Bovine type I interferon receptor protein BoIFNAR-1 has high-affinity and broad specificity for human type I interferons

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Abstract The type I interferon receptor (IFNAR[1]) is composed of two transmembrane polypeptides, IFNAR-1 and IFNAR-2. Human IFNAR-1 has low intrinsic affinity for IFNs, but enhances the affinity for IFNs of the complex over that of HuIFNAR-2 alone, and modulates the ligand specificity. Bovine cells respond to human alpha interferons. The bovine homologue of HuIFNAR-1, BoIFNAR-1, when expressed in heterologous cells, confers high-affinity binding and broad specificity for human type I IFNs. A soluble fusion protein of the ectodomain of BoIFNAR-1 and an immunoglobulin Fc domain was produced. In contrast to HuIFNAR-1, this protein competes strongly with human cells for IFN binding, and directly binds a wide spectrum of human type I IFNs, including diverse IFN-αs, IFN-β and IFN-ω, with moderate to high affinity. This accounts for much of the specificity for human IFNs possessed by bovine cells, with several exceptions. The BoIFNAR-1 ectodomain, in contrast to HuIFNAR-1, may be useful for studies of binary and ternary complexes with IFNs and IFNAR-2, and for purification, assay and biological neutralization protocols.

Key words: Interferon; Interferon receptor; Immunoadhesin; Fusion protein

1. Introduction

The type I interferon receptor (IFNAR) is believed to be a heterodimer of two transmembrane polypeptides, IFNAR-1 and IFNAR-2 [2–6]. The complex binds all type I IFNs: IFN-αs, IFN-β, IFN-ω, and, in ruminants, IFN-τ [7–10]. In human cells, IFNAR-2 has moderate to high affinity for diverse IFNs [3,11–16]. HuIFNAR-1 has low intrinsic affinity for IFNs ([2,17–19]; Langer, unpublished data), but contributes to ligand binding by increasing the affinity and modulating the specificity from that which is seen with IFNAR-2 alone [3,12–16,20,21]. Both subunits are required for high-affinity ligand binding and physiological responses (reviewed in [5,6]).

Bovine cells are highly sensitive to human IFN-αs and have often been used in the study of human IFN-αs [10,22–24]. Curiously, a single protein from bovine MDBK cells or tissues, but not from human Daudi cells, was sufficient for binding radiolabeled human IFN-α2 [25,26]. The broad ligand specificity of bovine cells motivated the cloning of bovine IFNAR-1 (BoIFNAR-1) [27,28], which has about 68% amino acid identity with human IFNAR-1 (HuIFNAR-1).

Data from experiments where BoIFNAR-1 is expressed on heterologous cells is consistent with BoIFNAR-1 having a major role in the broad specificity of bovine cells for HuIFN-αs. Thus, human cells transfected with BoIFNAR-1 have a bovine-like specificity, characterized by a greatly enhanced response to HuIFN-α1 (= IFN-αD), which has low binding affinity for, and activity on human cells [27]. Similarly, BoIFNAR-1 transiently expressed on COS cells or *Xenopus laevis* oocytes efficiently promoted the binding of HuIFN-α2 and IFN-α8 [17,28]. In contrast, under similar conditions HuIFNs bind weakly to HuIFNAR-1. Weak binding and covalent crosslinking of IFN was demonstrated to HuIFNAR-1 expressed on *Xenopus* oocytes [17], and a weak affinity of about 10^{-7} M was estimated for the interaction of the soluble ectodomain of HuIFNAR-1 with a fluorescent derivative of IFN-α8 [18].

It is a reasonable to hypothesize that a small number of amino acids in the ectodomain of human and bovine IFNAR-1 determine the respectively weak and strong binding of human type I IFNs. Although cellular IFNAR-1 is part of a functional receptor complex with IFNAR-2, the study of ligand interactions with isolated subunits has been a productive approach with other heteromeric cytokine receptors, such as those for IL-2 and TNF, and it would be desirable to reconstitute the ternary complex of IFN with IFNAR-1 and IFNAR-2. Human IFNAR-1 is a particular challenge because of its weak interaction with ligands, so that a study of human IFN interactions with BoIFNAR-1 may be quite useful in identifying critical regions or amino acids in IFNAR-1/IFN interactions, and in studying the stoichiometry and structure of the ternary complex.

