Mapping Human Interferon-alpha (IFN-α2) Binding Determinants of the Type I Interferon Receptor Subunit IFNAR-1 with Human/Bovine IFNAR-1 Chimeras

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ABSTRACT: Type I interferons bind to a common receptor (IFNAR), composed of two transmembrane polypeptides, IFNAR-1 and IFNAR-2. Although human IFNAR-1 has a weak intrinsic affinity for human Type I interferons (IFNs), bovine IFNAR-1 binds human Type I IFNs with moderate (nM) affinity, and can be conveniently used to investigate the regions of IFNAR-1 involved in ligand binding. We have constructed 14 bovine/human IFNAR-1 chimeras by exchanging homologous subdomains in the extracellular portion of the receptor. These chimeras were expressed at very high levels on COS cells, and their ability to bind HuIFN-α2 was measured. No single bovine subdomain substituted into human IFNAR-1 could confer moderate-affinity ligand binding on the resulting chimera. Simultaneous substitution of bovine IFNAR-1 subdomains 2 and 3 for the homologous human subdomains resulted in a dramatic increase in the binding of IFN-α2, suggesting that critical determinants for moderate-affinity ligand binding by BoIFNAR-1 reside in these two subdomains. Bovine subdomains 1 and/or 4 each further enhanced IFN-α2 binding in the presence of bovine subdomains 2 and 3. Thus, the binding interactions of BoIFNAR-1 with IFNs appears to be more complex than that of other class II cytokine receptors with their ligands.

The human Type I interferon (IFN)[1] family is comprised of 13 to 14 nonallelic IFN-αs, one IFN-*β*, and one IFN-*ω*, all of which are structurally and functionally related (*1*, *2*). These IFNs can cross-compete for receptor binding, showing that they bind to a common cell surface receptor (*1*, *3*−*5*). Thus, the Type I IFN receptor has the largest number of natural ligands of all known cytokine receptors. The Type I interferon receptor complex (IFNAR)[2] consists of at least two subunits, IFNAR-1 and IFNAR-2 (*6*−*10*). Human IFNAR-1 (HuIFNAR-1) has low affinity ($K_d \geq 10^{-7}$ M) for IFNs ((*6*, *11*−*13*); Langer, unpublished data), but contributes to both the affinity and specificity of Type I IFN binding (*7*, *14*−*20*). HuIFNAR-2 contributes more strongly to IFN binding, with moderate and differential affinity for Type I IFNs (e.g., $K_d \sim 2-8$ nM for HuIFN-α2) (*7*, *15*−*18*, *20*, *21*). Both subunits are required for specificity and participate directly in ligand binding and signaling (reviewed in refs *9*, *10*).

In contrast to the human IFNAR-1, the bovine homologue (BoIFNAR-1) serendipitously has moderate affinity for human Type I IFNs, an observation which may largely explain the high biological activity of human IFNs on bovine cells (*1*, *22*−*24*). We and others cloned the BoIFNAR-1 cDNA and showed that bovine IFNAR-1 can confer on murine or human cells a bovine-like specificity for HuIFN-αs (*11*, *25*, *26*). Thus, the bovine IFNAR-1, in the context of endogenous murine or human IFNAR-2, can dominate the specificity of the receptor complex for ligand binding and biological activity. Furthermore, when expressed on *Xenopus* oocytes or COS cells under conditions where other receptor subunits are likely not to contribute to ligand binding, BoIFNAR-1, but not HuIFNAR-1, can mediate moderate-affinity binding of human IFNs to these cells (*11*, *26*). Finally, a purified, soluble BoIFNAR-1/immunoglobulin fusion protein directly binds diverse human IFN-αs, IFN-*β*, and IFN-*ω* with moderate affinity (e.g., ∼10 nM for IFN-α2), whereas IFN binding was essentially undetectable with a HuIFNAR-1/immunoglobulin fusion protein (*27*).

IFNAR-1 is in the class II cytokine receptor superfamily (Figure 1) (*28*, *29*), with BoIFNAR-1 and HuIFNAR-1 having 68% amino acid identity (*25*, *26*). IFNAR-1 consists of a large extracellular region ("ectodomain"; ∼400 amino acids), a single transmembrane domain, and a cytoplasmic domain (∼100 amino acids). The ectodomain of IFNAR-1 is larger than that of most other cytokine receptor subunits, consisting of four fibronectin type III (FBN−III; in the immunoglobulin superfamily) subdomains of about 100 amino acids each (here denoted SD1-SD4), which can be organized into two major domains (∼200 amino acids) (*28*,

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[1] The abbreviations used are: IFN, interferon; IFNAR, Type I interferon receptor; Bo, bovine; Hu, human; FBN-III, fibronectin type III.; Ig, immunoglobulin; SD, subdomain.

[2] Nomenclature for interferons and interferon receptors is in accordance with recommendations of the Nomenclature Committee of the International Society for Interferon and Cytokine Reseach (Lundgren, E., and Langer, J. A. (1997). *J. Interferon Cytokine Res. 17*, 315−316). IFNAR-1 has also been referred to as IFNAR, IFN-αR1, and IFN-Rα.

