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Correspondence of the Functional Epitopes of Poxvirus and Human Interleukin-18-Binding Proteins

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Molluscum contagiosum virus, a human poxvirus that causes persistent small benign skin tumors, encodes a variety of putative immune defense proteins. Three such proteins, MC51L, MC53L, and MC54L, have 20 to 35% amino acid sequence identities with human interleukin-18 (hIL-18)-binding protein (hIL-18BP), a naturally occurring antagonist of the proinflammatory cytokine IL-18. We previously demonstrated that seven amino acids within the immunoglobulin-like domain of hIL-18BP were important for high-affinity binding to hIL-18. Model building indicated that MC54L, which has been shown to bind hIL-18, contains five of the seven amino acids at corresponding positions in its immunoglobulin-like domain, the exceptions being the conservative substitution of isoleucine for a leucine and the nonconservative substitution of valine for a phenylalanine. We found that individual alanine substitutions for these six identical or highly conserved amino acids of MC54L caused changes in affinity and binding free energy for hIL-18 that were quantitatively similar to those produced by mutagenesis of hIL-18BP. Furthermore, when the nonconserved valine of MC54L was mutated to phenylalanine, making it more like hIL-18BP, its affinity for hIL-18 increased more than 10-fold. In addition, the carboxyl-terminal half of MC54L, which has no similarity with hIL-18BP, was dispensable for hIL-18 binding. Thus, despite their relatively low overall sequence identity, MC54L and hIL-18BP have similar hIL-18 binding sites and functional epitopes. On the other hand, MC51L and MC53L have nonconservative substitutions of three to six of the seven critical amino acids of hIL-18BP and neither protein bound hIL-18, suggesting that they may interact with unidentified ligands.

Molluscum contagiosum virus (MCV) has a worldwide distribution, and with the eradication of smallpox it is the only member of the poxvirus family that relies exclusively on a human host. A high incidence of MCV infection was documented in a report that 39% of individuals over 50 years of age in a representative Australian population tested seropositive (16). Unlike smallpox virus, which causes a rapidly progressive and frequently lethal disease, MCV causes small, benign, papular skin lesions in children and young adults and a more extensive disease only when there is a concurrent immunodeficiency such as AIDS (12). Even in immunocompetent individuals, however, the virus-filled skin lesions frequently persist for many months without signs of inflammation, suggesting local immune suppression (5, 13, 36). Due to the lack of a useful animal model or tissue culture system (7, 11), MCV host defense mechanisms have been difficult to investigate. However, determination of the complete MCV genome sequence (24, 25) and subsequent functional studies revealed several potential immune-evasion mechanisms, some of which had not previously been described for any virus (21). MCV immune-evasion genes include a viral homolog of cellular chemokines with chemokine antagonist activity (9, 17, 19), a major histocompatibility complex class I homolog that binds β2 microglobulin (26), a selenocysteine-containing glutathione peroxidase that inhibits peroxide- and UV-mediated apoptosis (28), a death effector domain protein that inhibits tumor necrosis factor alpha- and Fas ligand-mediated apoptosis (4, 14, 27, 29, 33, 34), and an interleukin-18 (IL-18)-binding protein that inhibits the gamma interferon (IFN-γ)-inducing activity of IL-18 (39).

IL-18 is a proinflammatory cytokine that was formerly known as the IFN-γ inducing factor (22, 35). Acting synergistically with IL-12, IL-18 induces IFN-γ production from T lymphocytes and macrophages, enhances the cytotoxicity of natural killer cells, is required for an effective T helper 1 response, and protects against bacterial and viral infections (reviewed in reference 10). Based on its amino acid sequence and predicted secondary structure, IL-18 was recognized as a member of the IL-1 family (3). The recently discovered IL-18-binding proteins of humans (hIL-18BP) and mice bind IL-18 with a subnanomolar K_d and prevent IL-18 from binding to its receptor (15, 23, 39). MC54L, an MCV homolog of hIL-18BP, has been shown to bind IL-18 and inhibit IL-18-mediated IFN-γ production in a dose-dependent manner (39). Functional homologs of IL-18BP were subsequently found in orthopoxviruses, including vaccinia virus, cowpox virus, and ectromelia virus (6, 8, 32), and yet-uncharacterized homologs are present in additional poxvirus genera (18, 20) but not in other virus families.

Approximately 60% of the mature hIL-18BP consists of an immunoglobulin (Ig) domain that includes a highly conserved pair of cysteines and a tryptophan residue. Through site-directed mutagenesis and binding studies, we identified a small number of amino acids of hIL-18BP that were important for binding IL-18 (37). Those amino acids, collectively called functional epitopes, cluster in the predicted β-strands *c* and *d* of the Ig domain. Three amino acids with aromatic side chains, phenylalanine 93, tyrosine 97, and phenylalanine 104, were the

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most critical and together contributed to about 65% of the total binding free energy. Here we show that despite a relatively low overall sequence identity, MC54L and hIL-18BP have similar IL-18 binding sites. In contrast, MC51L and MC53L, the other two hIL-18BP homologs of MCV, lack three or more of the critical amino acids and do not bind IL-18.

