Communication

Reconstitution of a High Affinity Binding Site for Type I Interferons*

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The type I interferon (IFN) receptor complex is assumed to be composed of multiple protein subunits. Recently, two proteins have been identified as potential receptor components, both of which share a high degree of structural homology with the immunoglobulin superfamily. One of these proteins, referred to as the human interferon α receptor (IFNAR), has been shown to be involved in interferon signal transduction, but it does not bind IFN with high affinity. A second putative receptor protein, named FLP40, has been cloned from human Daudi cells. Transfection of FLP40 into murine NIH 3T3 cells does not result in high affinity IFN binding. In this study, we demonstrate that when expressed in murine L929 cells neither IFNAR nor FLP40 by themselves are capable of binding human IFN-α8. Co-expression of IFNAR and FLP40 results in cells capable of binding IFN-α8 and IFN-α2. Scatchard analysis of binding demonstrated the presence of high (Kd, 350 pm) and low (Kd, 4.0 nm) affinity binding sites. Binding of radiolabeled IFN-α8 can be competed with either unlabeled IFN-α8 or a recombinant form of human interferon β, IFN-β2b, but not with IFN-γ. Ligand binding of IFN-α8 can be inhibited by antibodies directed against IFNAR providing further support for a role for this protein in the formation of a ligand binding site. This is the first demonstration indicating that two previously identified IFN receptor proteins, which individually do not bind type I IFN with high affinity, cooperate in the formation of a type I IFN receptor ligand binding complex.

Ligand-receptor cross-linking experiments (1, 2) suggest that a functional type I interferon (IFN) receptor complex is comprised of multiple components much like that of the previously described IFN-γ, interleukins, and granulocyte-macrophage colony-stimulating factor receptor families (3–6). However, the identity and mechanism of interaction between proteins comprising the human type I IFN receptor complex is unclear. Two proteins have recently been identified as potential components of the human type I IFN receptor complex (7, 8). One, the human interferon α receptor (IFNAR) was identified by its ability to reconstitute an antiviral response to IFN-α8 in a murine cell line (7, 9). Monoclonal antibodies directed against the extracellular region of IFNAR have been shown to block the antiviral activity of a number of type I IFNs (10). It appears, however, that neither human IFNAR nor the purified extracellular domain of IFNAR is able to mediate high affinity binding (11, 12). Although Daudi cells, from which IFNAR was cloned, bind high levels of type I IFN at 20°C, murine cells expressing human IFNAR could only be demonstrated to bind ligand at 37°C in which binding values were expressed as cellular uptake rather than direct receptor binding (7). Indeed, it was later shown that murine cells expressing IFNAR did not bind detectable levels of type I IFN when ligand binding was measured under conditions which minimized endocytosis (11). Recent studies in which human IFNAR was expressed in Xenopus laevis oocytes (13) confirmed the notion that IFNAR alone binds type I IFN with very low affinity. Finally, it has been shown that IFNAR becomes phosphorylated by IFN-α and IFN-β but not IFN-γ in a ligand-dependent manner demonstrating again its involvement in type I IFN signaling (14).

A second IFN receptor protein, P40, identified by Novick et al. (8), was purified as a soluble protein from human urine by its ability to bind to an IFN-α2 affinity column. A variation of this protein also appears as a membrane-bound form in Daudi cells where it contains a transmembrane and cytoplasmic domain (8). We refer to the membrane-bound form of this protein as full-length P40 (FLP40). Antibodies directed against the soluble form of this protein block the antiviral activity of a number of human type I IFNs on human WISH cells (8). Although P40 binds to immobilized IFN-α2, it appears to do so with only low affinity. This is implied by the observation that the membrane-bound form of this protein when expressed on murine NIH 3T3 cells could only be demonstrated to bind low levels of ligand after extensive cross-linking (8).

Neither IFNAR nor FLP40 alone appears to be able to act as a receptor capable of high affinity ligand binding. Therefore, it seems likely that in the case of the type I IFN receptor a ligand-induced association between two or more proteins would be required to form a functional type I IFN receptor complex capable of specific high affinity ligand binding. In this report we describe the results of co-expressing IFNAR and FLP40 in a L929 murine cell line. The co-expression of these two proteins in L929 cells results in the formation of a high affinity ligand binding site for human type I IFNs in a cell type initially lacking the ability to bind type I IFNs.