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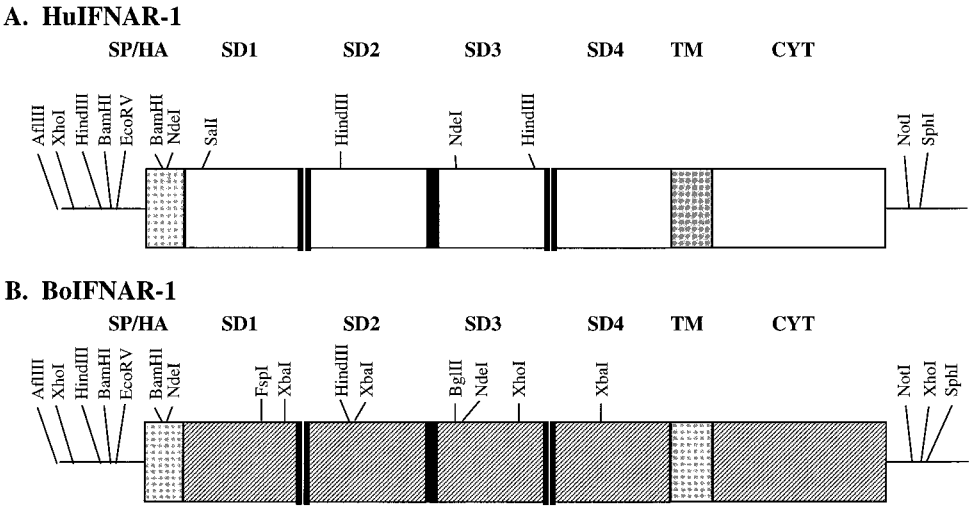

FIGURE 1: Common and useful enzyme sites used in IFNAR-1 chimeras. The restriction enzyme sites used for generating and analyzing some of the chimeras are shown for (A) HuIFNAR-1 and (B) BoIFNAR-1. Abbreviations: SP/HA, signal peptide and hemagluttinin epitope; SD1, SD2, SD3 and SD4: subdomains 1, 2, 3, and 4 of the extracellular ligand binding domain; TM, transmembrane region; CYT, cytoplasmic region. Domain boundaries corresponding to di- and triproline sequences are shown as thick vertical black lines.

29). Each subdomain is separated by conserved di- or triproline motifs.

With such different binding properties for the human Type I IFNs, the human and bovine IFNAR-1 provide a potentially valuable pair of molecules for studying the interactions of IFNs with IFNAR-1, and for examining the interactions involved in the ternary complex of IFNs with IFNAR-1 and IFNAR-2. In addition, BoIFNAR-1 is intrinsically interesting as a moderate-affinity protein capable of binding a broad array of human Type I IFNs, including diverse IFN-αs, IFN-β and IFN-ω (*27*).

To begin dissecting the molecular determinants responsible for moderate-affinity IFN binding by BoIFNAR-1, we examined a series of bovine/human IFNAR-1 chimeras to analyze the contributions of the four subdomains of the BoIFNAR-1 ectodomain to the binding of human Type I IFNs. These studies implicate subdomains 2 and 3 as necessary but not sufficient for moderate-affinity binding, and therefore suggest a somewhat different motif for ligand−receptor interactions than previously seen for other class II cytokine/receptor structures.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies. Human and bovine IFNAR-1 (HuIFNAR-1 and BoIFNAR-1) cDNAs were previously characterized (*20*, *26*). IFN-α2 (=IFN-αA; 2×10^8 U/mg) was provided by Dr. Sidney Pestka (this department). The HuIFN-α2 analogue, IFN-α2-P1, was phosphorylated to high radiospecific activity (routinely 30−40 μCi/μg, corresponding to 600−800 Ci/mmol) with [γ-^{32}P]ATP (6000 Ci/mmol, DuPont-New England Nuclear) using bovine heart cAMP-dependent protein kinase (*30*); the phosphorylated product is denoted [^{32}P]IFN-α2. Anti-HuIFNAR-1 monoclonal antibodies FB2, AA3, GB8, DB2, and EA12, generously provided by Dr. Christopher Benjamin (Biogen, Inc), do not recognize BoIFNAR-1, and their epitopes have been localized to different subdomains of HuIFNAR-1 (*31*; Goldman et al., manuscript in preparation).

Nomenclature of Human/Bovine IFNAR-1 Chimeras. Human/bovine IFNAR-1 chimeras are named for the origin of each subdomain of the extracellular ligand-binding domain from the human ("H") or bovine ("B") molecules (Figure 1). There are four subdomains in the extracellular region of the receptor subunit, here denoted SD1-SD4, with SD1 being at the N-terminus and distal from the membrane. Thus, HHHH and BBBB represent constructs where all four subdomains of the extracellular domain are of human or bovine origin, respectively. The species origin of the signal peptide (SP) matches the origin of the first extracellular subdomain. Similarly, the origin of the transmembrane and cytoplasmic regions of the receptor match the origin of the fourth subdomain. Thus, for the chimera BHBH, the signal peptide and first and third subdomains (SD1 and SD3) are bovine, while the second and fourth subdomains (SD2 and SD4) are human, as are the contiguous transmembrane segment and the cytoplasmic portion of the receptor.

Construction of Human/Bovine IFNAR-1 Chimeras. HHBB and BBHH were constructed using a two-step polymerase chain reaction (PCR) procedure (*32*). Chimeras with one human and three bovine subdomains (the "1H3B" set: HBBB, BHBB, BBHB, and BBBH) were constructed using a combination of asymmetric PCR (*33*, *34*) and subcloning. Chimeras with three human and one bovine subdomains (the "3H1B" set: BHHH, HBHH, HHBH, HHHB) were constructed by subcloning restriction fragments from other chimeric IFNAR-1 cDNAs. The chimeras BHHB, HBBH, HBHB, and BHBH (which, along with HHBB and BBHH, comprise the "2H2B" set) were also constructed by subcloning fragments from other chimeric IFNAR-1 cDNAs. Key enzyme sites used for subcloning and for diagnostic mapping are illustrated in Figure 1.

