The human interleukin (IL)-18-binding protein (hIL-18BP) is a naturally occurring antagonist of IL-18, a proinflammatory cytokine that is related to IL-1β and has an important role in defense against microbial invaders. As its name implies, the hIL-18BP binds to IL-18 with high affinity and prevents the interaction of IL-18 with its receptor. We genetically modified the C terminus of hIL-18BP by appending a 15-amino acid biotinylation recognition site and a six-histidine tag and then performed site-directed mutagenesis to determine the functional epitopes that mediate efficient binding to IL-18. The mutated IL-18BPs were secreted from mammalian cells, captured by metal affinity chromatography, and most had hydrophobic side chains. Just two amino acids, tyrosine 97 and phenylalanine 104, contributed ~50% of the binding free energy. Information obtained from these studies could contribute to the design of molecular antagonists of IL-18 for treatment of inflammatory diseases.

EXPERIMENTAL PROCEDURES

Construction of the Fusion Vector—For other purposes, we had appended a DNA segment encoding the biotinylation site and a six-histidine tag to the 3′-end of the molluscum contagiosum virus MC51L open reading frame. This was accomplished by polymerase chain reaction (PCR) using DNA containing the MC51L open reading frame as a template (19) and the primers 5′-GGATCC-TAAATGGATGATGGTGATGG-3′ and 5′-GGATCC-TTAATGGATGATGGTGATGGTGTTCGTGCCATTC-GATTGTTGTTCGAGATGTTGCGTGCAGCCCCAGGTTTCT-CAACAT-3′. The PCR product was ligated to the pTarget TA cloning vector (Promega) to form pYX26. To recover the DNA containing the biotinylation site and six-histidine tag and to add a flexible linker GGSGGGS in front of the biotinylation site GLNDIFEAQKIEWHE, another PCR was made using pYX26 as the template with primers 5′-ATAGGATCCGGGGGAAGGGTGGTGGCTTGGCTTGAGCACAGCAT-CTT-3′ and 5′-CGGATCCCTCTGAGTATGCGTTCGACG-3′. The PCR product 5′-ATTAGGATCCGGGGGAAGGGTGGTGGCTTGGCTTGAGCACAGCATCTTCTCTGGTCTTCACAGAAAGTCTGAGGAATTCGATGATGGTGATGGTGTTCGTGCCATTC-GATTGTTGTTCGAGATGTTGCGTGCAGCCCCAGGTTTCT-CAACAT-3′ was amplified by PCR with the pTarget TA cloning vector (Promega) to form pYX26. To recover the DNA containing the biotinylation site and six-histidine tag and to add a flexible linker GGSGGGS in front of the biotinylation site GLNDIFEAQKIEWHE, another PCR was made using pYX26 as the template with primers 5′-ATAGGATCCGGGGGAAGGGTGGTGGCTTGGCTTGAGCACAGCAT-CTT-3′ and 5′-CGGATCCCTCTGAGTATGCGTTCGACG-3′. The PCR product 5′-ATTAGGATCCGGGGGAAGGGTGGTGGCTTGGCTTGAGCACAGCATCTTCTCTGGTCTTCACAGAAAGTCTGAGGAATTCGATGATGGTGATGGTGTTCGTGCCATTC-GATTGTTGTTCGAGATGTTGCGTGCAGCCCCAGGTTTCT-CAACAT-3′ was amplified by PCR with the pTarget TA cloning vector (Promega) to form pYX26. To recover the DNA containing the biotinylation site and six-histidine tag and to add a flexible linker GGSGGGS in front of the biotinylation site GLNDIFEAQKIEWHE, another PCR was made using pYX26 as the template with primers 5′-ATAGGATCCGGGGGAAGGGTGGTGGCTTGGCTTGAGCACAGCATCTT-3′ and 5′-CGGATCCCTCTGAGTATGCGTTCGACG-3′. The PCR product 5′-ATTAGGATCCGGGGGAAGGGTGGTGGCTTGGCTTGAGCACAGCATCTTCTCTGGTCTTCACAGAAAGTCTGAGGAATTCGATGATGGTGATGGTGTTCGTGCCATTC-GATTGTTGTTCGAGATGTTGCGTGCAGCCCCAGGTTTCT-CAACAT-3′ was amplified by PCR with the pTarget TA cloning vector (Promega) to form pYX26.