MATERIALS AND METHODS

Molecular modeling of MC54L Ig domain. The Homology and Builder modules in the Insight II program (Molecular Simulations Inc., San Diego, Calif.) were used for model construction. Similar to the procedure used for modeling of hIL-18BP, the N terminus of the MC54L Ig domain was based on the constant domain of the light chain anti-T-cell receptor antibody H57 (PDB entry no. 1nfd), and the rest of the MC54L Ig domain was based on domain III of IL-1R (PDB entry no. 1itb). The entire structure was then optimized through energy minimization with the Builder module.

Site-directed mutagenesis. When amplifying the MC54L open reading frame (ORF) by PCR under standard conditions, a repetitive GC-rich sequence near the C terminus of the MC54L gene was consistently deleted, resulting in the removal of codons 142 to 173 without changing the remainder of the ORF. The resulting MC54L ORF was fused in frame with DNA encoding a flexible linker (GGGGSGGGGS), a biotinylation site (GLNDIFEAQKIEWHE), and a six-histidine tag in a manner similar to that previously reported for the hIL-18BP ORF (37). This fusion plasmid was named pYX52. A derivative of pYX52 was constructed by introducing a silent mutation at residue 70, which created a unique *Hpa*I site. This unique *Hpa*I site, together with either a 5′ *Nhe*I site or a 3′ *Bam*HI site, facilitated the insertion of PCR products containing substitutions at residue 63, 65, 67, 69, 74, 76, 78, or 84. For each mutant, the entire MC54 coding sequences of both strands were confirmed.

Protein purification and in vitro biotinylation. Proteins were expressed in mammalian cells, purified, and biotinylated in vitro as previously reported for recombinant hIL-18BP (rhIL-18BP) (37). Briefly, 293T cells were transfected, and after 3 days the serum-free medium was harvested and incubated with Ni-nitrilotriacetic acid resin (Qiagen, Valencia, Calif.). The resin was then washed and resuspended in 10 mM Tris-HCl (pH 8.0), and *Escherichia coli* biotin holoenzyme synthetase (BirA; Avidity, Denver, Colo.) was added. The resin was incubated with shaking at 37°C for 1 to 4 h, packed into a column, and washed with 15 mM imidazole in phosphate-buffered saline containing 150 mM NaCl. The recombinant protein was eluted with 300 mM imidazole in phosphate-buffered saline. Fractions containing the biotinylated proteins were used for analysis on a BIAcore 2000 sensor (BIAcore, Piscataway, N.J.).

Surface plasmon resonance (SPR). Biotinylated MC54L was captured onto a BIAcore SA chip via the strong and virtually irreversible interaction of biotin with streptavidin. The chip was then washed several times by injections of 10 mM glycine (pH 1.5) to remove any loosely bound materials. Less than 300 response units (RU) of protein were captured to prevent any mass transport effect during the kinetic analysis. The maximal responses for IL-18 binding (R_{max}) were usually around 100 RU, which was close to the theoretical R_{max} calculated with an assumption that all captured MC54L proteins were active. The binding kinetics of MC54L with recombinant human IL-18 (rhIL-18; PeptroTech, Rocky Hill, N.J.) were determined as previously described.

Sequencing of MC51L, MC53L, and MC54L from different MCV strains. MCV type 2 and 4 genomic DNA samples were provided by C. H. Thompson (University of Sydney, Sydney, Australia) and used as templates for PCR. MCV type 4 was isolated from lesions on the face of a human immunodeficiency virus-infected male with AIDS, and the DNA had a *Bam*HI restriction pattern distinctly different from that of type 1/1v, 2, or 3. The primers used for PCR were derived from the 5′ and 3′ end sequences of MC51L, MC53L, and MC54L genes from MCV type 1. They were 5′-CTATCAGCTAGCATGAAGGAAAGTCTTTCTCG-3′ and 5′-TAATGGATCCACCACCTCCCCACAGGTTTCTCAACATGA-3′ for MC51L, 5′-CTATCAGCTAGCAATGTTCTTCGACAACATGC-3′ and 5′-TAATGGATCCACCACCTCCGCACCAGAAGTCTGCCAAG-3′ for MC53L, and 5′-CTATCAGCTAGCATGGCGCGTGGCGGAAAAAGC-3′ and 5′-TAATGGATCCACCACCTCCCCTGACATGTCGATTCTTGG-3′ for MC54L. PCRs were performed with a Clontech Advantage-GC cDNA PCR kit, which allowed accurate amplification of GC-rich templates (Clontech, Palo Alto, Calif.). The cloned PCR products were sequenced using a model 310 automated instrument (Applied Biosystems, Foster City, Calif.).

TABLE 1. Kinetics and affinity constants of MC54L proteins[a]

Mutations[b]	Binding with hIL-18			
	K_{on} (10^4/M s)	K_{off} (10^{-4}/s)	K_d (nM)	$\Delta\Delta G°_{(mut-WT)}$ (kcal/mol)
Δ(142–173)	5.6 ± 0.9	1.0 ± 0.3	1.7 ± 0.3	0
Δ(140–235)	6 ± 1	0.9 ± 0.1	1.5 ± 0.2	−0.09
V63A	7 ± 1	1.47 ± 0.08	2.0 ± 0.4	−0.1
V63F	8 ± 2	0.12 ± 0.01	0.15 ± 0.02	−1.5
L65A	8 ± 1	2.42 ± 0.09	2.9 ± 0.4	0.3 (0.9)[c]
I69A	4.6 ± 0.6	24 ± 1	53 ± 8	2.0 (1.5)[c]
E76A	2.6 ± 0.6	17 ± 2	70 ± 30	2.2 (1.6)[c]
L78A	6 ± 1	5.1 ± 0.6	9 ± 2	1.0 (1.2)[c]
E84A	1.9 ± 0.6	6.7 ± 0.8	40 ± 10	1.8 (1.7)[c]

[a] The kinetics and affinity constants were derived from four independent experiments similar to those shown in Fig. 4. *K* values are means ± standard deviations.