In all cases, subdomains were joined at the di- or triproline motifs which are taken as the subdomain boundaries. All chimeras were tagged at the 5′ end of the cDNA between the signal peptide and the first codon of subdomain 1 with a tandem trimer of the hemagluttinin (HA) epitope of influenza virus to allow visualization of expressed protein (*35*).

The chimeric cDNAs were verified by a combination of restriction mapping, sequencing at the sites of manipulation

(Sequenase 2.0 system, USB), and Southern dot blot hybridization (ECL system, Amersham Life Sciences) with oligonucleotides specific for each human and bovine subdomain.

Expression Plasmid Construction and DNA Transfections. HuIFNAR-1, BoIFNAR-1, and chimeric human/bovine IFNAR-1s were transiently expressed from the EF-1α promoter in vectors pcDEF1 or pcDEF3 (*36*). For analysis by flow cytometry, some experiments utilized BoIFNAR-1 and HuIFNAR-1 constructs modified to include a hydrophilic FLAG epitope (*37*) at their amino terminus, engineered by the polymerase chain reaction (PCR) and verified by DNA sequencing (E. C. Cutrone and S. Kotenko, unpublished). These constructs were expressed from the pcDEF3 vector (*36*), modified by the substitution of a zeocin resistance marker for the published neomycin resistance gene (E. C. Cutrone and S. Kotenko, unpublished). COS-1 cells (*38*), derived from a simian kidney cell line, were transfected using a DEAE-Dextran/DMSO shock procedure (*26*, *39*, *40*) and assayed for ligand binding and receptor expression after growth for 2 to 3 days. Typically, 20−40% of cells showed significant expression of transfected receptors (see below), consistent with previous estimates from expression of bacterial β-galactosidase (unpublished data). This fractional expression of introduced receptors was sufficient to increase the binding of IFN to 10-to-50 times higher than that found in control COS cells, thereby allowing the measured IFN binding to be attributed almost entirely to the ectopically expressed receptors (see below).

Receptor Binding Assays. Receptor binding studies using transfected COS cells were performed as previously described (*20*, *26*), with some modifications. Transfected COS cells were trypsinized, pelleted, and resuspended. (Trypsinization, a convenient way to collect cells, did not noticeably decrease ligand binding, and trypsin-treated cells showed high surface expression of the transiently expressed receptors, as monitored by flow cytometry.) To obtain accurate binding curves approaching ligand saturation on cell lines that vary 20 to 40-fold in the amount of IFN bound per cell, it was necessary to adjust cell concentrations, ranging from 0.5×10^6 cells/mL (for cells that bind large amount of [^{32}P]IFN-α2, such as BoIFNAR-1-transfected COS cells), to $(4-6) \times 10^6$ cells/mL (for HuIFNAR-1-transfected cells or vector control-transfected cells). Cells (230 μL) were added to wells of a 96-well plate (Falcon U bottom Microtest III assay plate, Becton Dickinson). [^{32}P]IFN-α2 was then added to the cells in the first well for each concentration series (∼5000 cpm/μL cells) and samples were serially diluted; alternatively, appropriate amounts of [^{32}P]IFN-α2 were added directly. Samples were then incubated for 1 h at room temperature (control experiments showed negligible internalization of ligand at this temperature). [^{32}P]IFN bound to cells was separated from unbound by sedimentation through a 10% sucrose cushion. Nonspecific binding of [^{32}P]HuIFN-α2 (generally ≤5−10% of total binding) was determined in a parallel series of tubes containing ≥200-fold excess nonradioactive recombinant HuIFN-α2.

Specific binding was calculated as a point-by-point subtraction of nonspecific binding from total binding. Data were analyzed by nonlinear regression to one-site and two-site binding models using the program Prism v.2.01 (Graphpad Software, Inc., San Diego, CA); one-site models were sufficient to produce good fits to the data. Data are presented as the calculated specific binding for one experiment, representative usually of at least three independent experiments (see figure legends for details). To allow visual comparison of vastly different binding curves on the same figures, some binding curves were normalized to different cell concentrations, as indicated in the figure legends.

Analysis of Receptor Surface Expression. Cell surface expression of receptors was monitored by flow cytometry, using a panel of anti-human IFNAR-1 monoclonal antibodies which bind to different subdomains of the receptor ectodomain (see above). IFNAR-1 constructs with an amino-terminal FLAG epitope (see above) were detected with the M2 anti-FLAG antibody (Kodak). Briefly, transfected cells were resuspended in DMEM with 5% calf serum (DMEM-5) and 0.1 mg/mL human IgG. Cells (usually $\geq 10^5$ in 25 μL) were incubated for 1 h at 4 °C with 0.25 μg (25 μL) of primary antibody. The cells were washed with PBS and resuspended in 0.125 μg (50 μL) of secondary antibody (R-phycoerythrin-conjugated F(ab′)$_2$ goat anti-mouse IgG; Jackson ImmunoResearch) in PBS, and incubated for 1 h. Cells were washed and fixed in 3% paraformaldehyde (100 μL) for 1 h, washed again, and resuspended in 500 μL of PBS for analysis with a Coulter Epics Profile II cell sorter. Depending on which primary antibody was used, about 8−28% of COS cells transfected with HuIFNAR-1 showed an enhanced fluorescence of 0.5−2.0 orders of magnitude over what was observed with control COS cells (i.e., transfected with vector alone or with vector containing BoIFNAR-1) (Figure 2).