As a first step, we produced the BoIFNAR-1 extracellular domain ('ectodomain') as a soluble fusion protein ('immunoadhesin') with the human immunoglobulin heavy chain constant domain. Here we report the ligand binding properties of this protein, and compare them to the properties of bovine cells.

2. Materials and methods

2.1. Interferons

IFNs are designated by their preferred nomenclature [29], with original or laboratory nomenclature in parentheses. IFN-α2 (= IFN-αA), IFN-α8 (= IFN-αB2), and IFN-α1 (= IFN-αD) were provided by Dr. Sidney Pestka (this department). IFN-ω was the gift of Dr. G.R. Adolf (Bender Wien, Vienna, Austria). IFN-β derived from CHO cells was from Dr. Leona Ling (Biogen Corp., Cambridge, MA). For calculations, the suppliers' determination of protein concentration was used. Several IFN dilutions from competitive binding

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[1] IFN receptors are denoted as recommended [1]. IFNAR-1 is also called: IFNAR, IFN-αR1 and IFN-Rα. IFNAR-2 has been called IFN-α/βR and IFN-αR2, and IFN-Rβ.

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experiments were assayed for antiviral activity on MDBK cells with vesicular stomatitis virus (VSV) [30] or on human HeLa-229 cells with VSV. (Note: The relatively low antiviral activity of IFN-β on MDBK cells reported here has been noted previously, but the apparent antiviral activity can be increased about 10-fold by increasing the length of the initial incubation between cells and IFN-β from 4 h to 16 h; J.A.L., unpublished data.)

2.2. BoIFNAR-1/Fc fusion protein

A recombinant fusion protein ('immunoadhesin') of the extracellular domain of BoIFNAR-1 with a human constant heavy immunoglobulin domain was expressed in Cos7 cells and purified by affinity chromatography over Protein A-Sepharose 4B (Pharmacia), as described previously for the HuIFNAR-1/Fc fusion [31]. It is referred to as 'BoIFNAR-1/Fc'.

2.3. Phosphorylation of interferon

A HuIFN-α2 analogue possessing a phosphorylation site, originally designated IFN-αA-P1, was phosphorylated to high radiospecific activity with [γ-^{32}P]ATP and bovine heart cAMP-dependent protein kinase [32]. Human [^{32}P]IFN-αA-P1 is referred to here as [^{32}P]IFN-α2.

2.4. Solid phase radioligand assay

The protocol was modified from an ELISA protocol of Mohammad R. Zafari (Biogen, Inc.; see also [16]). Immulon 2 plates (Dynatech Laboratories) were coated with 50 μl of 5 μg/ml AffiniPure Goat Anti-Human IgG Fcγ fragment (Jackson ImmunoResearch) in PBS. After 1–2 h the wells were blocked with BSA (5 mg/ml) in PBS for at least 2 h. Wells were washed once with PBS containing 0.05% Tween 20 and twice with PBS. The recombinant BoIFNAR-1/Fc fusion protein was added (50 μl/well) at 1.25 μg/ml in PBS and incubated for ≥1 h at room temperature. Plates were then washed as above. For competition binding assays, 25 μl of specific dilutions of non-radioactive IFNs diluted in 5 mg/ml BSA/PBS (or DMEM with 5% iron-supplemented calf serum) was added, followed immediately by 25 μl of [^{32}P]IFN-α2 (∼0.75–1×10^5 cpm). After 1 h at room temperature, plates were placed on ice, and were washed as above (but with ice-cold solutions) to remove unbound [^{32}P]IFN-α2. Bound [^{32}P]IFN-α2 was eluted with 1% SDS at 55° for 10 min, and added to scintillation fluid for determination of radioactivity. Competition curves were analyzed by non-linear regression to one- or two-site competition models, using the program Prism v.2.01 (GraphPad Software, Inc., San Diego, CA).

