACV IFN-γBPs play a role in ECTV IFN-γBP binding to mIFN-γ with high affinity, we constructed point mutations in the ECTV IFN-γBP to the corresponding VACV residue wherever a difference occurred (Fig. 1). These point mutants were evaluated by SPR for their ability to bind mIFN-γ. A lowered affinity for mIFN-γ points to the importance of a residue in high-affinity mIFN-γ binding. Of the 17 point mutations screened, only three were found to have a statistically significantly negative impact on the affinity of ECTV IFN-γBP for mIFN-γ (P < 0.01 by ANOVA; Fig. 3; Table 1). ECTV R45K resulted in a 3.8-fold faster dissociation rate for this single amino acid substitution. Substitution of alanine at this position (R45A) resulted in a 200-fold lower affinity for mIFN-γ (10-fold slower k₄ and 20-fold faster k₃), pointing to the importance of this position in ligand binding. The combination of G68A and N71K also resulted in a fivefold faster k₄, although N71K alone had no effect on mIFN-γ binding (P > 0.05 by ANOVA). When the combination of R45K G68A and N71K was tested, a small additive effect of the three positions could be detected, although this was not statistically significant (P = 0.43 for R45K G68A and N71K, but the latter was not statistically significant (P = 0.0031 and P > 0.05 vs WT ECTV by unpaired Student’s t-test, respectively).
Following the observation that ECTV R45, G68 and N71 are involved in high-affinity binding for mIFN-α, we constructed reciprocal mutations in the VACV IFN-α BP (Fig. 4; Table 1). Whilst no mIFN-α binding could be detected from WT VACV IFN-α BP, substitution of a single amino acid (VACV K46R) resulted in a detectable SPR signal for mIFN-α (Fig. 5, right side). Increased activity was also observed for VACV A69G K72N, although to a lesser extent. Substitution of all three ECTV residues into VACV (K46R A69G and K72N) resulted in a remarkable increase in affinity for mIFN-α to within 3- to 5-fold of that observed for WT ECTV IFN-α BP.

ECTV IFN-α BP R45, G68 and N71 are important in the inhibition of the mIFN-α-induced antiviral state

To address the impact of altered affinity by SPR on biological activity, we tested the ability of each of the IFN-α BP mutants to inhibit mIFN-α in a bioassay (Fig. 6). The murine fibroblast cell line L-929 is responsive to the antiviral effects of IFNs. Pre-treatment of L-929 monolayers with mIFN-α induces an antiviral state and the cells are no longer permissive for VSIV infection. Inclusion of IFN-α BP in the pre-treatment conditions results in sequestration of mIFN-α and prevents induction of the antiviral state. VSIV can then replicate in the cell monolayer and the extent of this infection can be monitored by MTT metabolism to formazan in healthy cells. A protective dose of mIFN-α was premixed with twofold dilutions of the IFN-α BP constructs and applied to murine cells to induce an antiviral state. The cellular antiviral state was assessed following VSIV infection and MTT assay. Consistent with the data observed in SPR experiments, ECTV IFN-α BP inhibited mIFN-α with an EC50 of approximately 1 nM. Inhibition was not affected by VACV residue substitution at position N71; however, VACV residue substitution at R45
or G68 and N71 resulted in an approximately fivefold increase in the EC50. Combination of substitutions at all three positions resulted in a approximately 50-fold increase in the EC50. This is substantially greater than the approximately 7.5-fold lower affinity observed by SPR for this mutant. Consistent with previous studies, VACV IFN-γBP was unable to inhibit mIFN-γ in this assay. Reciprocal substitutions of ECTV residues into VACV IFN-γBP resulted in activity of VACV IFN-γBP in the bioassay; however, mIFN-γ inhibition was only observable when the critical residues at VACV 46, 69 and 72 were swapped for the corresponding ECTV sequence. The EC50 of the VACV K46R A69G K72N mutant was approximately 5 × 10⁻⁸ M, approximately 50-fold higher than the kₐ obtained by SPR.

**DISCUSSION**

In this study, we have begun to evaluate the nature of the expanded species specificities of the poxvirus IFN-γBPs. As a model for understanding this relaxed specificity, we chose the IFN-γBPs of ECTV and VACV. The IFN-γBP of the former has been shown to bind and inhibit the biological activity of mIFN-γ, whereas the latter is unable to interact with mIFN-γ with high affinity. There are 17 amino acid changes between the ECTV and VACV IFN-γBPs (excluding the signal sequence and VACV C-terminal extension). To address which of these substitutions confer the ability to bind mIFN-γ, we constructed ECTV/VACV IFN-γBP chimeras, as well as ECTV and VACV IFN-γBPs with point mutations, and looked for loss or gain of mIFN-γ binding by SPR and bioassay.