Since all chimeras and parental molecules were constructed with a tandem trimer of the influenza hemagluttinin (HA) epitope (*35*) at the amino terminus (see above), total ectopic receptor expression was monitored semiquantitatively by Western blotting of detergent extracts of transfected cells, using the 12CA5 anti-HA monoclonal antibody. (Unfortunately, the trimeric HA epitope was not recognized well on the cell surface by the 12CA5 antibody, and could not be used in flow cytometry as a shared epitope for all IFNAR-1 constructs.) These results were in qualitative agreement with the flow cytometric data on the relative expression of various IFNAR-1 constructs.

RESULTS

Properties of the Expression System. The transient, high-level expression of cell surface receptors on COS cells provides a convenient and sensitive system for the analysis of ligand-binding properties of interferon and other cytokine receptors (*41*). One advantage for mutagenesis studies is that cell surface expression is itself a first-order indication of proper protein folding, since eukaryotic cells efficiently trap or degrade most misfolded proteins. In addition, since the high level expression of ectopically expressed cytokine receptors under appropriate promoters is many times higher than the endogenous expression of these molecules, one essentially measures the ligand-binding properties of the ectopically expressed receptors, as though in isolation.

Although high-level expression of BoIFNAR-1 in COS cells from vectors utilizing the CMV promoter was previously documented by ligand binding (*26*), expression of human IFNAR-1 from these vectors was poor (*36*). The EF-

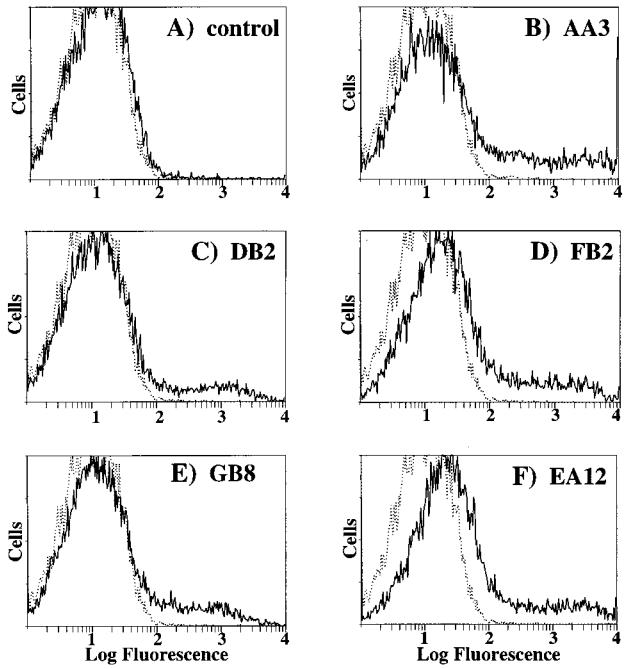

FIGURE 2: Flow cytometric analysis of the reactivity of anti-HuIFNAR-1 monoclonal antibodies with COS cells transfected to express HuIFNAR-1. Transfected cells were reacted as in Experimental Procedures with primary anti-HuIFNAR-1 antibodies (identified in each panel), and then with an R-phycoerythrin-conjugated goat antimouse antibody, fixed and analyzed. Each panel shows cell number vs logarithm of fluorescence (4 decades): solid lines; experimental antibody; dotted lines; control reaction lacking primary antibody. Panel A used an unrelated anti-IFN mAb (IgG1 subclass) as the primary antibody. In this figure, the percent of cells shifting to the highest two logarithms of fluorescence varied from 10% for DB2 (panel C) to 19% (AA3 and EA12; panels B and F, respectively).

1α promoter provided high-level expression of bovine and human IFNAR-1 and most chimeric constructs (*36*), as measured by ligand binding, flow cytometry, and/or western blotting with antibody 12CA5 against the hemagglutinin epitope tag on all IFNAR-1 constructs (see below, and *36*).

Cells transfected with BoIFNAR-1 display an average of 50 to 100-fold higher levels of [^{32}P]IFN-α2 binding at saturation than do cells transfected with HuIFNAR-1 (Figure 3 and Table 1; see also *26*), or 20−40-fold higher binding below saturation (at about 2 nM IFN, a typical input concentration). HuIFNAR-1-transfected COS cells showed on average a small increase in [^{32}P]IFN-α2 binding above the levels seen with COS cells transfected with control vector (Figure 3), as reported previously (*13*). This is consistent with HuIFNAR-1 having a weak intrinsic affinity for human [^{32}P]IFN-α2 (*6*, *11*−*13*, *20*), but could represent a complex of ectopic HuIFNAR-1 with endogenous IFNAR-2 (*13*). For the cells whose ligand binding is illustrated in Figure 3, comparable levels (within 2-fold) of BoIFNAR-1 and HuIFNAR-1 expression were verified by flow cytometry using the anti-FLAG M2 antibody to detect the common N-terminal FLAG epitope (Figure 3, panel C). In this example, about 19% of cells transfected with FLAG-tagged BoIFNAR-1 or HuIFNAR-1 were shifted into the upper two logarithms of fluorescence, and cells with either construct had a similar distribution of fluorescence. Thus, the weak binding of [^{32}P]IFN-α2 by HuIFNAR-1-transfected COS cells is not explained by a failure to express HuIFNAR-1.