[b] The single amino acid mutations were all made in the MC54LΔ(142–173) backbone. No measurement of the affinity constant was attempted for Y67A or F74A because of their poor binding with IL-18.

[c] Number in parentheses is the free energy change caused by the corresponding mutation in hIL-18BP.

RESULTS

The carboxyl-terminal half of MC54L was dispensable for IL-18 binding. The MC54L protein consists of two domains: the amino-terminal portion (residues 1 to 140) comprises an Ig-like domain with homology to hIL-18BP, whereas the carboxyl-terminal half (residues 141 to 235) contains a large number of positively charged amino acids with no sequence similarity to any known protein. When the MC54L ORF was PCR amplified under standard conditions, a repetitive and highly GC-rich sequence was consistently deleted, resulting in the removal of codons 142 to 173. As this in-frame deletion turned out to have no effect on IL-18 binding and the DNA could be faithfully amplified, it was used as the starting sequence for further mutagenesis. The strategy for purification and immobilization of proteins on BIAcore chips was similar to that described elsewhere for rhIL-18BP (37). PCR was used to fuse the MC54L ORF to a DNA fragment encoding a flexible peptide linker followed by a 15-amino-acid BirA biotinylation-recognition site and a six-histidine tag. After expression by mammalian cells, the secreted protein was bound to Ni-nitrilotriacetic acid resin and biotinylated in situ with BirA. Upon removal of excess biotin, the histidine-tagged protein was eluted with imidazole and the biotinylated species was captured on a streptavidin-coated BIAcore sensor chip. In this way, we took advantage of secretion, metal chelation, and streptavidin binding to purify the MC54L protein. The real-time association and dissociation of rhIL-18 were determined by SPR using a BIAcore apparatus. To determine affinity and kinetic constants, various concentrations of rhIL-18 were injected over the sensor chip, and the sensorgrams were globally fitted to a 1-to-1 binding model as previously described (37). Similar experiments were performed at least four times, and the kinetic and affinity constants that were obtained are listed in Table 1. MC54LΔ(142–173) bound rhIL-18 with a K_d of 1.7 nM, approximately five times lower than previously reported for the full-length MC54L protein immobilized by amine coupling (39). This increase in affinity may be due to the more defined method of immobilization through the C-terminal biotin tag or to a different source of rhIL-18, as a similar im-

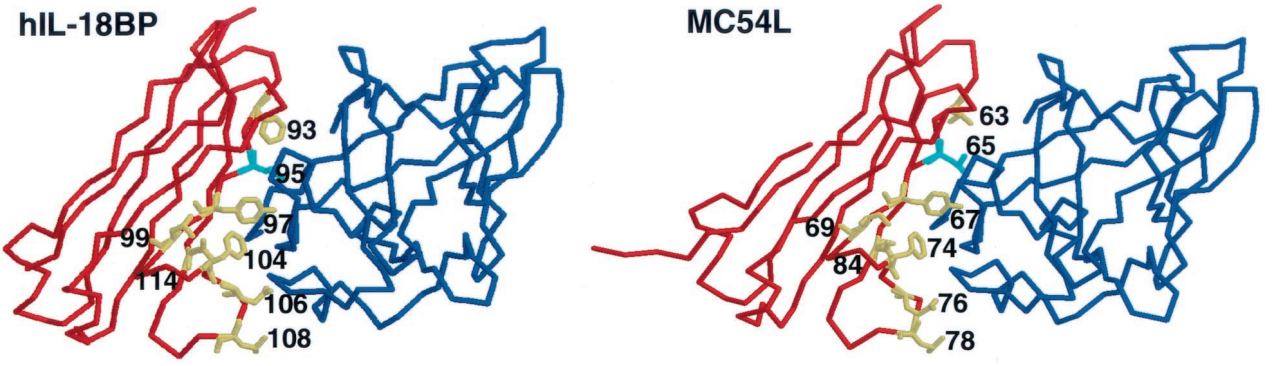

FIG. 1. Structural models of hIL-18BP and MC54L. The previously reported model of hIL-18BP interacting with IL-18 was based mostly on the X-ray structure of the complex of IL-1 and IL-1R1 (15) (left panel). The backbones of hIL-18BP and hIL-18 are red and blue, respectively. The side chains of seven amino acids of hIL-18BP that are the most important for IL-18 binding are shown in yellow, and the side chain of a control amino acid that is not important for IL-18 binding is shown in cyan. (Right panel) The model of MC54L interacting with IL-18 was constructed similarly to that of hIL-18BP. Also shown in yellow or cyan are the side chains of those MC54L residues that correspond to the hIL-18BP residues.

provement was observed for rhIL-18BP with the present protocol (37).

