Identification of Critical Residues in Bovine IFNAR-1 Responsible for Interferon Binding*

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Interferons have antiviral, antigrowth and immunomodulatory effects. The human type I interferons, IFN-α, IFN-β, and IFN-ω, induce somewhat different cellular effects but act through a common receptor complex, IFNAR, composed of subunits IFNAR-1 and IFNAR-2. Human IFNAR-2 binds all type I IFNs but with lower affinity and different specificity than the IFNAR complex. Human IFNAR-1 has low intrinsic binding of human IFNs but strongly affects the affinity and differential ligand specificity of the IFNAR complex. Understanding IFNAR-1 interactions with the interferons is critical to elucidating the differential ligand specificity and activation by type I IFNs. However, studies of ligand interactions with human IFNAR-1 are compromised by its low affinity. The homologous bovine IFNAR-1 serendipitously binds human IFN-ω with nanomolar affinity. Exploiting its strong binding of human IFN-ω, we have identified residues important for ligand binding. Mutagenesis of any of five aromatic residues of bovine IFNAR-1 caused strong decreases in ligand binding, whereas mutagenesis of proximal neutral or charged residues had smaller effects. These residues were mapped onto a homology model of IFNAR-1 to identify the ligand-binding face of IFNAR-1, which is consistent with previous structure/function studies of human IFNAR-1. The topology of IFNAR-1/IFN interactions appears novel when compared with previously studied cytokine receptors.

The type I interferon receptor, IFNAR, mediates the binding of all type I interferons (IFNs), which, in the human, derive from genes for 13 IFN-αs, one IFN-β, and one IFN-ω (1–6). The type I IFNs produce differential activation of genes and cellular activities (7–10). This may arise from differential binding to the receptor or differences in receptor subunit recruitment by the various type I IFNs. IFNAR consists of two cloned subunits denoted IFNAR-1 and IFNAR-2. On its own, human IFNAR-2 (HuIFNAR-2) has moderate intrinsic affinity for the range of human IFNs (HuIFNs) (11–17), whereas its partner, human IFNAR-1 (HuIFNAR-1), alone binds IFNs weakly (Kₐ = 10⁻⁷ M) (6, 11, 13–22). However, IFNAR-1 plays an essential role by contributing to both the final high affinity and the differential specificity of the IFNAR complex (11, 23) in addition to its role in signaling (24–26).

IFNAR-1 and IFNAR-2 are members of the cytokine receptor superfamily. All shared conserved structural fibronectin type III (FNIII) “building blocks” forming the extracellular ligand-binding domain (27–29). Therefore, much can be learned about IFNAR-1 and IFNAR-2 from the structurally well-defined related receptors, including the interferon-γ receptor (IFNGR-1) (30, 70), human growth hormone receptor (31–34), tissue factor (35, 36), and erythropoietin receptor (37). In the cases of IFNGR-1, IFNGR-2, growth hormone receptor, tissue factor, and IFNAR-2 there are two FNIII domains, each containing ~100 amino acids with seven β-strands and connecting loops. The extracellular domain of IFNAR-1 is atypical, consisting of a tandem array of four FNIII domains, here denoted subdomains 1 through 4 (SD1–4; beginning from the N terminus). The four-domain structure of IFNAR-1 appears to represent a tandem duplication of the more common two-domain structure (27, 38, 39).

The low intrinsic affinity of HuIFNAR-1 for IFNs has hampered studies seeking to identify residues involved in ligand binding and specificity. Previously, the identity of elements of the ligand binding site of HuIFNAR-1 could only be deduced indirectly from studies involving antibody epitope mapping (40–42) or homology modeling based on other cytokine receptors (40, 43). Without large-scale mutagenesis and ligand binding analysis, key residues could not be identified in HuIFNAR-1.

The bovine IFNAR-1 homologue is an attractive target for mutagenesis and analysis of the IFN binding site. Although the type I interferons are predominantly species-specific, human interferons display uniformly high binding and biological activity on bovine cells (3, 44–46). This appears to reflect the ability of bovine IFNAR-1 (BoIFNAR-1) to bind human type I IFNs with moderately high affinity. Thus, human or murine cells expressing BoIFNAR-1 greatly increase their responsiveness to a variety of human type I IFNs (18, 47, 48). The nanomolar binding affinity of BoIFNAR-1 for HuIFN-ω provides an elegant way to circumvent difficulties in the studies of the human IFN type I receptor complex (47–50).