Of the point mutations tested, only three amino acid substitutions demonstrated altered affinity for mIFN-γ by SPR. Substitution of the corresponding VACV residue at ECTV position 45 (R to K) alone or positions 68 (G to A) and 71 (N to K) in combination resulted in approximately fivefold-reduced affinity for mIFN-γ. Interestingly, the dissociation rate of the complex was affected predominantly, implying that these amino acid substitutions are involved in stability of the IFN-γBP–mIFN-γ complex. The importance of ECTV 45 could have been predicted from the structure and alignment with the hIFN-γR1 (Walter et al., 1995). Like the hIFN-γR1, the IFN-γR1 (GenBank accession no. NP_034641) and VACV IFN-γBP both encode lysine at this position, whereas the ECTV IFN-γBP uniquely encodes arginine at this position. ECTV R45K G68A N71K gave a similar shift in mIFN-γ binding to the VACV I chimera (containing eight of the 17 amino acid changes), demonstrating that these three ECTV amino acids account for a large portion of the ECTV IFN-γBP species specificitity. The combination of R45K G68A N71K with VACV III did not result in the additive effect observed for the combination of VACV I and III. The importance of ECTV R45, G68 and N71 on mIFN-γ binding became more apparent when the reciprocal mutations were made in the VACV IFN-γBP. VACV K46R A69G K72N was able to bind mIFN-γ with high affinity in SPR experiments, whereas wild-type VACV IFN-γBP had an affinity too low to measure by SPR. No amino acid substitutions or ECTV/VACV chimeras resulted in a complete loss of mIFN-γ-binding activity. This result suggests that many of the 17 point mutants participate in mIFN-γ affinity, possibly in a
Table 1. Summary of kinetics and affinity measurements of IFN-γ-BP constructs for mIFN-γ.

SPR curves were fitted to a 1:1 binding model and kinetic rate constants were determined by using BIAevaluation 4.1 software \((n \geq 3, \text{ except } R45A \text{ with } n=2)\). Values represent the mean ± SEM for the calculated rate constants and affinity from each cycle. ECTV to VACV point mutant and chimeric IFN-γ-BP rate constants were analysed by one-way ANOVA with Dunnett's post test vs WT ECTV IFN-γ-BP.

