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LIGAND-INDEPENDENT INTERACTION OF THE TYPE I INTERFERON RECEPTOR COMPLEX IS NECESSARY TO OBSERVE ITS BIOLOGICAL ACTIVITY

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Abstract

Ectopic coexpression of the two chains of the Type I and Type III interferon (IFN) receptor complexes (IFN- R1 and IFN- R2c, or IFN- R1 and IL-10R2) yielded sensitivity to IFN-alpha or IFN-lambda in only some cells. We found that IFN- R1 and IFN- R2c exhibit FRET only when expressed at equivalent and low levels. Expanded clonal cell lines expressing both IFN- R1 and IFN- R2c were sensitive to IFN-alpha only when IFN- R1 and IFN- R2c exhibited FRET in the absence of human IFN-alpha. Coexpression of RACK-1 or Jak1 enhanced the affinity of the interaction between IFN- R1 and IFN- R2c. Both IFN- R1 and IFN- R2c exhibited FRET with Jak1 and Tyk2. Together with data showing that disruption of the preassociation between the IFN-gamma receptor chains inhibited its biological activity, we propose that biologically active IFN receptors require ligand-independent juxtaposition of IFN receptor chains assisted by their associated cytosolic proteins.

Keywords

FRET; interferon receptor; spectral deconvolution; receptor reconstitution; confocal fluorescence spectroscopy; RACK-1

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DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

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POLICY AND ETHICAL STATEMENT

The work in this manuscript was carried out in accordance with 'The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans', with the 'EU Directive 2010/63/EU for animal experiments', and with the 'Uniform Requirements for manuscripts submitted to Biomedical journals'.

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1. Introduction

Type I interferons (IFNs), released in response to the detection of a viral infection, promote a virus-resistant state in cells and assist the immune system to detect and eliminate viruses and virus-infected cells. All activities of Type I IFNs are mediated by their interaction with the Type I IFN receptor complex [1;2], also known as the IFN- α or IFN- β receptor. The IFN- α R2c (also known as IFNAR-2C) chain interacts with not only the tyrosine kinase Jak1 but also the transcription factor Stat2. The IFN- α R2b splice variant encodes a transmembrane receptor chain with a truncated intracellular domain that binds neither Stat2 nor Jak1. The IFN- α R1 (also known as IFNAR-1) chain interacts with the tyrosine kinase Tyk2.

An important question is whether the Type I IFN receptor complex in its resting state is a preassembled entity or is dissociated prior to its nucleation by Type I IFN. Based on crosslinking studies, immunoprecipitations, and studies with soluble receptors, it is currently accepted that the Type I IFN receptor complex is dissociated until ligand is added, and IFN- α R1 and IFN- α R2c interact only indirectly through the ligand [3–7]. These studies are described in depth in Supplementary Text 2. Because these studies require analyzing the receptor outside of its native environment (where bias favoring high-affinity interactions that are environmentally insensitive is introduced), we wanted to use technologies that remove this bias by analyzing receptor structure in their physiological environment (i.e., within intact, where environmentally-sensitive and low-affinity interactions are maintained) in order to obtain more physiologically accurate data.

We successfully utilized fluorescence resonance energy transfer (FRET) to noninvasively probe the structure of the IFN- α and the interleukin-10 receptor complexes. Observing FRET between IFN- α R1 and IFN- α R2 and between IL-10R1 and IL-10R2 in the absence or presence of ligand, we concluded that the IFN- α and the IL-10 receptor chains interact in the absence of ligand, inferring that conformational changes accompany the activation of the receptor complex by ligand [8–10]. We confirmed the specificity of these interactions by coexpressing receptor chains from different receptor complexes and by mutating the IFN-receptor chains to inhibit biological activity; FRET between receptor chains was reduced substantially in most cases, providing evidence that mutations that eliminate biological activity also inhibit the interaction between IFN- α R1 and IFN- α R2.

Whether IFN- α and IFN- α receptor complexes are similarly associated in a ligand-independent manner is the subject of these current studies.

2. Materials and Methods

2.1 Genetic engineering and modification of proteins

All restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs and used according to the manufacturer's instructions. Primers were obtained from Integrated DNA Technologies (Coralville, IA) and were used as provided. All primers in this manuscript are written in the 5'-3' orientation. The synthesis of the various cDNA's used in this manuscript is described in Supplementary Text 1 of this manuscript and elsewhere [11].