BoIFNAR-1 cDNAs were cloned by independent laboratories (47, 48) and found to encode a transmembrane protein of 560 amino acids. The protein consists of a 24-amino acid signal sequence, a 414-amino acid extracellular domain, a 24-amino

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The abbreviations used are: IFNAR, type I interferon receptor; IFN, interferon; HuIFN, human IFN; IFNGR-1, interferon-γ receptor; SD1–4, subdomains 1 through 4; BoIFNAR, bovine IFNAR; DMEM, Dulbecco’s modified Eagle’s medium; SOE, splice overlap extension; HA, hemagglutinin; PBS, phosphate-buffered saline; mAb, monoclonal antibody.
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acid transmembrane sequence, and a 98-amino acid cytoplasmic domain. The human and murine IFNAR-1 proteins share 68 and 46% amino acid sequence identity, respectively, with BoIFNAR-1 (6, 48, 51). This high sequence identity, combined with an overall structural conservation, has allowed us to use BoIFNAR-1 in place of HuIFNAR-1 in studies directed at identifying structural features contributing to HuIFN ligand binding.

The ability of BoIFNAR-1 to bind HuIFNs in the nanomolar range has been well characterized using a variety of systems (18, 23). COS-1 cells transfected with BoIFNAR-1 cDNA express very high levels (0.5–1.0 × 10^10/cell) of nanomolar affinity binding sites for HuIFN-α2a, -αβ8, -αβ1b, -β, and -ω (11, 48, 49). Consistent with these results, a soluble BoIFNAR-1/Fc fusion protein bound HuIFN-α2a with an affinity of at most 10 nM (the true affinity is expected to be closer to a Kd of 0.14 nM (23)). All human type I IFNs tested were able to compete with HuIFN-α2a for binding to BoIFNAR-1/Fc, although they varied in affinity. Thus, the extracellular domain of BoIFNAR-1 by itself displays moderate affinity and differential binding of a broad range of human type I IFNs.

Previously, we used BoIFNAR-1 to regionally localize the determinants that confer strong binding of IFN (49). A series of 14 HuIFN-1/BoIFNAR-1 chimeric receptors representing various human/bovine subdomain substitutions were assayed for their ligand binding properties. Only when the two central domains of BoIFNAR-1 (SD2 and SD3) were simultaneously substituted into the HuIFNAR-1 was a significant increase in the binding of HuIFN-α2a measured over the low affinity binding characteristic of HuIFNAR-1, indicating that ligand binding was focused in this region. The BoIFNAR-1 N-terminal (SD1) and membrane-proximal (SD4) domains each further enhanced binding when transferred with SD2 and SD3 into HuIFNAR-1 (49). Thus, the IFN binding site is not localized on one FNIII subdomain or even on one tandem pair of FNIII subdomains, as previously predicted from other cytokine family members (43); instead the ligand-binding determinants of IFNAR-1 appear to be distributed in a more complex array centered on SD2 and SD3. Kumaran et al. (52) used murine/human IFNAR-1 hybrids to confirm that SD1 plays at most a minor role in species-specific ligand binding.

The current study of BoIFNAR-1 identifies amino acids that are critical for IFN binding, using a series of alanine substitutions throughout its extracellular domain. By presenting these determinants that confer strong binding of IFN (49). A series of various human/bovine subdomain substitutions were assayed for their ligand binding properties. Only when the two central domains of BoIFNAR-1 (SD2 and SD3) were simultaneously substituted into the HuIFNAR-1 was a significant increase in the binding of HuIFN-α2a measured over the low affinity binding characteristic of HuIFNAR-1, indicating that ligand binding was focused in this region. The BoIFNAR-1 N-terminal (SD1) and membrane-proximal (SD4) domains each further enhanced binding when transferred with SD2 and SD3 into HuIFNAR-1 (49). Thus, the IFN binding site is not localized on one FNIII subdomain or even on one tandem pair of FNIII subdomains, as previously predicted from other cytokine family members (43); instead the ligand-binding determinants of IFNAR-1 appear to be distributed in a more complex array centered on SD2 and SD3. Kumaran et al. (52) used murine/human IFNAR-1 hybrids to confirm that SD1 plays at most a minor role in species-specific ligand binding.

The current study of BoIFNAR-1 identifies amino acids that are critical for IFN binding, using a series of alanine substitutions throughout its extracellular domain. By presenting these results in the context of a new three-dimensional homology model of IFNAR-1, we shed light on the interaction of type I IFNs with IFNAR-1.