EXPERIMENTAL PROCEDURES

Cell Lines—Human Daudi cells and murine L929 cells were obtained from the American Type Tissue Culture (ATTC). Murine L929 cells were maintained in Dulbecco’s modified Eagle’s media and Daudi cells in RPMI 1640. Media in both cases were supplemented with 10% fetal bovine serum, 2.0 mM L-glutamine, 200 units/ml penicillin, and 60 μg/ml streptomycin. Following transfection, murine cells were selected in media supplemented with 1.5 mg/ml G418 (Life Technologies, Inc.) or 0.6 mg/ml hygromycin.

Plasmid Construction and Transfection Protocol—IFNAR cDNA was kindly provided by Dr. Gilles Uze, Institut de Genetique Moleculaire,
CNRS, Montpellier, Cedex 1, France and cloned into a plasmid containing the cytomegalovirus promoter, SV40 origin of replication (pb-SE9R99), and a neomycin gene for resistance to Geneticin (G418). FLP40 was cloned from Daudi cell mRNA using the reverse transcriptase-polymerase chain reaction (PCR). Single-strand cDNA was synthesized from mRNA isolated from Daudi cell using Superscript II (BRL).

Two primers (5'-GGCCAGACGCTCGAAGTTTAATT and 5'-AGAAA-CATTGGACAAACGGAGAAA) corresponding to the 5'- and 3'-untranslated region of P40 (8) were used to generate a full-length form of P40 cDNA (FLP40) by reverse transcriptase-PCR. The PCR fragment was then cloned into pTA vector (Invitrogen). Nucleotide sequence analysis of the cloned cDNA showed two nucleotide changes when compared to the published sequence (8) which resulted in an amino acid change in the signal peptide region (Val10 to Phe), and one in the intracellular region (Thr105 to Ala). For expression in mammalian cells, FLP40 cDNA was first cloned into an expression vector (pbSE9R97) which contains the myeloproliferative sarcoma virus promoter, a hygromycin resistance gene, and an SV40 origin of replication (15).

L929 cells were transfected using LipofectAMINE according to the protocol from the supplier (5 µg/ml LipofectAMINE for 5 h in Opti-MEM I media) (Life Technologies, Inc.). After transfection, cells were placed in selection media, and individual clones were selected by limited dilution. The resultant cell line expressing only IFNAR was referred to as L929R1, and L929R1 was expressed using the anti-human IFNAR monoclonal antibody, 4B1, to L929R1. Specific binding (open circles), nonspecific binding in the presence of 100-fold excess unlabeled monoclonal antibody (closed circles), and binding of $^{125}$I-4B1 to parental L929 cells (squares) is shown. Data represent mean values of $n = 2$, and variation between replicates was less than 15%.

Mouse L929 cells (L929) have previously been shown to be effective-antiviral assay (not shown). Direct binding assays on Daudi (10$^6$ cells/ml) were performed at 20°C for 90 min. After ligand binding, Daudi cells were collected by centrifugation through a cushion of silicone oil prior to determination of bound radioactivity. Binding assays using adherent mouse L929 cells were performed on 35-mm dishes in which cells were grown to near confluence. After ligand binding, cells were washed in cold media as described above and solubilized in 2.0% sodium dodecyl sulfate (SDS) prior to scintillation counting. Nonspecific binding was determined by incubation with 100-fold excess unlabeled ligand. Recombinant human IFN-α8 was kindly provided by Ciba-Geigy AG, Basel, and recombinant human IFN-β1b was purified as described previously (17).