2.5. Saturation binding and competition curves

Saturation binding assays to cells were done as described [16,28,33]. Briefly, MDBK cells were trypsinized, collected in medium, and resuspended at 0.5–1.0×10^7 cells/ml. Half of the cells were treated with ≥100-fold excess non-radioactive IFN-α2 (1–3 μg/ml). Aliquots of cells with and without excess non-radioactive IFN were then combined with serial dilutions of [^{32}P]IFN-α2, and incubated while rocking for 1 h at room temperature. Cell-bound [^{32}P]IFN-α2 was separated from unbound by brief centrifugation through a cushion of 10% (w/v) sucrose in PBS. Tubes were frozen, cut, and counted to determine bound and free radioactivity. For competition binding assays, cells were prepared as for saturation binding, except that cells were resuspended to 2× concentration (usually 1–2×10^7 cells/ml). In 96-well plates, cells were combined with specific dilutions (generally 4-fold serial dilutions) of different cold IFNs in addition to [^{32}P]IFN-α2. Cells were incubated and analyzed as above. For most competition assays, binding at each concentration of non-radioactive competitor was measured in triplicate or quadruplicate. Points more than 2 standard deviations from the mean were omitted from the analysis. Suppliers' determinations of IFN concentration (in mg/ml) were used for all calculations.

Data were analyzed by non-linear regression to one- or two-site binding or competition models, using the program Prism v.2.01 (GraphPad Software, Inc., San Diego, CA). For binding curves, specific binding is defined as total binding minus non-specific binding. Competition curves were analyzed in two ways: (1) without any constraints; and (2) with the constraint that all curves in a single experiment have the same total binding in the absence of competitive ligand, and the same non-specific binding in the presence of saturating amounts of any competitor. Since the constraints are biochemically reasonable, reported IC_{50} values and figures are for constrained fits, unless indicated.

2.6. Crosslinking of labeled IFN to BoIFNAR-1/Fc

Crosslinking of [^{32}P]IFN-α2 to BoIFNAR-1/Fc was initiated by incubating [^{32}P]IFN-α2 (15 μl containing ∼120 000 cpm≈1.5×10^{-8} M final concentration) with BoIFNAR-1/Fc (5 μl of 0.2 mg/ml) for 3 h at 4°C. Parallel control reactions contained, in addition, 10 μg/ml of non-radioactive IFN-α2 or 0.1% SDS. After incubation, samples were reacted with the bifunctional crosslinking reagent BS^3 (0.5 mM final concentration; Pierce Chemical Co.) for 20 min at 4°C [17]. Crosslinked products were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on a 10% acrylamide gel [34], with BioRad Kaleidoscope molecular weight standards.

3. Results

The BoIFNAR-1/Fc protein was expressed transiently in COS cells and purified by affinity chromatography on Protein A-Sepharose 4B. When analyzed by SDS-PAGE under reducing conditions, it migrates with a M_r of ∼118 000, compared with a mass of 62 019 calculated from the polypeptide sequence (assuming a mature amino terminus of Ser-Gly-Glu; data not shown). This substantial size difference presumably reflects post-translational modifications, particularly *N*-linked glycosylation. In the absence of reducing agents, BoIFNAR-1/Fc migrates with a M_r of >200 kDa. This is consistent with the native form being a disulfide-linked dimer, probably through the immunoglobulin heavy chain, as often seen with other immunoadhesins.

In solution, the BoIFNAR-1/Fc protein binds [^{32}P]IFN-α2 to which it can be covalently crosslinked in a high molecular weight complex (Fig. 1, lane 1). The size of the covalent complex suggests that it consists of a BoIFNAR-1/Fc dimer and one or two molecules of [^{32}P]IFN-α2. This complex is not formed when excess non-radioactive IFN-α2 is present, nor when the proteins are denatured by 0.1% SDS (lanes 2, 3).

As expected for an efficient IFN binding protein, BoIFNAR-1/Fc can compete in solution for the high-affinity binding of [^{32}P]IFN-α2 to human Daudi cell (Fig. 2). The IC_{50} for BoIFNAR-1/Fc was 1.3–1.7 nM under these conditions. Using an approximate value of 1×10^{-10} M for the K_d of [^{32}P]IFN-α2 on Daudi cells, the calculated K_i for BoIF-

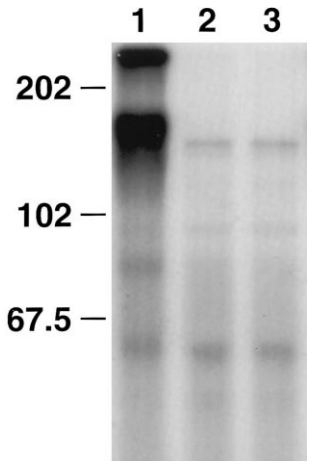

Fig. 1. Covalent complex of [^{32}P]IFN-α2 and BoIFNAR-1/Fc. BoIFNAR-1/Fc was incubated with [^{32}P]IFN-α2 in the absence (lane 1) or presence (lane 2) of 10 μg/ml nonradioactive IFN-α2, or under denaturing conditions (0.1% SDS; lane 3). Samples were covalently crosslinked with BS^3 reagent (Pierce Chemical Co.), analyzed on a 10% acrylamide gel, and autoradiographed. M_r values were determined relative to prestained protein standards (GIBCO) and 'Kaleidoscope' prestained markers (Biorad).