Using the procedures described above, we also expressed a form of the MC54L protein that had the entire carboxy-terminal half (residues 140 to 235) deleted. MC54LΔ(140–235) bound rhIL-18 almost identically to MC54LΔ(142–173) with a K_d of 1.5 nM (Table 1), indicating that the entire carboxy-terminal half of MC54L was dispensable for IL-18 binding.

IL-18 binding sites of MC54L are similar to those of hIL-18BP. A previously described structural model of the hIL-18BP interaction with hIL-18, based mostly on the crystal structure of IL-1R1 complexed with IL-1β (15), is shown in Fig. 1 (left panel). The side chains of residues 93, 97, 99, 104, 106, 108, and 114 were identified by mutagenesis as the important functional epitopes for IL-18 binding (37). Similarly, we constructed a model of the Ig domain of MC54L (Fig. 1, right panel). The side chains of residues 63, 67, 69, 74, 76, 78, and 84, corresponding to the functional epitopes of hIL-18BP, are also shown in Fig. 1. Even though MC54 and hIL-18BP have a relatively low overall sequence identity, the same amino acids were present at all but two of these seven positions. The major difference was the substitution of valine for phenylalanine at residue 63 of MC54L; at residue 69 there was a conservative substitution of isoleucine for leucine. To ascertain the validity of the structural model, we mutated the seven amino acids of MC54L individually and measured the affinities of the mutated MC54Ls with rhIL-18. As a control, we also mutagenized residue 65 of MC54L, which corresponds to an amino acid of hIL-18BP that has little effect on IL-18 binding. All site-directed mutagenesis was performed on MC54LΔ(142–173), and the mutated proteins were synthesized, purified, and biotinylated as described above. The mutated proteins were expressed at similar levels, and they had electrophoretic mobilities equivalent to that of the parental MC54LΔ(142–173) protein and

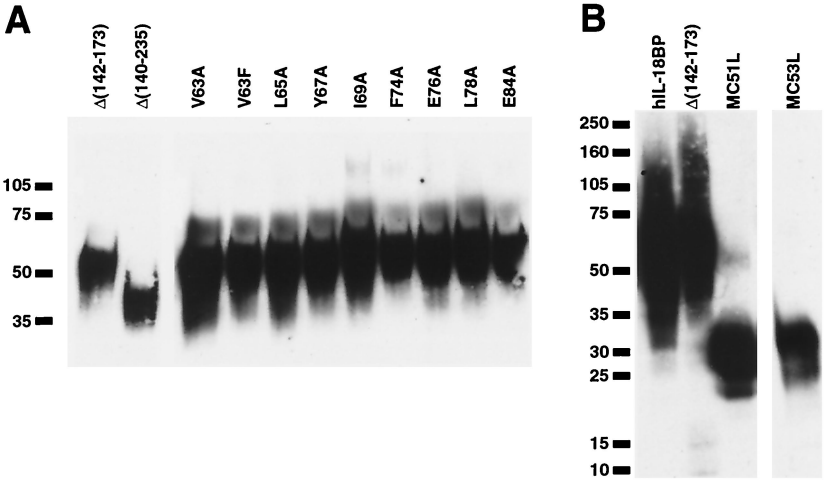

FIG. 2. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of expressed proteins. Media from cells transfected with plasmids containing the indicated mutated or wild-type MCV ORFs were incubated with Ni-nitrilotriacetic acid resin. After washing, the resin-bound proteins were biotinylated with BirA, washed, and then eluted with imidazole. The eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. (A) Biotinylated species were detected by chemiluminescence with a streptavidin-horseradish peroxidase conjugate. (B) Histidine-tagged proteins were detected with a monoclonal antibody against four consecutive histidines (Qiagen). The positions and masses in kilodaltons of the protein markers are shown on the left.

slower than that of MC154LΔ(140–235) (Fig. 2A). The broadness of the bands is consistent with the known glycosylation of MC54L (38).

The biotinylated proteins were captured on streptavidin-coated BIAcore sensor chips and the binding of rhIL-18 was measured. rhIL-18 bound rapidly to MC54LΔ(142–173) during the association phase (which reflects both on and off rates) and dissociated slowly during the dissociation phase (which reflects the off rate) (Fig. 3A). As occurred with the corresponding mutations of rhIL-18BP, alanine substitution for tyrosine 67 (Y67A) or phenylalanine 74 (F74A) caused a profound decrease in affinity for rhIL-18. Even when a high concentration of rhIL-18 was injected (111 nM), F74A and Y67A showed very little binding compared to MC54LΔ(142–173) (Fig. 3A). Indeed, the binding of rhIL-18 was too low to determine an accurate affinity constant by this technique. Thus, these two amino acids have critically important roles in both hIL-18BP and MC54L.

MC54L contains a valine in place of the important phenylalanine 93 of hIL-18BP. Although mutagenesis of phenylalanine 93 to alanine increased the K_d of rhIL-18BP for rhIL-18 by approximately 100-fold, substitution of alanine for valine 63 caused little change in binding of MC54L to rhIL-18 (Fig. 3B). However, when phenylalanine was substituted for valine, making MC54L more like hIL-18BP, a significant decrease in the rhIL-18 off rate was observed (Fig. 3B), indicating an increase in affinity. Kinetic analysis with a series of concentrations of rhIL-18 (Fig. 4; Table 1) indicated that the K_d of MC54L(V63F) for rhIL-18 was approximately 10 times lower than that of MC54LΔ(142–173) and was similar to that of rhIL-18BP.