**RESULTS AND DISCUSSION**

Selection of Murine Cells Expressing Type I IFN Receptor Proteins—Mouse L929 cells (L929) have previously been shown not to be able to bind or produce an antiviral response to human type I IFNs at concentrations which induce such responses in human cells (7, 11, 19). Therefore, this cell line was chosen for transfection studies in which the function of individual human type I IFN receptor subunits could be examined. As a first step in studying the role of IFNAR and FLP40 in ligand binding, stable L929 cell lines expressing only IFNAR (L929R1) were generated. The surface expression of IFNAR on L929R1 cells was confirmed by making use of a monoclonal
antibody (4B1) which is specific for human IFNAR (12, 14). Iodinated 4B1 was used in direct binding studies to demonstrate the presence of IFNAR on the surface of L929R1 cells (Fig. 1, A and B). Scatchard analysis of binding curves revealed that binding of 4B1 detected 17,000 ± 2000 antibody sites/cell (mean ± S.E., n = 2) on L929R1 cells. Scatchard analysis of binding curves was also performed on L929R12 cells (Fig. 1C) and showed that binding of 4B1 (10,234 ± 3,000 antibody sites/cell, mean ± S.E., n = 2). Therefore, L929R1 and L929R12 cells express similar amounts of IFNAR on their cell surface. The expression of IFNAR on these cells was also confirmed by fluorescent-activated cell sorting (FACS) analysis using the monoclonal antibody 4B1 (not shown).

Next we generated L929 cell lines expressing either FLP40 (L929R12) or both IFNAR and FLP40 (L929R12). L929R12 cells were produced by transfecting into L929R1 cells a plasmid containing only the cDNA encoding FLP40. In Daudi cell mRNA (8) and was not detected in cells transfected with a plasmid containing a gene encoding FLP40 as described under “Experimental Procedures.” FLP40 mRNA in L929R1 and L929R12 cells was confirmed by demonstrating the presence of a 1.5-kb band corresponding to the expected size of FLP40 mRNA (Fig. 2) (8). As expected, the 4.3-kb band, presumed to represent a soluble truncated receptor (8), was visible only in blots using Daudi cell mRNA (8) and was not detected in cells transfected with a plasmid containing only the cDNA encoding FLP40. In all L929R12 cell lines tested (8 individual clones), it appeared that co-expression of IFNAR and FLP40 resulted in an apparent increase in the amount of FLP40 mRNA. A second faint variable band of 3.0 kb was sometimes observed in L919R12 cells. However, the presence or absence of this band did not correlate with ligand binding. The monoclonal antibody 4B1 was used to confirm that the expression of IFNAR in L929R12 cells was similar to that observed in L929R1 cells (Fig. 1).

Human Type I IFN Binding to Murine Cells—L929R1, and L929R12 cells were evaluated for their ability to bind IFN by measuring the binding of increasing concentrations (25–750 pM) of phosphorylated IFN-α8 and IFN-α2 in the presence or absence of 100-fold excess unlabeled ligand (Fig. 3, A and B). We could not demonstrate ligand binding to either L929R1 or L929R12 cells. The lack of ligand binding to L929R1 cells confirms previous results in which the binding of type I IFNs could not be detected in L929 cells expressing IFNAR (11). Next, we determined the ability of L929R12 cells, which express both IFNAR and FLP40, to bind labeled ligands. Both IFN-α8 and IFN-α2 specifically bound to L929R1 cells (Fig. 3, A and B). Saturation ligand binding could be demonstrated, and Scatchard analysis of the binding data demonstrated the presence of 31,000 ± 5,000 high affinity and 125,640 ± 20,000 low affinity sites/cell (mean ± S.E., n = 2). The Kd of IFN-α8 binding for the high affinity site was 350 pM ± 80 pM (mean ± S.E., n = 2) whereas the Kd of binding to the high affinity site was 4.5 nm ± 1.0 nm (mean ± S.E., n = 2). The affinity of ligand binding to L929R12 cells was similar to that observed on Daudi cells (20).

Both IFN-α8 and IFN-β1b were able to compete binding of radiolabeled IFN-α8 (100 pm) to L929R12 cells (Fig. 4). Unlabeled IFN-α8 exhibited an IC50 of 500 pm, while unlabeled IFN-β1b competed for the binding of radiolabeled IFN-α8 with an IC50 of 50 pm. A similar result was observed using Daudi cells (not shown).

To investigate further the role of IFNAR in the formation of a ligand binding complex, we performed studies using a polyclonal antisera directed against the extracellular region of IFNAR. This antiserum reacts with IFNAR present on the surface of Daudi, L929R1 and L929R12 cells as judged by FACS analysis (not shown). Preincubation of cells with this antisera inhibited IFN-α8 binding to both Daudi and L929R12 cells by 80–90% (Fig. 5A). A dose-response curve demonstrating the antibody induced inhibition of IFN-α8 binding to L929R12 cells is shown in Fig. 5B. The blocking of ligand binding by the anti-IFNAR antibody supports the suggestion that IFNAR interacts with an additional component(s) which together form a receptor complex that can bind with high affinity either IFN-α8, IFN-α2, or IFN-β1b.