<table>
<thead>
<tr>
<th>Construct</th>
<th>(k_a) (× 10^8 M^-1 s^-1)</th>
<th>(k_d) (× 10^-5 s^-1)</th>
<th>(K_D) (× 10^-10 M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECTV</td>
<td>2.24 ± 0.25</td>
<td>7.65 ± 1.17</td>
<td>3.98 ± 0.56</td>
</tr>
<tr>
<td>ECTV T17K</td>
<td>3.13 ± 0.37</td>
<td>7.91 ± 1.91</td>
<td>2.82 ± 0.80</td>
</tr>
<tr>
<td>ECTV N37D</td>
<td>1.99 ± 0.36</td>
<td>4.43 ± 1.63</td>
<td>2.63 ± 1.51</td>
</tr>
<tr>
<td>ECTV R45K</td>
<td>3.25 ± 0.37</td>
<td>29.2 ± 4.62*</td>
<td>10.8 ± 3.58</td>
</tr>
<tr>
<td>ECTV R45A</td>
<td>0.22 ± 0.08</td>
<td>151 ± 9.46*</td>
<td>787 ± 242</td>
</tr>
<tr>
<td>ECTV N71K</td>
<td>3.27 ± 0.79</td>
<td>9.51 ± 2.81</td>
<td>3.36 ± 0.84</td>
</tr>
<tr>
<td>ECTV G68A N71K</td>
<td>3.07 ± 1.63</td>
<td>32.3 ± 5.91*</td>
<td>16.6 ± 5.23</td>
</tr>
<tr>
<td>ECTV R45K G68A N71K</td>
<td>1.56 ± 0.48</td>
<td>39.2 ± 11.5*</td>
<td>33.6 ± 8.73</td>
</tr>
<tr>
<td>ECTV D89E</td>
<td>3.44 ± 0.57</td>
<td>1.96 ± 0.16</td>
<td>0.59 ± 0.12</td>
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<tr>
<td>ECTV D89E K90E</td>
<td>5.83 ± 0.71*</td>
<td>1.93 ± 0.20</td>
<td>0.34 ± 0.07</td>
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<tr>
<td>ECTV 197V</td>
<td>1.43 ± 0.24</td>
<td>8.67 ± 0.67</td>
<td>6.37 ± 1.38</td>
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<tr>
<td>ECTV N137G</td>
<td>0.74 ± 0.15</td>
<td>1.77 ± 0.13</td>
<td>2.59 ± 0.68</td>
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<tr>
<td>ECTV N160T</td>
<td>3.23 ± 0.11</td>
<td>4.65 ± 0.14</td>
<td>1.44 ± 0.01</td>
</tr>
<tr>
<td>ECTV M186T</td>
<td>3.27 ± 0.35</td>
<td>5.43 ± 0.45</td>
<td>1.67 ± 0.04</td>
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<tr>
<td>ECTV L194F D196E</td>
<td>3.94 ± 0.14</td>
<td>7.18 ± 0.29</td>
<td>1.82 ± 0.03</td>
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<tr>
<td>ECTV E229D</td>
<td>3.77 ± 0.09</td>
<td>6.00 ± 0.12</td>
<td>1.59 ± 0.01</td>
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<tr>
<td>ECTV T232A</td>
<td>2.24 ± 0.60</td>
<td>4.17 ± 1.89</td>
<td>2.36 ± 0.91</td>
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<tr>
<td>ECTV H244Q</td>
<td>3.49 ± 0.63</td>
<td>4.69 ± 1.72</td>
<td>1.86 ± 0.87</td>
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<tr>
<td>ECTV I258V</td>
<td>1.55 ± 0.10</td>
<td>4.58 ± 1.88</td>
<td>2.66 ± 0.71</td>
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<tr>
<td>VACV I</td>
<td>2.48 ± 0.72</td>
<td>34.9 ± 2.86*</td>
<td>21.7 ± 5.85</td>
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<tr>
<td>VACV I and II</td>
<td>4.33 ± 0.95†</td>
<td>66.1 ± 7.85*</td>
<td>21.4 ± 5.69</td>
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<tr>
<td>VACV I and III</td>
<td>3.72 ± 1.77</td>
<td>96.5 ± 15.1*</td>
<td>197 ± 137</td>
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<tr>
<td>VACV II</td>
<td>3.63 ± 0.71</td>
<td>18.1 ± 3.59</td>
<td>6.13 ± 1.37</td>
</tr>
<tr>
<td>VACV II and III</td>
<td>3.14 ± 0.38</td>
<td>41.9 ± 7.85*</td>
<td>15.2 ± 3.79</td>
</tr>
<tr>
<td>VACV III</td>
<td>3.20 ± 0.37</td>
<td>17.4 ± 2.64</td>
<td>5.77 ± 0.96</td>
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<tr>
<td>VACV K46R</td>
<td>2.52 ± 0.22</td>
<td>90.1 ± 4.68</td>
<td>37.0 ± 3.08</td>
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<tr>
<td>VACV A69G K72N</td>
<td>1.88 ± 0.50</td>
<td>149 ± 19.7</td>
<td>96.4 ± 12.0</td>
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<tr>
<td>VACV K46R A69G K72N</td>
<td>2.33 ± 0.32</td>
<td>25.3 ± 4.15</td>
<td>19.9 ± 4.31</td>
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</tbody>
</table>

Constructs with statistically significant alterations in the \(k_a\) or \(k_d\) vs those of WT ECTV are marked: *\(P<0.01\), †\(P<0.05\).

cooperative or additive fashion that would not be apparent through single amino acid substitutions.

The combination of substitutions at ECTV 45, 68 and 71 gave a slight additive effect by SPR; however, this effect was more dramatic in the bioassay, where an approximately 50-fold shift in the EC\(_{50}\) was observed. The nature of the inconsistency between the SPR affinity and the EC\(_{50}\) values from the bioassay remains unclear. We have investigated the possibility that the oligomerization of the IFN-γ-BP constructs has been affected by both reducing and non-reducing Western blots, as well as gel filtration for the ECTV and VACV constructs with three point mutations. We found no difference in the expression levels or oligomerization state of any of the mutants tested compared with WT ECTV and VACV IFN-γ-BPs (Supplementary Figs S1 and S2, available in JGV Online). The use of IFN-γ-BP as the injected analyte in the SPR experiments limits the possibility that there are large, specific activity differences between constructs, as this would probably have been reflected in the association-rate constants. Whilst both SPR and bioassay support the same observations with regard to critical residues, we find it important to point out the differences between measurements made on a covalently linked SPR surface versus a biological system. Unfortunately, we have been unsuccessful in establishing reliable conditions for SPR with hIFN-γ-BPs. Based upon prior studies demonstrating that both ECTV and VACV IFN-γ-BPs bind to hIFN-γ with high affinity, we would speculate that the substitution of ECTV residues into VACV would have little effect on hIFN-γ affinity (Mossman et al., 1995a).