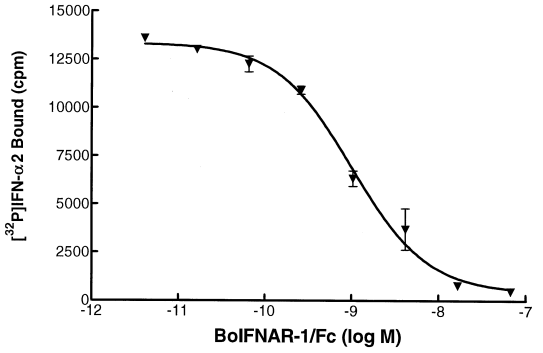

Fig. 2. Inhibition of [^{32}P]IFN-α2 binding to Daudi cells by BoIFNAR-1/Fc. Daudi cells were incubated with the indicated concentrations of BoIFNAR-1/Fc (assuming a mass of 1.2×10^5 for the covalent dimer) for 15 min at room temperature. [^{32}P]IFN-α2 was added (about 7×10^4 cpm; ca. 6.2×10^{-10} M) and incubated at room temperature for 1 h.

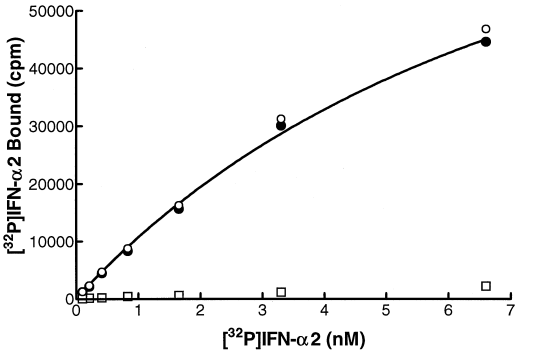

Fig. 3. Binding of [^{32}P]IFN-α2 by immobilized BoIFNAR-1/Fc. Increasing amounts of [^{32}P]IFN-α2 were added in the absence and presence of 2 μg/ml non-radioactive IFN-α2. The solid line is a non-linear regression fit to the calculated specific binding, with a one-site binding model. (○) Total binding; (□) non-specific binding; (●) specific binding.

NAR-1/Fc is 1.4×10^{-10} M, demonstrating high affinity. This competition is as efficient or more so than several well characterized neutralizing monoclonal antibodies (LI-1, LI-8; [35]) directed against the ligand IFN-α2 (data not shown).

A convenient radioligand binding assay (RLA) was developed with BoIFNAR-1/Fc immobilized non-covalently on a microtiter plate through goat anti-human IgG adsorbed to the plate. In this format, [^{32}P]IFN-α2 was efficiently bound to the immunoadhesin (Fig. 3), with very low non-specific binding. From these data, an affinity of BoIFNAR-1/Fc for IFN-α2 was estimated at about 10 nM. However, the high amount of BoIFNAR-1/Fc bound to the plates, with an attendant high capacity for IFN binding, makes this an upper estimate for the true affinity, which is likely to be closer to the K_i of 0.14 nM.

The RLA provides a convenient format to explore the ligand specificity of BoIFNAR-1. Diverse non-radioactive type I IFNs were tested for their ability to compete with [^{32}P]IFN-α2 for binding to BoIFNAR-1/Fc. All human ligands tested – IFN-α2, IFN-α1, IFN-α8, IFN-β, and IFN-ω – competed for binding, with IC_{50} values ranging from 7×10^{-10} to 8×10^{-8} M (Fig. 4A,B; Table 1). The affinity of the ligands varied in the order: IFN-α2 ≈ IFN-α1 > IFN-ω > IFN-α8 > IFN-β. The same ligands were tested for their ability to compete with [^{32}P]IFN-α2 for binding to bovine MDBK cells, and for their antiviral activity on MDBK cells (Table 1).