The data presented here indicate that IFNAR and FLP40 cooperate to form a high affinity ligand binding site for type I IFNs. Previous examples of other cytokine receptors, such as interleukin receptors, suggests this class of cell surface receptors form multicomponent complexes (5, 6, 21). Such a receptor complex is thought to bring together proteins which act to form a high affinity ligand binding complex. Once this occurs, cytosolic protein kinases (eg. Jak, Tyk2) are activated and phosphorylate specific tyrosines located within the cytoplasmic C-terminal region of the receptor (22, 23). The potential interaction between various proteins forming such a receptor

![Figure 3](image-url)
A Type I IFN High Affinity Ligand Binding Site

have been suggested in structural models for the type I IFN receptor (24, 25). In line with other known cytokine families, it is likely that the human type I IFN receptor is comprised of multiple protein subunits and that the interaction of these subunits is a prerequisite for ligand binding or signaling. We have tested this hypothesis by first demonstrating that murine cell lines L929R1 and L929R2, which stably express either IFNAR or FLP40, respectively, do not specifically bind IFN-α or IFN-β at levels detectable by saturation binding. However, when we attempted to establish ligand binding by producing an additional cell line, L929R12, expressing both IFNAR and FLP40, we observed specific binding of IFN-α and IFN-β to this cell. There are several findings that suggest the possibility that other proteins may be involved in the specific binding of some type I IFNs. First, our finding that IFN-β1b appears to be more efficient in competition binding than IFN-α (Fig. 4). Second, the recent description (26, 27) and our finding (28) of a IFN-β-specific IFNAR-associated surface protein. It appears likely that although IFNAR does not bind ligand with high affinity, it is capable of interacting with other receptor components. Such an interaction between IFNAR and ligand is further suggested by the observation that IFNAR can become phosphorylated in a ligand-dependent manner by both IFN-α and IFN-β.

One final issue to be addressed is that of biological response in our L929R12 cells. To address this, we are currently investigating the functional response of L929R12 cells to various human type I IFNs in order to correlate ligand binding with biological activity.

Understanding the process by which these components interact with each other will help to shed some light on the mechanism by which IFNAR and FLP40 form a ligand binding site on L929R12 cells. Such studies can serve as a starting point for identifying the protein components of the human type I IFN receptor. Studying the mechanism in which receptor proteins assemble to form a multicomponent type I IFN receptor complex in different cells will yield valuable insights into early events of type I IFN signaling in addition to helping define tissue-specific IFN responses.

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Note Added in Proof—A paper appeared after the submission of this Communication (29) in which a somewhat similar result in murine NIH 3T3 cells was observed. In agreement with our results, co-expression of IFNAR and FLP40 in murine NIH 3T3 cells generated a high affinity ligand binding site.

Fig. 4. Binding of IFN-α8 to L929R12 cells in the presence of unlabeled IFN-α8 or human IFN-β1b. Radiolabeled IFN-α8 (150 pm) was allowed to bind to L929R12 cells in the presence of increasing concentrations of unlabeled IFN-α8 (circles) or IFN-β1b (squares). IFN-β1b appears to compete for the binding of radiolabeled IFN-α8 more efficiently than does IFN-α8 itself.

Fig. 5. Inhibition of IFN-α8 binding to Daudi and L929R12 cells using a polyclonal antibody recognizing IFNAR. A, IFN-α8 (250 pm) binding to 105 L929R12 cells/ml and 105 Daudi cells/ml was performed in the presence of a polyclonal antiserum (1:100 dilution, solid bars) directed against IFNAR. The antiserum inhibited the binding of phosphorylated IFN-α8 to L929R12 cells (L929R12) and Daudi cells. Values shown are the mean for three experimental points ± S.E. Premimmune serum (1:100 dilution, open bars) did not inhibit ligand binding to Daudi or L929R12 cells. Percent of control binding of IFN-α8 is equal to the amount of IFN-α8 bound in the absence of antiserum. B, dose-response curve for the antibody induced inhibition of IFN-α8 (250 pm) binding to L929R12 cells. Data represent the mean of two experimental points (S.E. = ± 10%). Antiserum concentration is represented as the log of the dilution.

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