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1 The abbreviations used are: IL, interleukin; hIL-18BP, human IL-18 binding protein; IL-1R1, type 1 IL-1 receptor; PCR, polymerase chain reaction; rmIL-18, recombinant murine IL-18; RU, response units; rhIL-18, recombinant human IL-18.

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Multiple alignment of the Ig domains of hIL-18BP, MC54L, evp16, and IL-1R1. The multiple alignment was performed with the ClustalW program in the MacVector computer software package and adjusted manually. Amino acids that are identical or similar in three or more positions are boxed. The dark and light shading indicates amino acids that are identical or similar, respectively. The lines and the characters underneath the alignment indicate the locations and the names of the β-strands in IL-1R1 crystal structure. The numbers above the alignment indicate the residue numbers of hIL-18BP which are mutated in this study.
the selected amino acid was changed to alanine. Additional mutations were made at residues 114 and 130, the only two predicted by the structural model to participate in electrostatic interactions; one substitution maintained the same kind of charge, another changed it to the opposite charge, and the third changed it to a polar residue. Mutations at residue 93, 95, 97, 99, 104, 106, 107, 108, 112, and 114 also contained a conservative threonine to serine substitution at residue 103 which was introduced to facilitate PCR mutagenesis.

Expression and in Vitro Biotinylation of hIL-18BPs—Because of the high affinity between hIL-18BP and hIL-18 and the predicted minimal structural perturbation of alanine substitutions, the sensitive and quantitative method of surface plasmon resonance was used to determine binding constants. The hIL-18BPs were expressed by mammalian cells as secreted, glycosylated, C-terminal fusion proteins with a flexible linker, followed by a 15-amino acid biotin holoenzyme synthetase biotinylation recognition site and a six-histidine tag (Fig. 2A). The secreted hIL-18BPs were captured on Ni-nitrilotriacetic acid resin via the six-histidine tag. While on the resin, the proteins were biotinylated at a specific lysine by biotin holoenzyme synthetase (24) and then washed and eluted with imidazole. The only biotinylated protein detected after this purification step ran as a broad 50-kDa band on SDS-polyacrylamide gel electrophoresis (Fig. 2C), consistent with the size and glycosylation state of hIL-18BP. This procedure had a number of advantages over previous methods because no dialysis step was necessary before the biotinylation reaction, and excess biotin, which could interfere with binding to the streptavidin chip, was removed by washing prior to elution. Approximately 90% of the protein was biotinylated by this procedure.

Surface Plasmon Resonance Analysis of IL-18 Binding to Mutated hIL-18BPs—hIL-18BPs were captured individually on streptavidin chips, and the real time association and dissociation of rhIL-18 and rmIL-18 were monitored in a BIAcore 2000 sensor. Sensorgrams for all proteins with single amino acid substitutions were obtained by injecting 14 nm rhIL-18, and the responses were normalized to a maximum of 100 RU. As reported previously (17), the binding of rhIL-18 to unmutated hIL-18BP was characterized by a rapid association phase (which is affected by both on- and off-rates) and an extremely small decrease in response during the dissociation phase (which is only affected by the off-rate) (Fig. 3A). The association phases of the sensorgrams of all of the mutated proteins with single substitutions were similar to each other (Fig. 3, A–E). By contrast, the dissociation phases of several of the mutated proteins differed from unmutated hIL-18BP. Alanine substitutions of some of the selected individual residues on the predicted β-strand c and d caused increased rates of dissociation (Fig. 3, A–D). Among these, substitution of the tyrosine at residue 97 or the phenylalanine at residue 104 caused the largest change. Alanine substitutions at residues 93, 99, 106, 108, or 114 caused smaller but significant increases in the off-rates (Fig. 3, A–D). Outside of the predicted c and d strands, no single mutation was found to affect the binding significantly (Fig. 3E).