For comparison, and as an internal control for the integrity and activity of the interferons, binding and antiviral activities are also presented for human Daudi cells and HeLa cells, respectively (Table 1). On human cells, the human IFNs display their typical activities and affinities in the general order IFN-α8 > IFN-α2, IFN-ω and IFN-β. IFN-α1 showed considerably lower biological activity and receptor affinity, as consistently reported.

4. Discussion

Because of the serendipitous finding of broad specificity and high affinity of bovine cells for human type I IFN-αs, the bovine homologue of IFNAR-1 was cloned and characterized [17,27,28]. The ectopic expression of BoIFNAR-1 in mouse and human cells, which have endogenous type I IFN receptors, conferred on these cells a specificity similar to that of bovine cells [27,28]. With COS cells expressing BoIFNAR-1 at levels about 100-fold above the endogenous type I receptor, or with *Xenopus* oocytes, which show no evidence of an interferon system, BoIFNAR-1 confers high affinity binding of HuIFN-α2 and IFN-α8 [17,28]. This contrasts starkly with

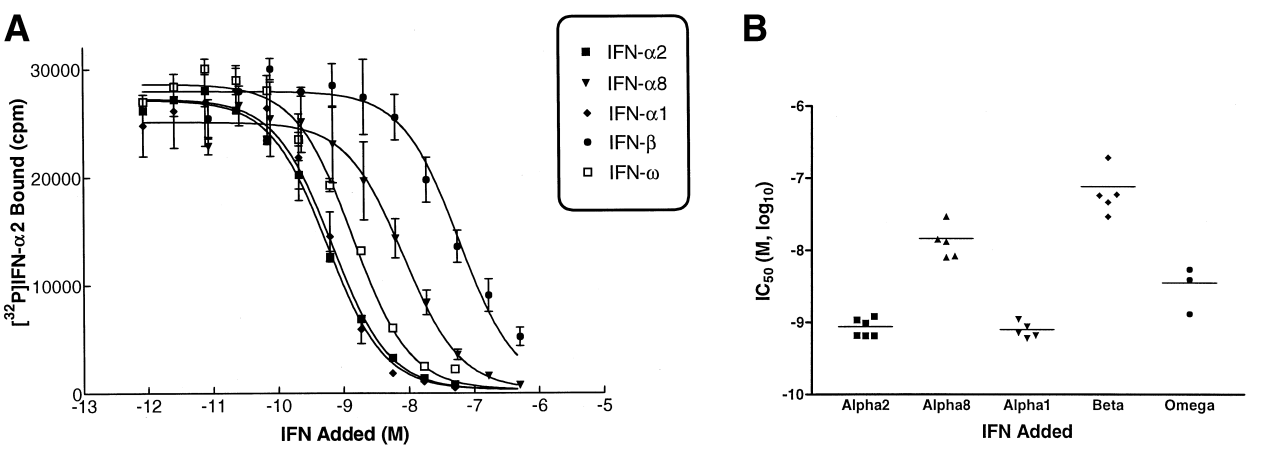

Fig. 4. Competition of type I IFNs for [^{32}P]IFN-α2 binding to BoIFNAR-1/Fc. A: Representative data. B: Compilation of IC_{50} values from independent competition experiments for each ligand. Each point represents an independent determination of the IC_{50}. Horizontal lines represent the average of the values.

Table 1
Activity of human type I interferons

Substrate[a]	Assay[b]	IFN-α2	IFN-α8	IFN-α1	IFN-β	IFN-ω
BoIFNAR-1/Fc	IC_{50} (M)	7.6×10^{-10}	1.5×10^{-8}	7.7×10^{-10}	8×10^{-8}	4.6×10^{-9}
MDBK	IC_{50} (M)	1.1×10^{-10}	1.5×10^{-10}	7.6×10^{-11}	1.1×10^{-8}	9.3×10^{-11}
MDBK	AVA (u/mg)	2.3×10^{8}	5.1×10^{8}	2.4×10^{8}	1.9×10^{6}	1.9×10^{8}
Daudi	IC_{50} (M)	7.6×10^{-10}	3.6×10^{-10}	$>3.8\times10^{-9}$	6.9×10^{-10}	1.8×10^{-10}
HeLa	AVA (u/mg)	8.6×10^{7}	4×10^{8}	5.6×10^{6}	2.4×10^{7}	n.d.
HuIFNAR-2/Fc[c]	IC_{50} (M)	$2–4\times10^{-10}$	6.3×10^{-9}	1.1×10^{-6}	1.3×10^{-8}	1.4×10^{-9}