2.2 Interferons

Human IFN- α 2a (also known as IFN- α A, #11100-1) and human IFN- α (#11500-1) were provided by PBL Biological Laboratories (Piscataway, NJ). The biological activity of human IFN- α 3 (recombinant, from conditioned medium of 293T cells, a gift from PBL Biomedical Laboratories) was assayed by the cytopathic effect inhibition assay in HepG2

cells challenged with EMCV. This assay was validated with human IFN- γ -His (#11820-1). From these assays, half-maximal cytopathic effect inhibition is defined as one unit/mL (U/mL).

2.3 Cell culture

Cell lines were grown in HEPA-filtered incubators at 37 °C, 5% CO₂, and 95% relative humidity. CHO-q3 cells [8] were grown in F12 medium containing 10% fetal calf serum (FCS), while 293T cells were grown in DMEM medium containing 10% FCS and 4 mM glutamine.

To obtain stably transfected cell lines, geneticin (350 µg/mL) was added to the medium three days after transfection to select for transfected cells harboring genomically integrated plasmid until expanded colonies were visible to the naked eye (two to three weeks). A sample of these cells were diluted to single cells in 96-well dishes to isolate clonal colonies. The clones were analyzed by light fluorescent microscope using the FITC and TR channels to visually identify colonies harboring GFP and OFP. The clones mentioned in this manuscript were green and orange when GFP or OFP were respectively directly excited (g +o), were weakly orange when OFP was directly excited (wo), or had such weak fluorescence that it was essentially nonfluorescent (nf). Selected clones were further amplified and screened for their ability to induce MHC Class I or be protected from viral cytotoxicity in response to human IFN- γ .

2.4 Confocal Fluorescence Spectroscopy

FRET between fluorescent protein donor:acceptor pairs ECFP and EYFP in intact cells was measured with an instrument in which a spectrometer is integrated into the sidebox of a confocal microscope, as described previously [9;11–13].

2.5 Data analysis

Spectral deconvolution (the mathematical separation of an observed emission spectrum into several spectra of homogeneous components) was described previously [10;12;13]. The improved analysis of deconvolved spectra, calculation of FRET efficiency, other cellular biophysical quantities, and the inter-FP distances are described elsewhere [11].

2.6 Interferon activity assays

The antiviral assay is based on cytopathic effect inhibition, and was performed as described previously with minor modifications [14;15]. Briefly, 50,000 cells are plated in each well of a 96-well plate. IFN is next added in two-fold serial dilutions throughout a row, and is incubated with the cells for 24 hours. Afterward, vesicular stomatitis virus (VSV, Indiana Strain) is added at 1250 pfu/well. The virus is incubated with the IFN-treated cells for 48 hours or until nearly complete killing of negative-control cells (that were not incubated with IFN) but not of positive control cells (that were incubated with neither IFN nor virus) is observed. Media is removed by inversion of the plates, and cells that survived viral infection are stained with crystal violet. Results are scored visually.

IFN also induces MHC Class I surface antigen in a dose-dependant fashion. The induction of MHC Class I Surface antigen by IFN was performed as previously described [8;15–18].

2.7 Fluorescence-Activated Cell Sorting

Flow cytometry was performed with a Becton-Dickinson FACScan as previously described [13]. In two-color experiments, the FL1 (530/30 nm barrier emission filter) and FL3 (650 long-pass emission filter) channels were compensated against the FL2 (585/42

barrier emission filter) channel prior to data collection to minimize the contribution of EGFP fluorescence in the FL2 channel and the contribution of StFP fluorescence into the FL1 channel. A positive gate on a forward scatter:side scatter dotplot was used to isolate intact cells.

Cells expressing StFP-labeled or EGFP-labeled receptors that were not treated with antibodies were identified and gated in a FL1:FL2 dotplot as events that were found at different dispersion angles from cells exhibiting only endogenous fluorescence. Gates were drawn to fully exclude cells that contain only endogenous fluorescence, accepting that cells with insignificantly low levels of acceptor and donor fluorescence will also be excluded with this negative gate. These gates were not changed among the various transfected populations. Because CHO-q3 cells constitutively express human Class I MHC surface antigen HLA-B7 on their cell surface, cells treated with primary anti-MHC Class I antibodies and FITC-conjugated secondary antibodies were shifted considerably along the FL1 axis whether they responded to IFN or not; induction by IFN further shifted only the subpopulation that is sensitive to IFN. To score for induction of MHC by IFN, a gate was created so that the left edge of the gate was positioned between the densest part of the IFN-inducible population and the densest part of the uninduced population, where the event density was minimal. The gate was held stationary between untreated and treated cells, and the percentage of cells within each gate was measured. The fraction of cells that induced MHC Class I surface antigen was taken as the difference in the percentage of gated cells in the ligand-treated and ligand-untreated populations. Data were collected and pre-processed with the CellQuest 3.3 software package (Becton-Dickinson) and processed with WinMDI2.9 (Joseph Trotter, The Scripps Research Institute, La Jolla, CA USA) for presentation in this manuscript.