Of the seven residues that were shown to affect binding, only two (106 and 114) had charged side chains, whereas the rest had hydrophobic side chains. The effects of mutations at residues 106 and 114 on IL-18 binding were relatively small compared with mutations at 97 and 104. When the charged residues 107, 119, 121, or 130 were mutated, there was little effect on binding even though residue 130 was predicted to interact with hIL-18 (18). In an effort to assess whether the charged amino acids as a group might contribute to IL-18 binding, we constructed four sets of mutations that combined several single
amino acid substitutions. Mutants c1, c2, c3, and c4 each had four residues substituted with alanine. They were residues 106, 114, 119, and 130 for mutant c1; residues 107, 114, 121, and 130 for mutant c2; residues 106, 114, 121, and 130 for c3; and residues 107, 114, 119, and 130 for c4. Sensorgrams for these multiply substituted proteins, with the exception of c1, were also obtained by injecting 14 nM rhIL-18. Because c1 bound to rhIL-18 very poorly, 111 nM rhIL-18 was injected. Except for c1, which cannot be compared with the others, the association phases were similar to the unmutated hIL-18BP. There were, however, differences in the dissociation phases. Residues at 107, 121, and 130, as a group, contributed very little to IL-18 binding because mutant c2 only had a small increase in the dissociation rate compared with that of the E114A mutant.

For kinetic analysis, sensorgrams that were obtained with various concentrations of rhIL-18 and rmIL-18 were globally fitted with BIAEVALUATION software to a 1-to-1 binding model, in which one molecule of IL-18 binds with one molecule of hIL-18BP. Both the actual data and fitted curve are shown for the mutated proteins with alanine substitutions at amino acids 93 and 97 (Fig. 4). Similar experiments were performed at least four times with each of the mutated proteins, and the kinetic and affinity constants that were obtained are listed in Table I. The relative changes of Gibbs free energy change (ΔΔG) caused by the mutations are also shown in the table. Alanine substitution of the tyrosine at residue 97 or the phenylalanine at residue 104 increased the Kd more than 100-fold above that of the unmutated hIL-18BP and together contributed to ~50% of the binding energy. Alanine substitution of residue 93 increased the Kd more than 50-fold and contributed about 15% of the binding energy. In addition, substitutions of residues 99, 106, 108, and 114 all increased the Kd by more than 5-fold. The substitution of lysine or glutamine for aspartic acid 114 caused a greater change than alanine in the Kd.

DISCUSSION
Crystal structures of protein-protein complexes usually reveal a large interface, suggesting the involvement of many side chains that are collectively called structural epitopes. However, the number of side chains actually shown to be important for binding by alanine mutagenesis is usually small. The latter amino acids are called the functional epitopes. We mutated 15 residues in the hIL-18BP which either had been predicted to be at the interface with IL-18 based on the crystal structure of the related IL-1β-IL-1R1 complex (18) or were conserved in Ig domains of hIL-18BP and two viral IL-18BP homologs (Fig. 5). When replaced by alanine, four of the mutated hIL-18BPs exhibited more than a 5-fold decrease in binding affinity with IL-18, and three exhibited more than a 50-fold decrease. The effects on binding caused by the single alanine substitutions are unlikely to have been caused by global changes in IL-18BP structure for the following reasons. Alanine scanning mutagenesis has been used successfully to probe many protein-protein interfaces. Statistically, alanine is not a β-strand breaker and is found in the middle of β-strands a, c, f, and g of the IL-1R1 domain 3. In fact, most of the alanine substitutions in IL-18BP

FIG. 3. Sensorgrams showing the binding of mutated hIL-18BPs to hIL-18. The mutated hIL-18BPs were biotinylated and captured individually on streptavidin chips. The injection of rhIL-18 started at 150 s and stopped at 750 s. The rhIL-18 concentration used was 14 nM in all cases except c1, where it was 111 nM. The colored lines are the responses obtained with the mutated hIL-18BPs and normalized to a maximum of 100 RU. A and B, single substitution at positions corresponding to strand c of IL-1R1. C and D, single substitution at positions corresponding to strand d of IL-1R1. E, single substitution at all other positions. F, multiple substitutions of charged amino acids.
caused small changes in affinity, and those that produced large changes caused a small or no effect on the on-rate. Furthermore, with the c2 and c4 mutations, the binding free energy changes were almost exactly the sum of free energy changes caused by the four individual substitutions. This result suggested that the mutations did not cause major structural changes and that the residues 107, 114, 119, 121, and 130 interact independently with IL-18. With the c1 and c3 mutations, the binding free energy changes were somewhat greater than the sums of those caused by the individual substitutions. However, because c1 and c3 bound poorly to IL-18, the surface plasmon resonance data did not have as good a fit to the 1-to-1 binding model as for the other mutations. We constructed structural models for each mutated hIL-18BP based on the published wild type hIL-18BP structural model (18). After energy minimization of the mutated hIL-18BP structure models, the root mean square deviations of the entire structure from the wild type structure were calculated (data not shown). There was little difference in the deviations between those mutations that caused more than a 5-fold change in affinity and those that did not, consistent with the absence of large structural perturbations caused by alanine mutagenesis.