The effects of alanine substitutions at residues 65, 69, 76, 78, and 84 were similar to the effects of corresponding substitutions in rhIL-18BP. Each mutation increased the off rate of rhIL-18 (Fig. 3C). The affinity constants, which were obtained from experiments similar to those shown in Fig. 4, ranged from 2.9 to 70 nM (Table 1). In most cases, the binding free energy changes caused by the substitutions were very close to those caused by similar substitutions in rhIL-18BP (37).

Undetectable binding of MC51L or MC53L to IL-18. The Ig domains of hIL-18BP and MC54L are 46% identical, partly due to 19 consecutive identical amino acids that lie outside of the mapped IL-18 binding site. The ectromelia and cowpox virus IL-18BP homologs have Ig domains that are about 29% identical to hIL-18BP, yet the proteins still bind IL-18 with high affinity (6, 8, 32). MC51L and MC53L encode secreted, glycosylated proteins with Ig domains that are 26 and 39% identical to that of hIL-18BP, respectively, but their binding affinities have not yet been determined (39). To investigate whether MC51L or MC53L also binds IL-18, we expressed the two proteins transiently in mammalian cells as histidine-tagged, biotinylated species (Fig. 2B) and immobilized them on streptavidin chips. While rhIL-18 bound to the control rhIL-18BP, no binding of rhIL-18 to MC51L or MC53L was detected (Fig. 5B), even though there were greater amounts of the latter proteins on the chip (Fig. 5A). When we compared MC51L and MC53L sequences with those of MC54L and hIL-18BP, we found that MC51L lacked six and MC53 lacked three of those seven amino acids that were important for IL-18 binding (Fig. 6). However, we failed to enhance IL-18 binding by inserting the critical amino acids of hIL-18BP into MC53L (data not shown), presumably because the amino acid changes were not compatible with the overall structure of the protein.

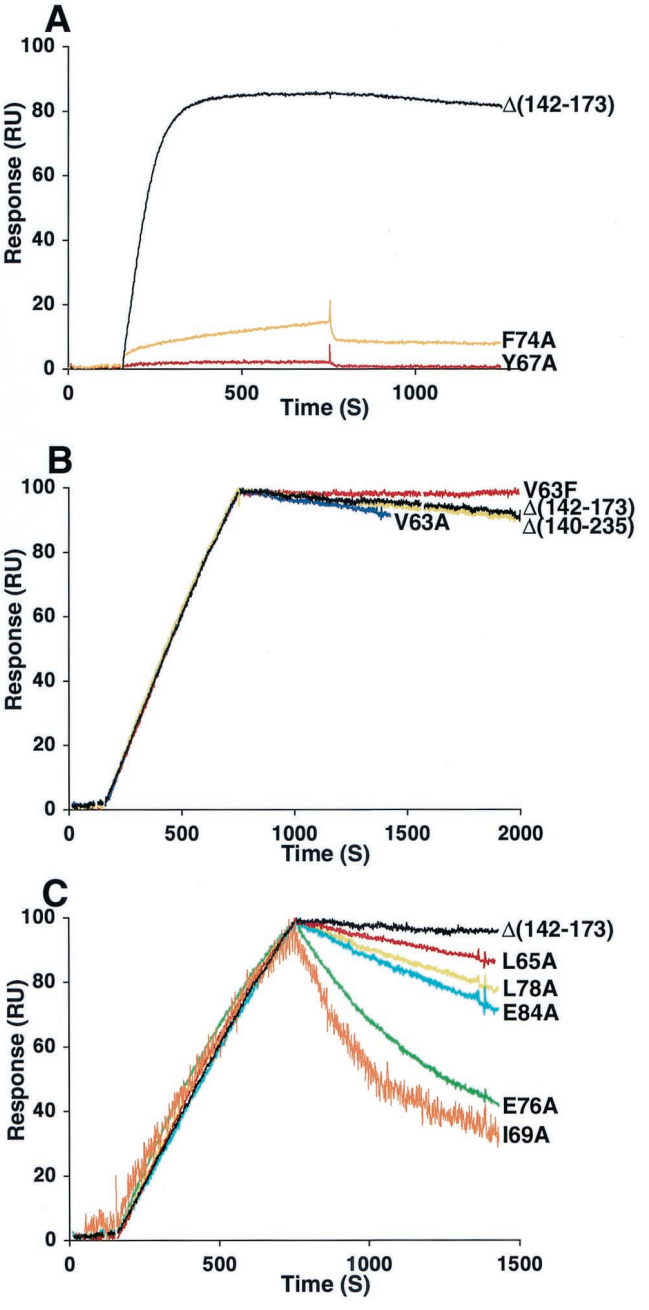

FIG. 3. Sensorgrams showing the binding of rhIL-18 to mutated MC54L proteins. The mutated proteins were expressed and purified as described in the legend for Fig. 2 and then captured on streptavidin chips. The injection of rhIL-18 started at 150 s and stopped at 750 s. The rhIL-18 concentrations used were 111 nM (A) and 14 nM (B and C). The colored lines are the responses obtained with the mutated proteins and normalized to a maximum of 100 RU in panels B and C for ease of comparison.