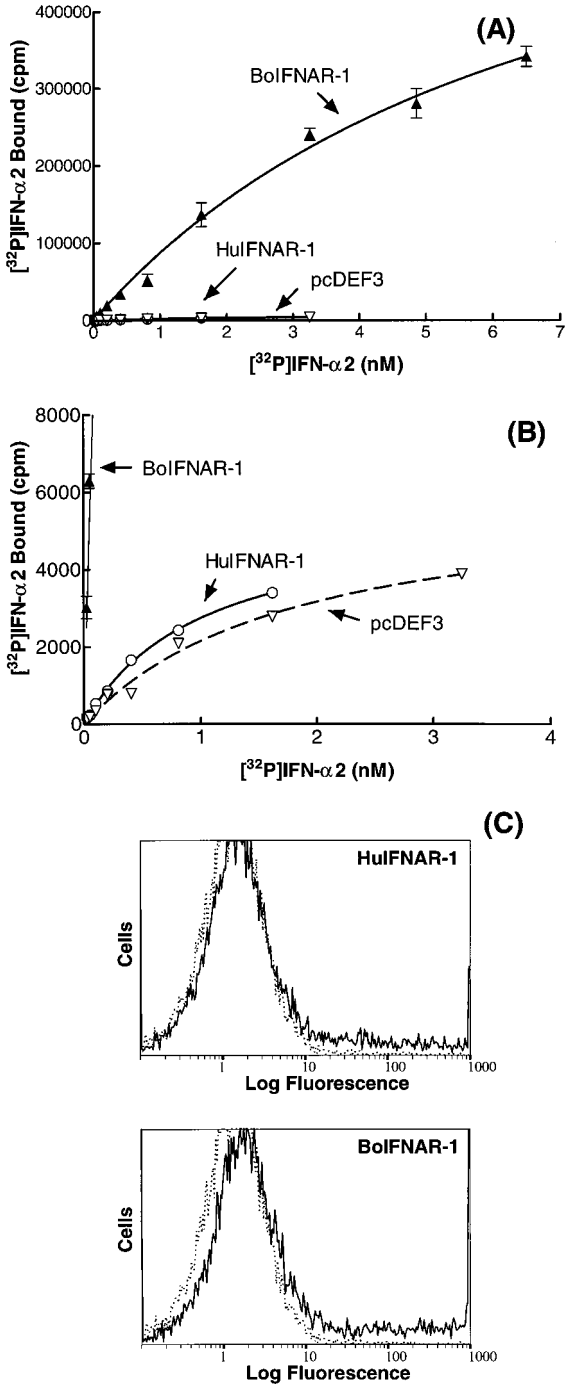

FIGURE 3: Specific binding of HuIFN-α2 to bovine and human IFNAR-1-transfected COS cells. COS cells transfected with the BoIFNAR-1/pcDEF3 or HuIFNAR-1/pcDEF3 plasmids or with pcDEF3 control plasmid were analyzed for their ability to bind [^{32}P]IFN-α2. Data are scaled to an input of 5×10^6 cells/mL. Binding to BoIFNAR-1 was measured in triplicate; averages and standard deviations are indicated. (A) Scaled to display all three curves. (B) The same data expanded to show cells transfected with HuIFNAR-1 or control plasmid. Data for BoIFNAR-1-transfected cells are the average and standard error of the mean (SEM) for triplicate samples; other data are for single samples. (C) Cells used in panels A and B analyzed by flow cytometry after reaction with an anti-FLAG mAb to detect surface expression of FLAG-tagged HuIFNAR-1 and BoIFNAR-1. Cells transfected with IFNAR-1 constructs (bold, continuous curves) were compared to cells transfected with pcDEF3 vector (dotted curve).

Nonlinear regression analysis of the BoIFNAR-1 binding curves provides an estimate for the K_d of BoIFNAR-1 and

Table 1: Binding Parameters for Transfected COS Cells

construct	K_d (nM)	receptors/cell
pcDEF1 (vector)	8 (±1; $n = 3$)	16×10^3 ($\pm 8 \times 10^3$; $n = 3$)
HuIFNAR-1	3 (±2; $n = 6$)	21×10^3 ($\pm 16 \times 10^3$; $n = 5$)
BoIFNAR-1	12 (±6; $n = 6$)	2.2×10^6 ($\pm 1 \times 10^6$; $n = 4$)
BBBH	7 (±4; $n = 5$)	3.7×10^5 ($\pm 1.6 \times 10^5$; $n = 2$)
HBBB[b]	18[b] (±15; $n = 3$)	4×10^5 ($\pm 0.6 \times 10^5$; $n = 2$)
HBBH[b]	20[b] (±6; $n = 3$)	6.9×10^5 ($\pm 4 \times 10^5$; $n = 2$)

[a] Binding parameters were calculated by nonlinear regression fit of a one-site binding model to the measured data (using the program Prism v. 2.01; Graphpad, Inc.). Numbers in parentheses are the standard deviation and the number of independent determinations (n). [b] For the constructs HBBB and HBBH, which have higher dissociation constants, the values are less reliable, since saturation conditions were not approached in the experiment.

[^{32}P]IFN-α2 of 12 nM, similar to that of a soluble BoIFNAR-1/Fc fusion protein (*27*). Furthermore, under the experimental conditions used, the observed binding of [^{32}P]IFN-α2 is essentially completely to BoIFNAR-1, and independent of the COS background; this is also indicated by the observation that human IFN-α1, which does not compete efficiently with binding of [^{32}P]IFN-α2 to human or COS cells, competes efficiently with [^{32}P]IFN-α2 for ligand binding to BoIFNAR-1 expressed on COS cells (data not shown), as it does on bovine cells and on the BoIFNAR-1/Ig soluble protein (*27*).