EXPERIMENTAL PROCEDURES

Interferons, Receptor cDNAs, and Antibodies—IFN-α2a (1.56 × 10^8 IU/mg) was provided by Dr. Sidney Pestka. The M2 anti-FLAG antibody was purchased from Sigma Chemical Co. R-Phycocerythrin-conjugated F(ab')2 goat anti-mouse IgG was purchased from Jackson ImmunoResearch.

Cells and Media—Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Hyclone) and 2 mm glutamine (Sigma) and 10 units/ml penicillin with 100 μg/ml streptomycin (Life Technologies, Inc.), was used to maintain all cell lines.

Phosphorylation of IFN—The HuIFN-α2a analogue IFN-α2a-P1 (53) was phosphorylated to a radioactivity level of about 10^10 Ci/mmol with [γ-32P]ATP (6000 Ci/mmol, PerkinElmer Life Sciences) and bovine heart CAMP-dependent protein kinase (Sigma). This corresponds to a fractional labeling of 0.15 μl of 32P/mmol of protein. For simplicity, [γ-32P]IFN-α2a-P1 is referred to as [32P]IFN-α2a.

Parental Plasmids and Expression—Bovine and Human IFNAR-1 were transiently expressed from the EP-1α promoter in the vector pcDEF3 (54). Unique restriction sites were engineered into the bovine IFNAR-1 cDNA by using oligonucleotides containing silent mutations RsrII, BspEI, AgeI, and NheI. These restriction sites were placed between each respective subdomain and at the beginning of the transmembrane domain, resulting in a modified version of BoIFNAR-1. This version of BoIFNAR-1 was also tagged with the FLAG epitope (55) between the signal peptide and the beginning of the first subdomain of the protein, allowing for its recognition on the cell surface by anti-FLAG antibody (23). Mutagenic-engineered in vitro receptor using either a two-step splice overlap extension (SOE) polymerase chain reaction method (56, 57) or the QuikChange site-directed mutagenesis kit (Stratagene). All clones were screened by restriction digests and confirmed by sequencing. In most cases altered receptors were analyzed using two independent clones, to eliminate false decreases in binding due to the engineered mutation. Mutants were made from the BoIFNAR-1 cDNA template in three different versions of pcDEF-3 at various stages in this project. The BoIFNAR-1 gene was the same for all three; however, the first had an HA tag at the N terminus (which was not effective for flow cytometric detection); in the second, the HA tag was replaced with a FLAG epitope to allow for cell surface confirmation by flow cytometry; the third version contained an internal ribosome entry site coupled to enhanced green fluorescent protein (ires-EGFP) (CLONTECH) after the FLAG-bovine IFNAR-1, and a zeocin marker in place of the original neomycin marker. These plasmids, when transfected into COS cells, were indistinguishable in their transient BoIFNAR-1 expression and IFN binding.

The two-step Splice Overlap Extension (SOE) polymerase chain reaction method (56, 57) was carried out using Elongase (Life Technologies, Inc.). SOE fragments were restriction-digested and then ligated into the proper vector. All reactions were carried out in a 50-μl volume in 0.5-ml thin-wall tubes in a PerkinElmer Life Sciences DNA thermal cycler. The QuikChange site-directed mutagenesis kit was used according to the manufacturer's directions (Stratagene), except that template and primer concentrations were doubled; extension time was increased to 3.5 min per kilobase; and DH10B Electromax competent cells (Life Technologies, Inc.) were used for transformations.

DNA Transfections—COS-1 cells (58), derived from a simian kidney CV-1 line, were transfected with 5–10 μg of plasmid using the DEAE-dextran/Me/SO shock protocol (48, 59, 60). Briefly, tissue culture dishes (10-cm dish, Falcon) containing 10 ml of 10% cosmic calf (Hyclone) serum-supplemented DMEM were seeded with 1.75–2.0 × 10^6 cells. Cells were incubated overnight at 37 °C before transfecting. Cells were harvested for assays after 48–72 h. Under our transfection conditions, we generally see about 0.25–1 × 10^6 receptors per cell, averaged over the entire cell population, as measured by ligand binding to BoIFNAR-1. This arises from high level expression on 20–50% of the cell population, as demonstrated by flow cytometry detection of the common FLA11 epitope at the N terminus. Much of this expression variability is between experiments; within any experiment, the variation of expression between constructs has a much smaller range. Each clone was transfected at least twice, and for most mutations binding was measured with transfections of two independent DNA clones.