Sequences of MC51L, MC53L, and MC54L of different MCV strains. We considered that the occurrence of a nonoptimal valine in MC54L and the absence of a functional IL-18

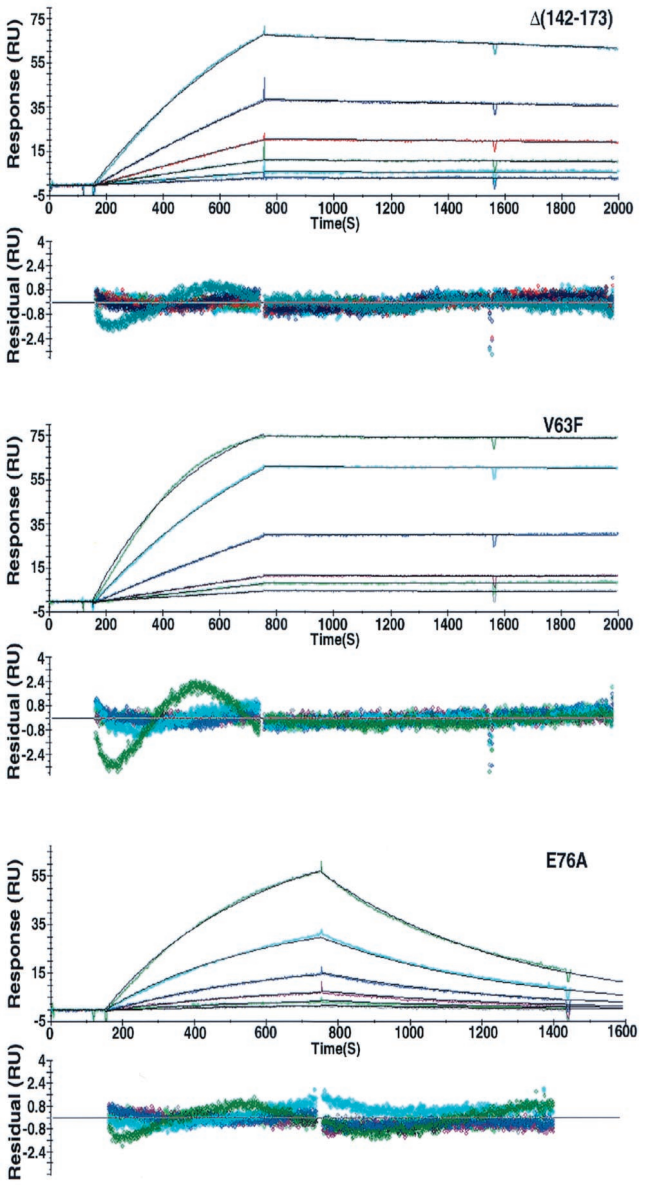

FIG. 4. Kinetic analyses of the binding of rhIL-18 to MC54L proteins. The biotinylated MC54L proteins were individually captured on streptavidin chips. Injection of rhIL-18 started at 150 s and stopped at 750 s. rhIL-18 concentrations used were 0.87, 1.7, 3.5, 6.9, 14, and 28 nM. The colored and black lines are the actual responses in RU and globally fitted curves, respectively. The residual responses, below each set of curves, represent deviations of the actual responses from the fitted curves. The root mean square deviations were 0.244 for Δ(142–173), 0.357 for V63F, and 0.276 for E76A.

binding site in MC51L or MC53L might be due to anomalies of the MCV type 1 isolate that was sequenced (24). We therefore acquired DNA from MCV type 2 and 4 strains. The MC54L sequences of the three strains were very similar, except for a 12-amino acid insertion within the C-terminal domain of the MCV-4 isolate (Fig. 7A). Significantly, the seven amino acids comprising the IL-18 binding sites of the three MC54L proteins were identical and each had the suboptimal valine at position 63.

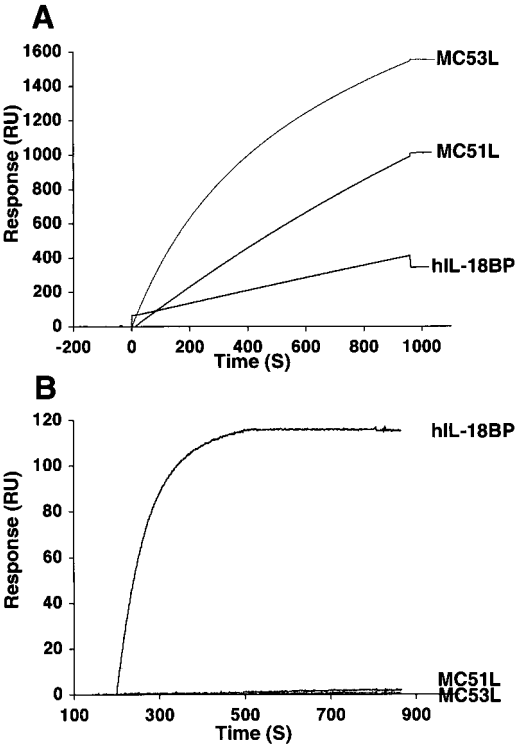

FIG. 5. MC51L and MC53L did not bind rhIL-18. (A) Capture of biotinylated MC51L, MC53L, and rhIL-18BP on a streptavidin-coated sensor chip. Biotinylated MC51L, MC53L, and rhIL-18BP were injected into flow cells starting at 0 s and stopping at 960 s. Approximately 1,550 RU of MC53L, 1,000 RU of MC51L, and 340 RU of rhIL-18BP were captured. (B) Binding of IL-18. rhIL-18 at a concentration of 111 nM was simultaneously injected to flow cells containing MC51L, MC53L, and rhIL-18BP. The injection started at 150 s and stopped at 500 s.