An examination of all of the sequenced orthopoxviruses (sampled in Fig. 1) reveals that only ECTV contains all of the residues identified in this study to be important in mIFN-γ binding (R45, G68 and N71). Although it is not practical to establish virus phylogeny from a single protein, this observation would support the notion that ECTV has
co-evolved with the mouse and adapted this immunomodulator to function within the murine host. Interestingly, both MPXV and CPXV have rodent hosts in nature (McFadden, 2005) and there is conservation of G68.
which contributes to mIFN-\(\gamma\) specificity, within both IFN-\(\gamma\) BPs. It is possible that conservation of this residue in MPXV and CPXV plays a role in maintenance of these viruses in rodents.

The importance of IFN-\(\gamma\) and a polarized type I immune response has been well-established for the clearance of poxvirus infections (Karupiah et al., 1990, 1993; Chaudhri et al., 2004); however, the importance of the IFN-\(\gamma\) BPs during poxvirus infections is still an issue of some debate. Early work with the leporipoxvirus MYXV showed that recombinant virus lacking M-T7 was attenuated dramatically with respect to mortality, disease severity and virus dissemination (Mossman et al., 1996). This result is difficult to interpret because of the additional role of M-T7 as a chemokine-binding protein and the lack of a revertant control virus (Lalani et al., 1997). Whilst disruption of the IFN-\(\gamma\)-BP in VACV resulted in attenuation in infected rabbits, there have been conflicting reports as to the importance of the IFN-\(\gamma\)-BP in VACV infection of the mouse (Sroller et al., 2001; Verardi et al., 2001; Symons et al., 2002). These conflicting results are puzzling, given the observation that VACV IFN-\(\gamma\)-BP is unable to bind mIFN-\(\gamma\) with high affinity. Given this observation, it is prudent to evaluate the importance of IFN-\(\gamma\) antagonism during infection with ECTV, whose IFN-\(\gamma\)-BP can bind to and inhibit the biological activity of mIFN-\(\gamma\), whereas introduction of ECTV residues into the VACV sequence results in increased affinity for mIFN-\(\gamma\).

Study of the poxvirus IFN-\(\gamma\)-BPs lends itself well to future anti-cytokine therapies. In this study, we have identified several residues within a virus IFN-\(\gamma\)-BP that can increase or decrease its affinity for mIFN-\(\gamma\). Elucidation of the binding relationships between IFN-\(\gamma\)-BPs and multiple species of IFN-\(\gamma\) may facilitate directed alteration of human IFN-\(\gamma\)R1 to generate a soluble cytokine trap with substantially higher affinity than the membrane-bound receptor. Such a molecule may prove clinically useful as an anti-IFN-\(\gamma\) treatment in a very similar manner to the anti-TNF-\(\alpha\) agents currently in clinical use.

Fig. 5. Sensorgrams of ECTV and VACV IFN-\(\gamma\)-BP point mutations. Affinity of IFN-\(\gamma\)-BP constructs for mIFN-\(\gamma\) was assessed by SPR. Expressed proteins were injected over an mIFN-\(\gamma\) surface at concentrations of 50, 25, 10 (\(\times\)2), 5 and 1 nM to determine the kinetics of binding. Each construct was tested at least three times against different mIFN-\(\gamma\) surfaces. Representative sensorgrams are shown for ECTV and VACV IFN-\(\gamma\)-BPs, as well as reciprocal mutations affecting mIFN-\(\gamma\) binding. Introduction of VACV residues into the ECTV sequence can be shown to decrease the affinity for mIFN-\(\gamma\), whereas introduction of ECTV residues into the VACV sequence results in increased affinity for mIFN-\(\gamma\).
Fig. 6. IFN-γBP point mutations affect the biological activity of the protein. Point mutants found by SPR to affect the binding of mIFN-γ were evaluated for their ability to sequester mIFN-γ in a bioassay. L-929 cells were incubated with a protective dose of mIFN-γ in the presence of varying amounts of IFN-γBP. Sequestration of mIFN-γ by IFN-γBP resulted in diminished antiviral effects and increased susceptibility to VSIV challenge. Cell viability 48 h after infection was assessed by using an MTT metabolic assay. Results are presented relative to uninfected (100% viable) and VSIV-infected (0% viable) controls. Consistent with SPR observations, ECTV IFN-γBP was able to bind mIFN-γ with an EC50 in the low-nanomolar range. Incorporation of VACV residues at positions 45 or 68 and 71 resulted in an EC50 shift of approximately fivefold. Substitution of all three residues shifted the EC50 by approximately 50-fold. Biological inhibition of mIFN-γ by the reciprocal VACV point mutations could only be detected with the substitution of all three critical residues and remained approximately 50-fold higher than that of ECTV WT.

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