The sequence alignment and the results of our binding data together suggest that the functional epitopes cluster in β-strands c and d of the Ig domain and point their side chains outward. Residues 93–99 of hIL-18BP were confidently predicted by the PhD program (25) to form a β-strand, and identical or conservative amino acids are present in corresponding positions of the MC54L and evp16 IL-18BPs. In addition, these sequences align very well with the sequences that form the β-strand c of IL-1R1 domain 3. Like the c-strand of nearly all Ig domains, a tryptophan is found at position 98 of hIL-18BP and at the corresponding positions of MC54L, evp16, and IL-1R1. In all of the Ig domain structures examined, this conserved tryptophan points its side chain inward, which is thought to be crucial for folding (26). As a result, residues 93, 95, 97, and 99 would point their side chains outward in a β-strand. This is consistent with our finding that residues 93, 97, and 99 all contributed significantly to binding, with residue 97 contributing around 25% of the binding free energy. A model, based on the known structure of the complex of IL-1α and IL-1R1, predicted that amino acids 93–99 of IL-18BP are in the β-strand and that residues 93, 95, and 97 would be important for binding to IL-18 (Fig. 5). Our results minimized the role of residue 95.

Compared with the confidence in predicting the locations of residues 93, 97, and 99 in a three-dimensional structure, the locations of residues 104, 106, 108, and 114 are less certain. This is because different Ig domains vary considerably in the length and the sequences of amino acids between the c- and e-strands (27). In our multiple alignment of the Ig domains of IL-18BPs and IL-1R1 domain 3, there is a gap of seven amino acids between residue 103 and 104 of hIL-18BP. This gap was created because the sequences before and after the gap are similar among the three IL-18BPs and because the additional seven amino acids in evp16 and IL-1R1 share three identical residues. This gap suggests that the β-turn between the c- and e-strands of IL-1R1 domain 3 is maintained in evp16 but is shortened in hIL-18BP and MC54L. Residues 103–107 and 111–115 of hIL-18BP were also predicted by the PhD program to form β-strands. We suggest, therefore, that the d-strand of hIL-18BP includes residues 103–115. This strand may be broken in the middle by proline at 109 and glycine at 110. This is similar to the e-strand of IL-1R1 domain 3, which has a proline in the middle of the strand which breaks the strand into two smaller but parallel strands. Residues 104, 106, 108, and 114 of the hIL-18BP all contribute significantly to the binding, suggesting that they face outward, whereas residue 107 does not. Our assignment of residues 104, 106, and 108 to the d-strand is different from the previous prediction (18). In that model, residues 102–110 form a β-turn that is similar in size to the β-turn between the c- and d-strands of IL-1R1 domain 3; consequently, residues 106 and 108 are located further away from the interface with IL-18 and would not seem capable of making contact with IL-18 (Fig. 5).

Phenylalanine 93, tyrosine 97, and phenylalanine 104 together contributed ~65% of the free energy for binding of hIL-18BP to IL-18. This is similar to the situation for human growth hormone binding to its receptor in which two tryptophans contribute three-quarters of the binding free energy (28). Such “hot spots” in protein-protein interactions frequently involve tryptophan, tyrosine, and arginine (29). Phenylalanine is also involved, although with lower frequency. Tryptophan and tyrosine can participate in hydrogen bonding, π-interactions, and van der Waals interactions, accounting for their important role in protein-protein interactions. Phenylalanine can participate in π- and van der Waals interactions but not hydrogen bonds. The tendency of hot spot residues to be at the center of a protein-protein interface reinforces the predicted locations of residues 93 and 97 in the c-strand and residue 104 in the d-strand. Our finding that a small number of residues of hIL-18BP contributed the majority of the binding free energy suggests that a small molecule mimicking the relative small func-