The MC53L sequences of the three MCV strains were also very similar, though again MCV-4 had the most variation (Fig. 7B). The important finding was that all three sequences lacked amino acid residues important for IL-18 binding. A similar result was found for the MC51L sequence of MCV-1 and MCV-2 (Fig. 7C). We failed to amplify the MC51L ORF from MCV type 4 when using primers derived from the MCV-1 sequence, presumably because of nucleotide variation at the primer binding sites.

DISCUSSION

Large DNA viruses such as poxviruses and herpesviruses subvert the host immune system by expressing proteins that antagonize the function of cellular immune defense molecules (1). In many cases, sequence homologies suggest that the viruses acquired these genes from their hosts and then modified and retained them by natural selection. In few cases, however, have mutagenesis studies been carried out on both the cellular and viral homologs to corroborate the structure-function relationships. In a previous report, we used SPR to map seven amino acids of hIL-8BP that are important for high-affinity binding to hIL-18 (37). Although the overall amino acid identity of MC54L and hIL-18BP is only 35%, model building suggested that five of the amino acids of hIL-18BP that con-

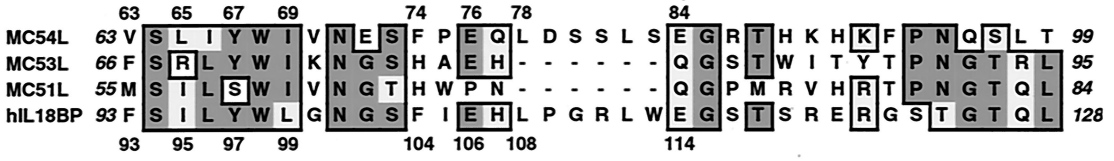

FIG. 6. Multiple sequence alignment of portions of hIL-18BP, MC54L, MC53L, and MC51L. Multiple alignment was performed with the Clustal W program in the MacVector computer software package and adjusted manually. Amino acids that are identical or similar in three or more proteins are boxed. The dark and light shading indicate amino acids that are identical or similar, respectively. The numbers above the alignment indicate the residue numbers of MC54L that were mutated in this study. The numbers underneath the alignment indicate the corresponding residue numbers of hIL-18BP.

tributed most of the binding free energy were present in MC54L. Of the two exceptions, one was a conservative substitution of isoleucine for leucine that should make little difference. The other, however, was more substantial: a valine substitution for phenylalanine. We found that alanine substitutions for the six identical or highly conserved amino acids caused very similar changes in affinity and binding free energy for MC54L and hIL-18BP. When we substituted phenylalanine for valine, the