Saturation Binding—Saturation binding assays were done as previously described (49). Briefly, COS cells were trypsinized and resuspended at 10^6 cells/ml. Vials of 10^5 cells/ml were incubated in 100 μl of cold IFN (1–3 μg/ml), combined with [32P]IFN-α2 (maximum concentration of 1.5–4 × 10^−9 M), serially diluted, and then incubated while rocking for 1 h at room temperature. Cell-bound [32P]IFN-α2 was separated from unbound by brief centrifugation of 100 μl of sample through a cushion of 10% (w/v) sucrose in PBS. Tubes were then frozen and cut, and the tips and tops were counted separately. Suppliers' determinations of IFN concentration (in mg/ml) were used for all calculations. Data were analyzed by non-linear regression to one-site binding using the program Prism v.2.01 (GraphPad Software, Inc., San Diego, CA). A mutant receptor that lost 75% of the binding seen for unaltered BoIFNAR-1 was defined as having a significant loss of binding activity. Among the mutants, there is a clear demarcation between those mutants that lost 75–100% and a secondary group of mutants, which lost 0–56% of binding. This criterion is consistent with thresholds used in studies of other receptors (61–63) and with the accuracy and precision of our data, thereby guarding against over-interpretation of marginal effects.

Flow Cytometric Analysis of Receptor Surface Expression—Transfected cells were harvested in DMEM supplemented with 10% (v/v) FBS (Life Technologies, Inc.) and washed with PBS and resuspended in 50 μl of 0.125 μg/ml of primary antibody (M2 anti-FLAG antibody, Sigma) or medium. The cells were washed with PBS and resuspended in 50 μl of 0.125 μg/ml of secondary antibody (R-phycocerythrin-conjugated F(ab')2 goat anti-mouse IgG, Jackson ImmunoResearch) and incubated for 1 h. Cells were again washed with PBS and then incubated in 100 μl of 3% paraformaldehyde...
at 4 °C for 1 h. The cells were washed once with 1 ml of PBS containing 50 mM Tris and then resuspended in 500 μl of PBS with 50 mM Tris and stored at 4 °C until analysis with a Coulter Epics Profile II cell sorter.

Model Building—Homology models were generated for the extracellular domain of human IFNAR-1 with the coordinates of the extracellular domain of IFNGR-1 (30, 70) graciously provided by Drs. Mark Walter (University of Alabama, Birmingham) and Steve Ealick (Cornell University). The homology model was created using GeneMine and Sybyl (Tripos, Inc.).

RESULTS

General Considerations—With 414 amino acids predicted for its extracellular domain, BoIFNAR-1 presented a large target for mutagenesis. We therefore created clusters of alanine mutations first, followed by individual alanine substitutions. Initially, we investigated loops whose amino acid sequences differ between BoIFNAR-1 and HuIFNAR-1, because their affinities for IFNs differ by 100-fold and because residues implicated in ligand binding are most often located in the loops of the cytokine receptors, rather than in the β-strands. The search for functionally important residues was aided by information about ligand/receptor interactions in other members of the cytokine receptor families, and by our three-dimensional homology model (27, 30, 37–39, 61, 66).

Transfected COS cells transiently expressing the receptors on the cell surface at very high levels serve as a reliable, efficient, and convenient platform for ligand binding experiments. With such high expression, the binding of IFN to BoIFNAR-1-transfected cells increases 15- to 40-fold over mock-transfected cells (48), providing a very strong signal. In the same system, surface expression of HuIFNAR-1, at levels comparable to that of BoIFNAR-1, increases binding of [32P]IFN-α2 to cells by 1- to 2-fold over COS cells transfected with empty vector (Table I; see also Ref. 49, Figs. 3–5; and Ref. 48). Thus, any excess endogenous (simian) IFNAR-2 does not make a substantial contribution to ligand binding. Cell surface expression levels of different constructs were compared through a common FLAG epitope. We thereby demonstrated that high expression of ectopic BoIFNAR-1. However, because the Simian-derived COS cells are biochemically responsive to human IFNs through their endogenous receptors, this system is useful only for ligand binding assays. Finally, the expressed receptors on the cell surface at very high levels serve as a reliable, efficient, and convenient platform for ligand binding experiments.
Mutagenesis—Previous work had emphasized the importance of subdomains 2 and 3 in generating the nanomolar affinity of BoIFNAR-1 (49), so our mutagenesis focused on these subdomains. In addition, a region corresponding to a well-characterized epitope of SD1 was examined in detail, as were several areas of SD4 whose sequences differ significantly between the bovine and human homologues. Finally, differences between human and bovine IFNAR-1 were investigated with several substitutions incorporating the amino acids found in HuIFNAR-1 into the BoIFNAR-1 structure (“homologue mutants”). The list of mutants (Tables I and II) is organized by subdomain, and denoted by the secondary structure location (e.g. 2L4–5 is a mutation in SD2 in the loop