**Fig. 4.** Kinetic analyses of the binding of IL-18BPs with alanine substitutions at amino acid 93 or 97. The biotinylated hIL-18BPs were captured individually on streptavidin chips. Injection of rhIL-18 started at 150 s and stopped at 750 s. For the hIL-18BP with alanine substitution at position 93, rhIL-18 concentrations were 0.22, 0.43, 0.87, 1.7, 3.5, 6.9, 14, and 28 nM. For the hIL18BP with alanine substitution at position 97, rhIL-18 concentrations were 1.7, 3.5, 6.9, 14, 28, 56, and 111 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 deviation were 0.0853 for alanine 93 and 0.108 for alanine 97.
|                 | hIL-18 |                | mL-18 |                |
|----------------|--------|----------------|-------|----------------|
|                | $K_m$  | $K_{off}$      | $K_d$ | $\Delta G^2_{mut/WT}$ | $K_m$  | $K_{off}$      | $K_d$ | $\Delta G^2_{mut/WT}$ |
|                | $10^4$/ms | $10^{-4}$/s | nM    | kcal/mol    | $10^5$/ms | $10^{-5}$/s | nM    | kcal/mol    |
| WT*            | 9 ± 3   | 0.05 ± 0.01   | 0.06 ± 0.03 | 0           | 7.96 ± 0.08 | 0.16 ± 0.01 | 0.019 ± 0.001 | 0 |
| F93A           | 3 ± 2   | 0.9 ± 0.05    | 0.5 ± 0.3   | 2.6         | 7 ± 1       | 0.5 ± 0.1   | 0.08 ± 0.003 | 0.8       |
| I95A           | 7.7 ± 0.5 | 0.2 ± 0.1   | 0.3 ± 0.1   | 0.9         | 2.3 ± 0.7   | 24 ± 1      | 11 ± 3       | 3.7       |
| Y97A           | 4 ± 1   | 19 ± 1        | 45 ± 11     | 3.9         | 7 ± 1       | 0.92 ± 0.08 | 0.14 ± 0.03  | 1.1       |
| L99A           | 8 ± 2   | 0.59 ± 0.06   | 0.7 ± 0.2   | 1.5         | 6 ± 2       | 20 ± 3      | 6 ± 1        | 3.1       |
| F104A          | 10 ± 5  | 9 ± 1         | 11 ± 5      | 3.1         | 12.0 ± 0.6  | 1.31 ± 0.01 | 0.11 ± 0.01  | 1.0       |
| E106A          | 11 ± 1  | 0.91 ± 0.07   | 0.9 ± 0.2   | 1.6         | 10.0 ± 0.8  | 0.19 ± 0.02 | 0.019 ± 0.002 | -0.03     |
| H107A          | 12 ± 2  | 0.19 ± 0.02   | 0.17 ± 0.04 | 0.60        | 7 ± 2       | 0.46 ± 0.05 | 0.07 ± 0.02  | 0.76      |
| L108A          | 7 ± 1   | 0.34 ± 0.05   | 0.5 ± 0.2   | 1.2         | 5 ± 1       | 0.19 ± 0.03 | 0.04 ± 0.01  | 0.4       |
| L112A          | 6 ± 2   | 0.09 ± 0.04   | 0.14 ± 0.02 | 0.50        | 6 ± 2       | 0.8 ± 0.2   | 0.13 ± 0.02  | 1.1       |
| E114A          | 5 ± 2   | 0.5 ± 0.1     | 1.2 ± 0.8   | 1.7         | 5 ± 1       | 0.7 ± 0.1   | 0.13 ± 0.01  | 1.1       |
| E114D          | 4 ± 2   | 0.38 ± 0.06   | 1.2 ± 0.7   | 1.7         | 4 ± 2       | 0.72 ± 0.07 | 0.6 ± 0.2    | 1 ± 1      |
| E114K          | 5 ± 3   | 1.72 ± 0.07   | 6 ± 6       | 2.8         | 4 ± 2       | 2.72 ± 0.02 | 1 ± 1        | 2.4       |
| E114Q          | 3 ± 2   | 2.0 ± 0.1     | 10 ± 7      | 3.0         | 4 ± 2       | 0.32 ± 0.03 | 0.13 ± 0.03  | 0.056     |
| R119A          | 7 ± 4   | 0.10 ± 0.07   | 0.2 ± 0.1   | 0.62        | 7 ± 2       | 0.19 ± 0.03 | 0.04 ± 0.01  | 0.4       |
| R121A          | 7 ± 3   | 0.04 ± 0.03   | 0.04 ± 0.02 | -0.22       | 11 ± 1      | 0.15 ± 0.03 | 0.014 ± 0.002 | -0.21     |
| K130A          | 5 ± 3   | 0.05 ± 0.03   | 0.10 ± 0.02 | 0.31        | 8 ± 2       | 0.17 ± 0.01 | 0.022 ± 0.004 | 0.06      |
| K130T          | 7 ± 3   | 0.05 ± 0.02   | 0.09 ± 0.08 | 0.23        | 8 ± 2       | 0.11 ± 0.02 | 0.016 ± 0.006 | -0.13     |
| K130R          | 4.5 ± 0.3 | 0.04 ± 0.02 | 0.10 ± 0.05 | 0.26        | 8 ± 3       | 0.13 ± 0.03 | 0.02 ± 0.01  | 0.056     |
| K130E          | ND*    | ND            | ND          | ND          | ND          | ND          | ND          | ND        |
| V151A          | 6 ± 1   | 0.05 ± 0.02   | 0.08 ± 0.05 | 0.22        | 6 ± 2       | 0.06 ± 0.04 | 0.011 ± 0.007 | -0.37     |
| P153A          | 5 ± 2   | 0.11 ± 0.04   | 0.25 ± 0.07 | 0.83        | 6 ± 2       | 0.06 ± 0.02 | 0.035 ± 0.007 | 0.34      |
| c1(E106A,E114A,R119A,K130A) | 2 ± 1 | (6 ± 2) × 10 | (9 ± 16) × 100 | 5.7 (4.2) | 3.1 ± 0.2 | 84 ± 2 | 27 ± 2 | 4.3 (2.0) |
| c2(H107A,E114A,R121A,K130A) | 3.0 ± 0.9 | 1.5 ± 0.3 | 6 ± 3 | 2.7 (2.4) | 6 ± 1 | 0.6 ± 0.2 | 0.11 ± 0.01 | 1.0 (0.9) |
| c3(E106A,E114A,R121A,K130A) | 1.2 ± 0.4 | 10 ± 1 | (9 ± 3) × 10 | 4.3 (3.4) | 5 ± 2 | 6.8 ± 0.2 | 1.5 ± 0.5 | 2.6 (2.0) |
| c4(H107A,E114A,R119A,K130A) | 3.5 ± 0.6 | 7 ± 1 | 20 ± 7 | 3.4 (3.3) | 7 ± 1 | 3.8 ± 0.6 | 0.54 ± 0.01 | 2.0 (1.6) |