2.8 Transfections

Several transfection systems were employed in this report, depending on the most cost-effective or favored transfection reagent or protocol at the time. In most cases, the expression was optimal after 24–48 hours (optimal OFP expression required 48–72 hours). We used transiently transfected cells two to four days after transfection only if the transfection efficiency exceeded 15%, judged visually by wide-field fluorescence of a sample of transfected cells. Stable transfections in CHO-q3 cells were done with either Polyfect (main manuscript) or custom liposomes (Supplementary Figure 3).

Transient transfections using Polyfect, Superfect (both from Qiagen, Inc.) or Fugene-6 and Fugene-HD (both from Roche) were performed according to the manufacturer's instructions. Transfections using DDAB:DOPE liposomes and polyethyleneimine (PEI) were performed as described elsewhere [11;19].

3. Results

3.1 Conditional FRET between IFN- α R1 and IFN- α R2c

We easily identified cells transfected with fluorescent IFN- R1 and IFN- R2 or with fluorescent IL-10R1 and IL-10R2 in which FRET between the two receptor chains was observed [8–10]. We hypothesized that we would also easily identify cells in which IFN- R1 and IFN- R2c exhibit FRET and biological activity. Initial studies using monomeric cyan and yellow fluorescent proteins (ECFP, EYFP) corroborated our hypothesis; these results are explained in Supplementary Text 3 and shown as Supplementary Figures 1–3.

Discovering that using non-jellyfish red-shifted truly monomeric fluorescent proteins gives more precise FRET determinations and removes artefacts arising from interactions between jellyfish fluorescent proteins [11], we used fluorescent protein pairs derived from distinct species (e.g., jellyfish-derived monomeric green or citrine fluorescent proteins, EGFP and

CiFP, or anemone-derived teal fluorescent protein, TFP, as donors, and coral-derived orange or strawberry fluorescent proteins, OFP and StFP, as acceptors).

Our initial studies using IFN- R1/EGFP and IFN- R2c/OFP argued against a simple preassociation. The results of several experiments overlapped and were pooled together. While most cells expressing IFN- R1/EGFP and IFN- R2/OFP exhibit FRET (Fig. 1A), only a fraction of cells coexpressing IFN- R1/EGFP and IFN- R2/OFP exhibited FRET efficiency over 10% (Fig. 1B). By resolving these data according to the molar ratio of the two chains, it was clear that while a stoichiometric excess of IFN- R2 did not inhibit FRET with IFN- R1, a stoichiometric excess of IFN- R1 inhibited FRET with IFN- R2c.

Currently available fluorescent proteins have diverse folding and maturation rates, and impart varying stabilities to fusion proteins. To ensure that these variables do not influence our results, we coexpressed either IFN- R1 and IFN- R2 or IFN- R1 and IFN- R2c tagged to various donor:acceptor pairs and analyzed these results in aggregate as a bubble plot. Whether the pair was TFP:CiFP (red circles), EGFP:OFP (black circles) or EGFP:StFP (green circles), excess relative levels of IFN- R2 did not inhibit FRET between IFN- R2 and IFN- R1 (Fig. 1C), while an excess of IFN- R1 rarely yielded strong FRET between IFN- R1 and IFN- R2c (Fig. 1D). Thus we conclude that excess levels of IFN- R1 inhibit FRET between IFN- R1 and IFN- R2c.

3.2 Fluorescent IFN- α R1 and IFN- α R2c are biologically functional

FRET was seen between IFN- R1 and IFN- R2c only when IFN- R1 levels do not exceed IFN- R2c levels. Because we found that mutations to the IFN- receptor complex that inhibited FRET also prevented its activation [9], we hypothesized that only in those few cells in which IFN- R1 and IFN- R2c interact will functional Type I IFN receptor complexes form. To test this, we transfected IFN- R1/EGFPm and IFN- R2c/OFP stably in hamster CHO-q3 cells and isolated individual clones. Because hamster cells do not respond to IFN- 2 unless both human IFN- R1 and IFN- R2c are coexpressed [20], CHO-q3 cells are useful to test the biological activity of two fluorescent receptor chains.