### Table I

| Mutant receptor          | Mutated bovine residues | Decrease from bovine | S.D. (N) | Bovine surface expression (avg) |
|--------------------------|-------------------------|----------------------|----------|--------------------------------|
| Bovine SD1               |                         |                      |          |                                |
| 1L3-4D                   | Asp44                   | 0                    | 74 (4)   | 70                             |
| 1L3-4W                   | Trp46                   | 32                   | 15 (2)   | 87                             |
| 1L5-6F                   | Phe62                   | 0*                   | 15 (4)   | 86                             |
| 1L5-6V                   | Val65                   | 0*                   | 49 (4)   | 94                             |
| 1L5-6                      | ELEN69                  | 57                   | 20 (4)   | 85                             |
| 1L5-6F2                  | Phe11                   | 47                   | 15 (4)   | 80                             |
| Bovine SD2               |                         |                      |          |                                |
| 2L1-2                    | EDK115                  | 93                   | 5 (4)    | 85                             |
| 2L2-3-2                  | DIMIMW113              | 93                   | 23 (15)  | 92                             |
| 2L2-3D                   | Asp126                  | 0                    | 39 (4)   | 77                             |
| 2L2-3W (Trp132)          | Trp132                  | 95                   | 4 (6)    | 126                            |
| 2L2-3                    | MXR136                  | 29                   | 21 (4)   | NT                             |
| 2B3F (Phe139)           | Phe139                  | 82                   | 1 (4)    | 81                             |
| 2B3Y (Tyr141)           | Tyr141                  | 91                   | 1 (4)    | 70                             |
| 2L4-5                    | EFPED143                | 75                   | 12 (12)  | 91                             |
| 2L4-5Y (Tyr160)         | Tyr160                  | 96                   | 4 (8)    | 95                             |
| 2L4-5-2                 | ETY145-2                | 90                   | 1 (4)    | 100                            |
| 2L6-7                    | EXRXRXX167             | 66                   | 32 (9)   | 104                            |
| 2L6-7R                  | Arg245                  | 29                   | 30 (6)   | 140                            |
| 2L7-P-1                 | EXXX196                | 6                    | 10 (2)   | NT                             |
| 2L7-P-2                 | EXRX204                 | 13                   | 28 (4)   | NT                             |
| Bovine SD3               |                         |                      |          |                                |
| 3L1-2                    | Asp216                  | 0                    | 39 (4)   | NT                             |
| 3L2-3                    | Glu229                  | 25                   | 4 (2)    | NT                             |
| 3L3-4:1                  | FXY244                  | 9                    | 21 (4)   | NT                             |
| 3L3-4:3                  | FXX243                  | 91                   | 10 (4)   | 90                             |
| 3L3-4:2                  | Asp251                  | 15                   | 5 (2)    | NT                             |
| 3L3-4W (Trp253)         | Trp253                  | 84                   | 10 (8)   | 105                            |
| 3L3-4K                  | Lyr254                  | 5                    | 2 (6)    | 124                            |
| 3L3-4Q                  | Gln255                  | 0                    | 123 (2)  | 107                            |
| 3L5-6                    | RR272                  | 16                   | 24 (5)   | NT                             |
| 3L5-6:2                 | SSR276                 | 9                    | 22 (6)   | 108                            |
| 3B6                     | YYV291                 | 76                   | 15 (10)  | 84                             |
| 3L6-7                    | XCGNG290               | 0                    | 13 (2)   | 91                             |
| 3B7                     | KFN301                 | 78                   | 12 (8)   | 80                             |
| 3L7-P-1                 | Lyr305                 | 0                    | 2 (2)    | NT                             |
| 3L7-P-2                 | Lyr309                 | 0                    | 0 (1)    | NT                             |
| Bovine SD4               |                         |                      |          |                                |
| 4L1-2                   | VTD320                 | 31                   | 2 (2)    | NT                             |
| 4L2-3                   | EYE324                 | 0                    | 13 (3)   | NT                             |
| 4L2-3V                   | Val325                 | 0                    | 25 (4)   | 95                             |
| 4L4-5                   | Arg365                  | 0*                   | 56 (2)   | NT                             |
| 4B5-1                   | NXY365                 | 47                   | 8 (4)    | NT                             |
| 4B5-2                   | EXD372                 | 56                   | 19 (4)   | NT                             |
| 4L6-7-2                 | DRR392                 | 0                    | 0 (1)    | NT                             |

* X represents an unmutated intervening residue.
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A variety of mutants are presented, as is the parental BovIFNAR-1 construct. IFN bound is from 100 μl of a binding reaction containing ~1 × 10⁶ cells.