* WT, wild type.

* ND indicates binding not detected.

* For c1, c2, c3 and c4, the $\Delta G$ values listed in the parentheses are the predicted $\Delta G$ values assuming each single substitution acts independently.
Functional Epitopes of Human IL-18-binding Protein

Figure 5. Locations of the mutated hIL-18BP residues in a predicted hIL-18BP/hIL-18 structure model. The model was reported previously (18) and was based on the x-ray structure of IL-1β/IL-1R1 complex. The backbone of hIL-18BP is red, and the backbone of hIL-18 is blue. The side chains of all of the hIL-18BP residues that are mutated are also shown. Those residues that cause more than 5-fold change in binding affinities when mutated to alanine are yellow, and those that cause less than 5-fold change in binding affinities when mutated are cyan.

Functional epitopes may be possible. One starting point for such an antagonist is a peptide comprising the c- and d-strands of hIL-18BP which have been constrained so as to bring them together side by side. Alternatively, a phage display library containing a C-terminal tail that included a flexible linker, a biotin holoenzyme synthesized, and biotinylated after which the hIL-18BP was eluted with imidazole and directly bound to a streptavidin-coated sensor chip. The coupling of the recombinant protein to the chip surface via a single biotin attached to a flexible tail should allow a uniform protein conformation and minimize steric hindrance to ligand binding. It is possible that this improvement was responsible for the $K_d$ of 0.06 nM obtained here compared with the higher value of 0.4 nM reported previously using protocols in which nonspecific amine coupling was used to attach hIL-18BP (17) or rhIL-18 (18) to the chip.

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