[a]Substrates are the BoIFNAR-1/Fc fusion protein, bovine MDBK cells, human Daudi and HeLa cells, and the HuIFNAR-2/Fc fusion protein (see [16]).
[b]Assays include competition of binding against [^{32}P]IFN-α2, to produce IC_{50} values (in units of molar concentration) and antiviral assays (AVA) measured as antiviral units/mg protein. Antiviral assays for MDBK cells represent an average of 3 determinations for IFN-αs, 2 for IFN-β, and 1 for IFN-ω. Binding assays for BoIFNAR-1/Fc represent 3–6 independent determinations (see Fig. 4B).
[c]Data from [16].
n.d., not done.

the weak binding of type I IFNs by HuIFNAR-1 [2,16–19], and the ability of HuIFNAR-1 to confer on murine cells significant response to HuIFN-α8, but not other human type I IFNs [2]. Indeed, binding experiments with a human IFNAR-1/Fc construct analogous to that described here for BoIFNAR-1 failed to detect significant binding of ^{32}P-labeled IFN-α2 or IFN-α8 (J.A.L. and L.E.L., unpublished data). Thus, BoIFNAR-1 plays a major role in differential binding of human IFNs on bovine cells, whereas, on human cells, HuIFNAR-2 plays the dominant role in differential ligand binding.

Here we have demonstrated that a soluble form of the extracellular domain of BoIFNAR-1 binds [^{32}P]IFN-α2 with high affinity (0.1–1 nM), and has a very broad specificity for human type I IFNs, including diverse IFN-αs, IFN-β and IFN-ω. A comparison of the binding activity of human type I ligands for BoIFNAR-1 with ligand specificity data toward the native receptor on bovine cells is instructive (Table 1); because of the different assay conditions for binding to MDBK cells and to immobilized BoIFNAR-1/Fc, the ratio of IC_{50} values for each substrate is more informative than their absolute values. In competition with [^{32}P]IFN-α2 for binding to bovine MDBK cells, four of the ligands (IFN-α2, IFN-α8, IFN-α1, and IFN-ω) demonstrate similar high affinity to that seen with BoIFNAR-1/Fc, while IFN-β has an affinity almost 100-fold lower. These results roughly correlate with the antiviral activity on these cells, which is high for the high-affinity ligands and much lower for IFN-β. For the ligands IFN-α2, IFN-α1, IFN-β, the inhibition constants are 7–10-fold higher for the BoIFNAR-1/Fc protein than for MDBK cells. In contrast, the IC_{50} values for IFN-α8 and for IFN-ω on the BoIFNAR-1/Fc protein are, respectively, 100 and 49 times higher than the IC_{50} values on MDBK. These enhanced differences for IFN-α8 and IFN-ω between MDBK cells and the soluble BoIFNAR-1/Fc protein suggest a stronger dependence on another cellular component (BoIFNAR-2?) to approximate the ligand specificity of bovine cells.

Expression in heterologous cells of BoIFNAR-1 and BoIFNAR-2, singly and together, will permit further dissection of the relative contributions to the ligand specificity, complementing our previous studies of the contributions of human IFNAR-1 and HuIFNAR-2 to ligand specificity [16]. The comparison of the ligand specificity of human IFNs toward bovine components and human receptor components may allow us to determine, in principle, whether the proper functioning of a two-component receptor requires one high-affinity and one low-affinity component, as seen with the native human type I IFN receptor (HuIFNAR) and with many other cytokine receptors, or whether the system can operate efficiently with two moderate-to-high affinity receptor chains.

The ability of BoIFNAR-1/Fc to bind a wide array of human type I IFNs contrasts strongly with the weak binding of these same ligands to HuIFNAR-1. We are exploiting the differential ligand binding by human and bovine IFNAR-1 to map the ligand binding site on IFNAR-1 (L.A. Goldman et al., submitted for publication).

BoIFNAR-1 is a useful alternative to the low-affinity HuIFNAR-1 for studying the binary and ternary complexes of IFNs with IFNAR-1 and IFNAR-2. Moreover, in binding all the human type I IFNs examined, it has a broader specificity than either the soluble ectodomain of human IFNAR-2 (Table 1), or than various anti-IFN monoclonal antibodies. BoIFNAR-1 may thus provide a broad-spectrum reagent for the assay, purification, and inhibition of human type I IFNs.

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