Mutagenesis resulted in altered receptors whose abilities to bind HuIFN-α2 ranged from the high levels equivalent to (or occasionally higher than) parental BovIFNAR-1, to receptors whose binding was low and indistinguishable from COS background (Table I, Fig. 2). However, all receptors were present on the cell surface at levels similar to parental (70–140% of BovIFNAR-1), as monitored by flow cytometric detection of a common FLAG epitope (55) at the N terminus (the FLAG epitope was previously shown to have no effect on ligand binding (49)). The expression of BovIFNAR-1 gave very high levels of ligand binding as shown previously; unfortunately, like parental BovIFNAR-1, all receptors were present on the cell surface at levels similar to parental (70–140% of BovIFNAR-1).

The majority of mutations showed minimal changes in ligand binding (0–29% decrease; see Table I). Three alanine clusters, 3B6, 3L3–4:3, and 3B7, resulted in drastic decreases of 76–91%, as did the single point mutation 3L3–4W (Trp253) (Table I; Figs. 2, 3). Although it lost 84% of it’s binding, the Trp253 mutant had a cell surface expression level of 105% of parental receptor.

Because our previous studies implicated SD1 as having a lesser effect on ligand binding than SD2 and SD3 (49), a full study of this domain was not undertaken. A more focused series of mutants came from the finding that the epitope for the anti-human IFNAR-1-neutralizing monoclonal antibody 64G12 (67) maps to the human IFNAR-1 sequence 62FSSLKLNVY70 in SD1 (sequence numbering of the mature protein; Fig. 1) (41, 42). We therefore explored the effect of mutations in the equivalent BovIFNAR-1 sequence 62FSSLKLNVY70, focusing on the large aromatic and charged residues (Table I). Alanine substitutions for Phe62 or Val65 actually produced an increase in ligand binding (Table I). A single alanine substitution at Phe131 (1L5–6F2) caused a moderate decrease of ligand binding. A slightly larger decrease (57%) was seen for the 64G12 epitope cluster mutant 1L5–6, where the ELEN sequence is substituted by four alanines.

In SD2 we generated eight alanine clusters, six single alanine substitutions, and three homologous human substitutions (Tables I and II). All mutants were expressed efficiently, and their levels of IFN binding fell into three groups: 1) mutants showing small or no decreases in binding (0–29% decrease; Table I); 2) mutants with binding decreases in the range of 67–75% (2L4–5, 2L6–7); 3) a third and most dramatic series of mutants with decreases of 82–96%. The clusters in this group were 2L1–2, 2L2–3:2, and 2L4–5:2. Most striking was the single substitution mutants, 2L2–3W, 2B3F, 2B5Y, 2L4–5Y, corresponding, respectively, to alanine substitutions at Trp132, Phe139, Tyr141, and Tyr160, which individually produced decreases in binding of 82–96% (Fig. 3) while retaining cell surface expression levels of 126%, 81%, 70%, and 95% of parental receptor, respectively.

SD3 was examined with a series of 12 cluster mutants, 3 single alanine mutants, and 3 human substitutions. The majority of mutations showed minimal changes in ligand binding (0–25% decrease; see Table I). Three alanine clusters, 3B6, 3L3–4:3, and 3B7, resulted in drastic decreases of 76–91%, as did the single point mutation 3L3–4W (Trp253) (Table I; Figs. 2, 3). Although it lost 84% of it’s binding, the Trp253 mutant had
Mutants are arranged by subdomain. Column 1 has the designation of mutants, based on their originally predicted secondary structure. The original and mutated sequences are in columns 2 and 3, with residue numbers based on the mature protein. Results are tabulated as the percent decrease from the level of IFN binding by BoIFNAR-1 (normalized within each experiment), with the standard deviation (S.D.) for the total number of experiments (N).