Identification of Domains Involved in Moderate-Affinity Ligand Binding by BoIFNAR-1. To exploit the considerable differences in the binding of [^{32}P]HuIFN-α2 by bovine and human IFNAR-1, we contructed a series of human/bovine IFNAR-1 chimeras and examined their ligand-binding properties to determine which subdomains of BoIFNAR-1 are responsible for the moderate affinity ($K_d \sim 10$ nM) IFN-α binding. The expression of each chimera was monitored to ensure that constructs displaying low levels of IFN binding showed surface receptor expression comparable to chimeras that conferred high levels of IFN binding. Cell surface expression was measured by flow cytometry, using a panel of anti-HuIFNAR-1 antibodies whose epitopes we have mapped to the different extracellular subdomains of HuIFNAR-1, but which did not cross-react with BoIFNAR-1 (Figure 2; and Goldman, L. A., et al., manuscript in preparation). (Note: More than 90−95% of the major peak at low fluorescence levels in all panels is attributable to the autofluorescence of COS cells and is not due to cross-reaction with IFNAR-1 on COS cells; i.e., this peak is seen even in the absence of primary and/or secondary antibody.) In addition, total cellular expression of IFNAR-1 constructs was monitored by Western blotting with an anti-HA antibody, and was completely consistent with the results of flow cytometry (data not shown).

Of the 14 chimeras, only 3 (BBBH, HBBB, and HBBH) showed binding of [^{32}P]HuIFN-α2 at levels significantly higher than HuIFNAR-1-transfected cells (Figures 4 and 5A; Table 1), although no chimera was fully comparable to BoIFNAR-1. Chimeras with human SD1 or SD4 in an otherwise bovine background (HBBB and BBBH) efficiently bound [^{32}P]HuIFN-α2 (Table 1). However, the affinity of HBBB was generally lower than those of BBBH and BoIFNAR-1, which were more similar to each other (Figure 3; Table 1; because the binding experiments utilized radiolabeled IFN in the range 0.1−8 nM, calculated K_d values ≥10 nM should be viewed as lower estimates, rather than precise numbers). In contrast, the substitution of human SD2 or SD3 into an otherwise bovine background (BHBB and

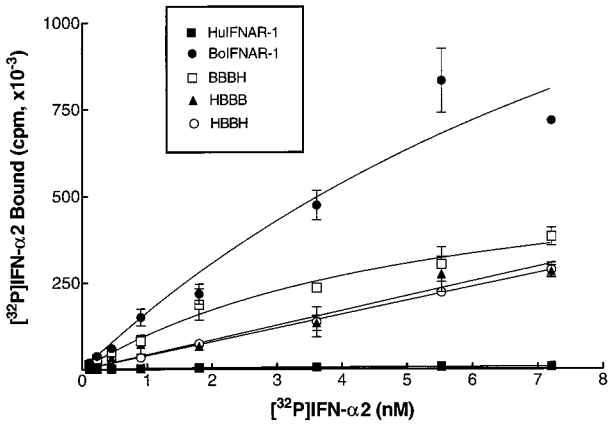

FIGURE 4: Binding of HuIFN-α2 to chimeras containing bovine subdomains 2 and 3. COS cells transfected with the moderate-affinity chimeras HBBB, BBBH, and HBBH, as well as the parental HuIFNAR-1 and BoIFNAR-1, were analyzed for their ability to bind [^{32}P]IFN-α2. The specific binding for one experiment is shown (average values from several experiments are presented in Figure 5A and Table 1), normalized to 5×10^6 cells/mL. Nonspecific binding varied from 5−10% of total binding (at low IFN concentrations) to 5−15% of total (at high IFN concentrations).

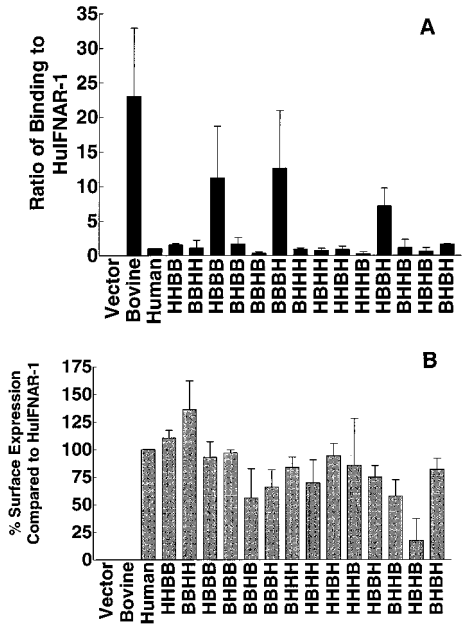

FIGURE 5: IFN binding and cell surface expression for all IFNAR-1 chimeras and parental constructs. (A) Summary of receptor binding for all chimeras. Bars represent specific bound IFN-α2 at an input of 2.25 nM [^{32}P]IFN-α2, derived from nonlinear regression analysis of binding curves. Values for all samples were normalized to 3×10^6 cells/mL. Data presented for pcDEF1, BoIFNAR-1, and HuIFNAR-1 controls represent the mean bound IFN-α2 for six or more experiments. Data for chimeras with moderate-affinity binding are averaged from four to six independent experiments, and data for chimeras with low binding are averages of two independent experiments. (B) Summary of FACS analysis of surface receptor expression. Percent of expression compared to HuIFNAR-1 (set as 100%) where the background fluorescence for COS cells transfected with BoIFNAR-1 is set as 0% and has been subtracted from the value for all chimeras (see Experimental Procedures). Each bar represents the average and standard deviation of three to six independent transfections and cytometric analyses, with the indicated standard deviation.