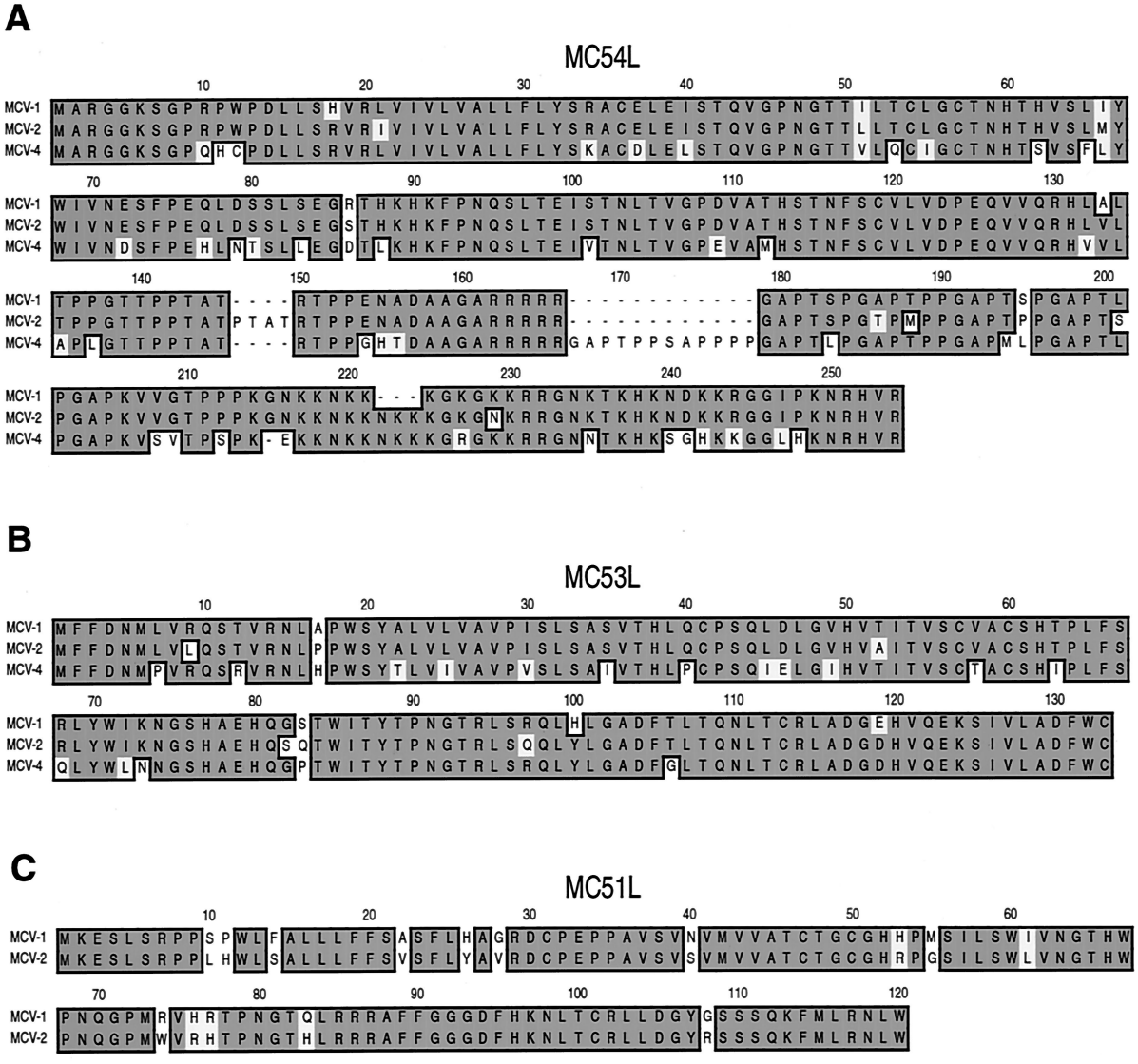

FIG. 7. Multiple sequence alignments of MC54L, MC53L, and MC51L of different MCV strains. MC51L, MC53L, and MC54L sequences from MCV type 2 and type 4 were determined and aligned with published sequences from MCV type 1. Identical or similar amino acids are boxed and indicated by dark or light shading, respectively.

binding affinity of MC54L increased 10-fold, a finding that is in accord with the frequent role of aromatic amino acids in protein-protein interactions. These results provided strong evidence that the binding interfaces of hIL-18BP and MC54L are very similar. Remarkably, outside the IL-18 binding sites there is a stretch of 19 or 24 consecutive amino acids in hIL-18BP that are identical to those in MC54L from type 1 (residues 113 to 131) or type 4 (residues 113 to 135), respectively. This amino acid stretch apparently has no role in IL-18 binding, since it is absent from the orthopoxvirus IL-18-binding proteins, and it either has another function or is an evolutionary remnant.

We have shown previously that the MC54L protein binds IL-18 with nanomolar affinity and is a potent inhibitor of IL-18-mediated IFN-γ induction (39). Nevertheless, our finding that MC54L contained a nonoptimal valine in place of phenylalanine was puzzling, since we intuitively expected a virus to encode the most potent antagonist. There are several possibilities that might explain this apparent discrepancy. Although MCV replicates exclusively in humans, MC54L could have been acquired at an earlier stage of evolution from a host that had a valine instead of a phenylalanine in the IL-18 binding site. However, even the mouse IL-18BP has phenylalanine in this position, and there is no evidence for an ancestral IL-18BP with valine. Furthermore, the orthopoxvirus IL-18BP homologs have phenylalanine, not valine, in corresponding positions. A second possibility is that the sequenced MC54L (24) was not representative of most strains of MCV. This was ruled out, however, by sequencing MC54L orthologs from MCV types 2 and 4: each had valine. Another possibility is that a less-efficient IL-18 antagonist is beneficial to MCV, as high expression of a more-potent antagonist might provoke systemic immunosuppression, which could be deleterious to the host or preferentially help other microbial invaders.

Whereas MCV is likely to have acquired the Ig domain of MC54L from its host IL-18BP, the origin and function of the C-terminal domain are mysteries. Deletion of the domain had no effect on IL-18 binding, no cellular homologs have been found in database searches, and the orthopoxvirus IL-18 binding proteins lack the domain. The C-terminal domain contains a high number of basic amino acids, and we are investigating the possibility that it binds negatively charged molecules on the cell surface. Tethering of MC54L might retain the protein at the site of infection, where it could antagonize IL-18. In this respect, the soluble orthopoxvirus complement control protein (31) and IFN-α/β receptor (2) bind to cell surfaces.

Although the overall amino acid identities of MC51L and MC53L with hIL-18BP were lower than that of MC54L, they were similar or higher than the values obtained by alignment of the orthopoxvirus homologs. Nevertheless, the orthopoxvirus homologs bound IL-18, whereas MC51L and MC53L did not. This difference can be readily explained, however, by analyzing the correspondence of the functional epitopes derived from mutagenesis of hIL-18BP and MC54L. Whereas the orthopoxvirus homologs contain all seven optimal amino acids, MC51L and MC53L lack three and six amino acids, respectively. The situation is more complicated, however, because we could not confer IL-18 binding to MC53L either by amino acid substitutions or by making hybrid proteins (unpublished data). Thus, if MC51L and MC53L were derived from an IL-18-binding protein, they have undergone considerable structural alterations. An intriguing idea is that these MCV proteins bind another ligand—possibly another member of the IL-1 family. Although we could not demonstrate binding of MC51L or MC53L to IL-1α or IL-1β, four new members of the IL-1 family recently discovered have not yet been tested (30).

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