**DISCUSSION**

BoIFNAR-1, whether as a purified BoIFNAR-1/Fc fusion protein or when expressed at high levels on COS cells, binds HuIFN-α2 with 1–10 nM affinity, and binds a broad selection of other type I IFNs (23). Based on subdomain substitutions between the human and bovine homologues, this strong binding, relative to the weak ligand binding by human IFNAR-1, was shown to reside predominantly in the two central subdomains, SD2 and SD3 (49). Here, through a series of alanine and homologue substitution mutations, we have identified residues in SD2 and SD3 of BoIFNAR-1 that strongly affect the binding of HuIFN-α2. The strongest decreases in binding came from the substitution of any of five hydrophobic residues, conserved in bovine, human, and murine IFNAR-1. Smaller effects came from mutating charged residues proximal to those hydrophobics.

The mutagenesis results are best interpreted in the context of our three-dimensional homology model of IFNAR-1 derived from the atomic coordinates of the closely related, two-subdo-

![Graph](http://www.jbc.org/)

**Fig. 4.** Binding of [125I]IFN-α2 to COS cells expressing BoIFNAR-1 with clusters of residues from HuIFNAR-1. Sample size is the same as in Fig. 2.

main IFNGR-13 (70) (Fig. 5). As there is no related crystal structure on which to base the interaction of the two separate domains of IFNAR-1 (specifically, the SD2-SD3 junction), we have chosen to illustrate the four subdomains in an extended (array rather than, for instance, a semi-circular or strongly bent arrangement). To orient the two halves of IFNAR-1 (SD1+SD2 and SD3+SD4) to each other, we logically assume that residues of SD2 and SD3, which strongly affect ligand binding, are on the same face of the molecule.

Most mutations resulted in little significant change in binding of IFN-α2 (Table I; Fig. 5, blue). Where cluster mutations showed strong decreases in binding, the localization was generally refined to a single residue, which decreased IFN binding by >80% of native BoIFNAR-1 (red). Several clusters, which produced large decreases in IFN binding, were not refined to single residues (orange).

Alanine mutagenesis identified five hydrophobic residues that individually decreased the binding of HuIFN-α2 by 82–96% while retaining cell surface expression levels of 69–125% of parental receptor. The homology model presents the functionally important aromatic residues Trp132, Phe139, Tyr160, and Trp253 as well exposed on the receptor surface, easily accessible for protein interactions. The three residues in SD2 (Trp132, Phe139, Tyr160) are localized to a common region. Trp253, in SD3, is on the protein surface and can be aligned in

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**Table II**

Human substitution mutations created in BoIFNAR-1

| Mutant     | Bovine residues | New human residues | Decrease from bovine | S.D. | N  |
|------------|-----------------|--------------------|----------------------|------|---|
| 2L4–5H     | 160YPEDK164     | 160YSRHK164        | 31%                  | 19   | 4  |
| 2L6–7H     | 181ELRQSR187    | 181ALLTSWK187      | 0%                   | 5    | 2  |
| 2L4–5/6–7H | 271REVSSR276    | 271REVFOKR276      | 21%                  | 22   | 8  |
| 3L3–4:2H   | 246PGNHSDKWKQ255| 246PGNHLYKWKQ255   | 48%                  | 23   | 8  |
| 3L5–6:2H   | 181ELRQSR187    | 181ALLTSWK187      | 75%                  | 16   | 4  |
| Bo 3H      | 2L4–5H          | 160YPEDK164        | 79%                  | 26   | 4  |
| Bo 4H      | 2L6–7H          | 181ELRQSR187       | 79%                  | 26   | 4  |

* Mutations are underlined.
this extended view with the residues of SD2 to define a common binding surface for IFNs. In contrast, Tyr 141 appears buried beneath Phe 139 and Tyr 160, forming extensive β-strand contacts within SD2. Therefore Tyr 141, although not directly involved in ligand binding, is likely to be required for the proper presentation of Phe139 and Tyr160. Each of these critical hydrophobic residues is conserved in the human and murine IFNAR-1 homologues.

Two other alanine clusters involving residues 113EDK115 (2L1–2) and 157ETV159 (2L4–5:2) produced decreases of IFN-α2 binding of >75% (orange) (Fig. 5). 157ETV159 (ENI in HuIFNAR-1 and INS in murine) is proximal (4–14 Å) to both Phe139 and Tyr160 and could be involved in either positioning them or in direct ligand interactions. The highly charged 113EDK115 cluster is near the interface with SD3 and is conserved in human and murine IFNAR-1. This could be involved in important contacts with SD3 or with IFNs. Mapping within these clusters was not further refined.