BBHB) led to the complete loss of the moderate-affinity binding characteristic of BoIFNAR-1, with binding of [^{32}P]-HuIFN-α2 at or near the level of cells transfected with HuIFNAR-1 (summarized in Figure 5A).

In the set of chimeras with only one bovine subdomain introduced into a background of 3 human subdomains (BHHH, HBHH, HHBH and HHHB), no construct could direct the binding of [^{32}P]HuIFN-α2 above the low levels of COS cells expressing HuIFNAR-1 (summarized in Figure 5A). Thus, no single subdomain of BoIFNAR-1 is capable of converting the low-affinity HuIFNAR-1 to a moderate-affinity IFN binding protein.

Of the chimeras having two human subdomains and two bovine subdomains in the extracellular binding domain of the receptor, only HBBH showed significantly higher levels of binding than HuIFNAR-1; this chimera, with a K_d of about 20 nM, had similar binding characteristics to HBBB (Figures 4 and 5A). Significantly (see Discussion), neither HHBB nor BBHH, with a bovine membrane-proximal or membrane-distal half of the ectodomain, exhibited moderate-affinity binding of HuIFN-α2.

The cell surface expression of all chimeras was measured by flow cytometry, and was compared to the ligand binding results (Figure 5A,B). Anti-HuIFNAR-1 antibodies FB2, AA3, GB8, and DB2, which react specifically with SD1 through SD4, respectively (Goldman, L. A., et al., manuscript in preparation), were used to detect receptor on the transfected cells. Differences in antibody reactivity were accounted for by normalizing to the reactivity of each antibody with HuIFNAR-1 (Figure 2). All chimeras, except HBHB, showed levels of surface expression no less than 50% that of HuIFNAR-1 (Figure 5B). Only HBHB expresses poorly (generally ≤10% of HuIFNAR-1), so that no conclusions can be drawn about its ligand binding properties. The three chimeras with high levels of IFN binding showed averages of 66−93% of the surface expression of HuIFNAR-1, whereas the low binding chimeras displayed 56−136% of the surface expression of HuIFNAR-1. These results were consistent with Western blotting data on total cell extracts using the anti-hemagglutinin (HA) antibody which detected an HA epitope tag which had been placed at the amino terminus of the mature protein of all constructs (data not shown). In these semiquantitative Western blotting experiments, all constructs except HBHB displayed comparable expression (within about 50%) to BoIFNAR-1 and HuIFNAR-1. Thus, chimeras with low levels and high levels of ligand binding are expressed on the cell surface in comparable amounts (except for HBHB).

It thus appears that the bovine SD2 and SD3 domains together constitute the core of the region involved in moderate-affinity ligand binding by BoIFNAR-1: all three chimeras with moderate-affinity ligand binding share bovine SD2 and SD3, while substitution by the human homologue of either bovine SD2 or SD3 in any chimera eliminates bovine-like ligand binding. Although bovine SD1 and SD4 in the chimeras HBBB and BBBH enhance the binding above that seen with HBBH, neither bovine SD1 nor SD4, when taken in combination with either bovine SD2 or SD3, can produce moderate affinity [^{32}P]HuIFN-α2 binding.

DISCUSSION

Elucidating the detailed interactions of Type I IFNs with their receptor is important for understanding the organization of the ligand−receptor complex and the details of ligand-specific differential activation of target cells, as well as for the development of highly specific IFN agonists and antagonists. Although HuIFNAR-1 binds IFNs with low affinity (*6*, *11*−*13*), it modulates the affinity and ligand specificity of the IFNAR complex (*19*, *20*), and the presence of IFNAR-1 is crucial for cellular signaling and subsequent biological activity (*7*, *15*, *16*, *18*, *42*, *43*). A complex of IFN with HuIFNAR-1 is, under many conditions, the major product formed by covalently cross-linking IFNs to the receptor on human cells. Since low-affinity protein interactions are difficult to study, the moderate affinity of BoIFNAR-1 may facilitate an understanding of the IFN/IFNAR-1 interaction and the formation of the ternary complex of IFN, IFNAR-1, and IFNAR-2. Furthermore, the broad specificity and moderate affinity of BoIFNAR-1 for human Type I IFNs (*27*) makes the detailed analysis of BoIFNAR-1/IFN interactions inherently interesting and potentially useful. The high-level expression of BoIFNAR-1 and several chimeras in this system (ca. $(0.2-2) \times 10^6$ binding sites per cell vs $(1-2) \times 10^4$ sites per cell on untransfected of vector-transfected COS cells) effectively eliminates complicating effects of endogenous receptors, including the effects of endogenous IFNAR-2. The current focus on the IFN/IFNAR-1 interaction is admittedly "less physiological" than an analysis in the presence of IFNAR-2, but temporarily removes any complications from simultaneous IFN/IFNAR-2 and IFNAR-1/IFNAR-2 interactions, and is part of a long-range program to understand ligand recognition in the ternary IFN/IFNAR-1/IFNAR-2 complex.

We therefore constructed 14 bovine/human IFNAR-1 cDNAs and transfected them into COS cells for transient expression and analysis of ligand binding. All but one IFNAR-1 chimera were consistently expressed at high levels on the surface of COS cells (Figure 5B), as assessed by flow cytometry with a series of anti-HuIFNAR-1 monoclonal antibodies which bind to different subdomains of HuIFNAR-1. The reasons for low surface expression of HBHB are not known, despite several attempts to analyze and mitigate the problem. However, the binding data for the other 13 chimeras provides a consistent and redundant picture.

The experiments demonstrate the central importance of subdomains 2 and 3 for the binding of HuIFN-α2 by BoIFNAR-1: substitution by the human homologue of either subdomain, but not of subdomains 1 and 4, abolishes moderate-affinity binding. Since only the chimeras HBBH, BBBH and HBBB showed substantial ligand binding (Figures 4 and 5), it appears that SD2 and SD3 of BoIFNAR-1 contain residues necessary for moderate-affinity binding of HuIFN-α2. These results are corroborated by ongoing experiments where we have identified small clusters of amino acids in subdomains 2 and 3 with strong effects on ligand binding (Cutrone, E. C., unpublished data). Subdomains 2 and 3, however, are not sufficient to confer binding completely comparable to BoIFNAR-1. Although the BBBH chimera came closest in affinity to BoIFNAR-1, suggesting the interchangeability of human and bovine SD4, significant (>2-fold) decreases in affinity were observed for both HBBB

and HBBH (Table 1); i.e., bovine and human SD1 cannot be freely interchanged. Thus, residues in SD1 and SD4 may also contribute, either directly or indirectly, to ligand binding. Finally, preliminary ligand competition experiments suggest that the three moderate-affinity chimeras have a ligand specificity similar to BoIFNAR-1 for four diverse ligands (IFN-α1, IFN-α2, IFN-α8, and IFN-*β*) (Cutrone, E. C., unpublished; cf. ref *27*); this suggests the importance of SD2 and SD3 for ligand specificity.

The regions or amino acid residues involved in IFN binding by the moderate-affinity BoIFNAR-1 and by the low-affinity HuIFNAR-1 are likely to be homologous. Several studies have implicated various portions of IFNAR-1 in ligand binding. Lu et al. recently mapped the epitopes of several anti-HuIFNAR-1 mAbs that block and neutralize the activity of various IFN-αs on human cells (*44*). Amino acids in SD1 and SD3 were identified as being important for the binding of various neutralizing mAbs, but particular note was made of lysine 249 in SD3. By identifying amino acids of IFNAR-1 that are important in the epitopes for neutralizing antibodies, Lu et al. sought to infer a direct role for these residues in ligand interactions. Similarly, Eid and Tovey (*45*) localized the epitope of an anti-HuIFNAR-1 monoclonal antibody ("64G12") that blocked binding of IFN-α2 and IFN-α8 to Daudi cells to a region lying somewhere between amino acids 23−229, corresponding to SD1 and SD2. We recently demonstrated that a monoclonal antibody against HuIFNAR-1 ("EA12") can block binding and cellular activation of human Daudi cells by HuIFN-α2; however, the epitope appears complex, and was not readily localized to one or another subdomain (Goldman, L. A., et al., manuscript in preparation). Finally, Hirata et al. (*46*), using polypeptides derived from the extracellular domain of HuIFNAR-1 to compete for IFN-αB binding, localized the IFN binding region to somewhere between amino acids 261−410, corresponding to SD3 and SD4. Our data suggest that these diverse studies may not be contradictory, since segments across the ectodomain, particularly in subdomains 2 and 3, appear to be important in forming the ligand binding site.

Although the crystal structures of other cytokine/receptor complexes are often used as models for all cytokine/receptor interactions, our identification of SD2 and SD3 as the most relevant domains for ligand binding was surprising, as was the possibility of additional effects from SD1 and SD4. The ligand-binding ectodomains of receptors for growth hormone (*47*), IFN-*γ* (*48*), and erythropoietin (*49*) are composed of two Ig-like or FBN−III-like domains, which can be represented as a tandem domain structure, A:B. Similarly, the four FBN−III domains of IFNAR-1 (SD1 through SD4) might be represented as A:B:A′:B′, since the SD1 and SD3 domains are more closely related to each other, as are the SD2 and SD4 domains (*8*). Thus each of the half-structures of IFNAR-1 (SD1:SD2 and SD3:SD4) appears similar to an entire ectodomain of the receptors for growth hormone, IFN-*γ*, or erythropoietin. Similar reasoning and model-building exercises based on the growth hormone/receptor complex had suggested to others that IFN binding is likely mediated by the membrane-distal SD1:SD2 half, or the membrane-proximal SD3:SD4 half (*8*, *50*). We were thus surprised when neither of our initial two chimeras, HHBB and BBHH, possessed BoIFNAR-1-like affinity for IFN-α2 (Figure 5A). Our data suggest that IFNAR-1 may bind ligand in a manner distinct from the previously described structures of two-subdomain cytokine receptors with their ligands. Although IL-1 and its receptor are in different structural families than IFN and its receptor subunits, it is interesting that all three of the subdomains of the IL-1R ectodomain interact directly with its ligands (*51*, *52*).

In conclusion, we have found that subdomains 2 and 3 of the extracellular ligand-binding domain of BoIFNAR-1 are crucial for moderate-affinity binding of HuIFN-α2, with possible supporting roles for SD1 and SD4. Experiments are underway to more precisely define the regions and residues that play a role in binding Type I IFNs. Biochemical and biophysical studies of soluble ectodomain complexes and fragments are also necessary to elucidate the interaction between ligand and receptor and between the receptor subunits themselves, and to thereby reconstitute the essential features of the IFN/IFNAR-1/IFNAR-2 ternary complex.

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