The conservation of the critical large hydrophobic residues in bovine, human, and murine IFNAR-1 was surprising, because the strategy originated in our studies of bovine/human IFNAR-1 chimeras and had presumed that the key residues would be those that differ between bovine and human IFNAR-1. However, like IFNAR-1, IFNGR-1 and growth hormone receptor demonstrate the importance of aromatic residues (predominantly W and Y) combined with charged residues (K, R, E, and D) packing against the critical aromatics in forming a binding interface (65, 67, 68). In BoIFNAR-1, the aromatic/charged pairs found appear to be Asp128/Trp132, Arg136/Phe139, Arg140, Glu157/Tyr160/Glu162/Asp163, Phe241/Lys243, and Lys252/Trp253/Lys254. Therefore, we hypothesize that the conserved aromatic residues are the foci of the receptor/ligand interface and that the clusters of residues surrounding the key hydrophobic residues modulate ligand binding and distinguish the low affinity human from the high affinity bovine IFNAR-1. These surrounding residues may also help differentiate the binding affinities of the diverse type I ligands.

This hypothesis led us to substitute homologous human residues, which were predicted from the three-dimensional model to be proximal to the conserved critical aromatic residues and that the clusters of residues surrounding the key hydrophobic residues modulate ligand binding and distinguish the low affinity human from the high affinity bovine IFNAR-1. These surrounding residues may also help differentiate the binding affinities of the diverse type I ligands.

Because of its low affinity for IFN, HuIFNAR-1 is a poor target for structure/function studies. What little is known about the ligand binding site of HuIFNAR-1 has been derived
from indirect studies. Two neutralizing mAbs against HuIFNAR-1, EA12 (50) and 447T (40), seem to recognize epitopes that are difficult to map to a single domain. The epitopes for two other neutralizing anti-HuIFNAR-1 mAbs have been identified. The high affinity mAb 2E1 appears to recognize epitopes within the non-contiguous sequences 244HLKYK1249 and 289EEIKF12298, with strong contributions to antibody binding attributed to Lys249, Glu291, and Asp296 (40). This epitope includes Trp248, the human homologue of our critical bovine epitope Trp253 (Fig. 6). The epitope for the anti-human IFNAR-1 64G12 mAb (67) that neutralizes the activity of all tested human type I IFNs on human cells has been localized to a sequence in SD1, 6FSSKLKNVY70 (41, 42). However, our mutations in the homologous sequence (6F86VLEENFY72) in BoIFNAR-1 produced only insignificant to moderate effects on binding of IFN-$\alpha$ (Fig. 6). This is consistent with our observations and those of Kumaran et al. that SD1 plays at most a minor role in the species specificity of human versus murine IFN binding (49, 52).

The three-dimensional model places the 64G12 epitope within 9–17 Å of the critical Trp132 residue in SD2, we speculate that mAb 64G12 sterically occludes Trp132 and any neighboring residues involved in IFN binding.

On the three-dimensional IFNAR-1 model, the epitopes for these two independently produced neutralizing mAbs (40–42, 50, 67) are proximal to and located on the same face of IFNAR-1 as the residues that we identify in BoIFNAR-1 as being critical to the binding of IFN-$\alpha$; i.e., in both BoIFNAR-1 and HuIFNAR-1 there is one face of IFNAR-1 that is involved in ligand binding. Thus, our mutagenesis study of BoIFNAR-1 significantly enhances the interpretation of the currently available studies of HuIFNAR-1 and provides focus for future studies of HuIFNAR-1.

In conclusion, in BoIFNAR-1 we have identified five large hydrophobic residues, conserved in the human and murine homologues, whose substitution with alanine decreases ligand binding by 82–96%. Several clusters of amino acids, proximal to the important aromatic residues, have additionally been identified whose coordinated substitution by alanine produces large decreases in binding of human IFN-$\alpha$2. The critical residues of BoIFNAR-1 map to the same face and are proximal to amino acids independently identified as part of epitopes for anti-HuIFNAR-1 mAbs that efficiently neutralize IFN activity and block IFN binding, supporting the relevance of the current results to the human IFNAR-1.

The most straightforward interpretation of our binding, domain swapping, and alanine mutagenesis data (23, 48, 49) is that the single nanomolar affinity site for HuIFN-$\alpha$2 on BoIFNAR-1 primarily, but not exclusively, involves ligand interactions with SD2 and SD3. The finding that ligand binding appears focused in SD2 and SD3 is novel in terms of previous models (39, 43, 69) and presents a unique pattern of ligand-receptor interactions for the cytokine superfamily. Although confirmation of any model must await a crystal structure, this extensive analysis of BoIFNAR-1 enables the focused analysis of the human IFNAR-1.

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