Several clones expressing both IFN- R1/EGFP and IFN- R2c/OFP were isolated, expanded, and assayed for biological activity to human IFN- 2. Although IFN- 2 did not induce Class I MHC in one clone (clone 5.g+o, cyan, Fig. 2A, left), it induced MHC in a second clone (clone 6.wo, purple, Fig. 2A, right) with comparable relative levels of both IFN- R1/EGFPm and IFN- R2c/OFP. We conclude that simply having IFN- R1 and IFN- R2 present in the cell is not sufficient for IFN- 2 to induce signaling activity, even when all other intracellular components are present to propagate signaling. The induction in cells expressing IFN- R1/EGFPm and IFN- R2c/OFP was similar in magnitude to that induced in cells expressing IFN- R1 and IFN- R2c (Supp. Fig. 3C), suggesting that fluorescently tagged Type I IFN receptors retain full signaling potential.

Only some MHC-inducible clones exhibited antiviral activity in response to human IFN- 2. Though IFN- 2 did not protect clone 5.g+o (Fig. 2B, top) or clone 6.wo (data not shown) from vesicular stomatitis virus (VSV)-induced cytotoxicity, IFN- 2 protected clones 5.wo (Fig. 2B, middle) and 5.2 nf (Fig. 2B, bottom) from VSV-induced cytotoxicity (Fig. 2B). IFN- 2 had comparable specific activity to IFN- 2 in these cell lines. Among MHC-inducible clones, antiviral activity was not consistent, and was never complete. Nevertheless, IFN- R1/EGFPm and IFN- R2c/OFP form an active Type I IFN receptor complex under the proper conditions.

We analyzed FRET in clones 5.g+o and 6.wo and compared data obtained from those clones to data obtained from the transiently transfected population (Fig. 2C–2F, purple, cyan, and

unfilled circles, respectively). We found that receptors in most cells from the bioactive clone 6.wo exhibited more FRET than did receptors within the inactive clone 5.g+o. In other cells within clone 6.wo, FRET between the two receptor chains was not observed. Addition of 20 ng/mL IFN- α 2 to the cells did not appreciably change the FRET between receptor chains in cells of either clone (data not shown). We show representative spectra from two cells in each population in the absence of IFN- α 2, and two different cells in each population in the presence of 20 ng/mL IFN- α 2; Supplementary Figure 4 shows the spectra of one of the two cells from each clone with higher FRET efficiency, while Supplementary Figure 5 shows the spectra of the cells with a lower FRET efficiency.

The relative levels of both IFN- α 1/EGFPm (Fig. 2C) and IFN- α 2c/OFP (Fig. 2D) in the two clones were low compared with those seen in transiently transfected populations of 293T cells; this is also observed in hamster cells expressing other cytokine receptor pairs (unpublished observations). However, the FRET efficiency as a function of the molar ratio overlapped among the two clones and the transiently transfected populations (Fig. 2E). Thus even in stably transfected clones, the molar ratio between IFN- α 1 and IFN- α 2c dictated the interaction between IFN- α 1 and IFN- α 2c. Notably, in cells from the bioactive clone 6.wo, the IFN- α 2c chain was in molar excess, while the IFN- α 1/EGFP chain was in molar excess in the cells from the inactive clone 5.g+o (Fig. 2E). To ensure that affinity differences between IFN- α 1/EGFPm and IFN- α 2c/OFP did not correlate with conditional sensitivity, we replotted the data as a function of the number of IFN- α 1/EGFPm:IFN- α 2c/OFP pairs formed (Fig. 2F). The affinity of both clones was similar (~20 relative pairs) but significantly less than that found in most transiently transfected cells (~100 relative pairs), implying that the increased apparent affinity between IFN- α 1/EGFPm and IFN- α 2c/OFP observed in hamster cells does not explain biological sensitivity to the Type I IFN receptor complex. Overall, our data imply that a juxtaposition of IFN- α 1 and IFN- α 2c that produces FRET is a prerequisite to initiate signaling by Type I IFN in cells.

3.3 Coexpression of IFN- α 1 and IFN- α 2c does not guarantee a biological response

We observed that considerable FRET and biological sensitivity to IFN- α 2 was observed in only some cells. To confirm that biological activity was seen only in some cells expressing both IFN- α 1 and IFN- α 2c, we correlated the expression of receptor chains with biological functionality in expanded populations of stably transfected hamster CHO-q3 cells.

Because CHO-q3 cells respond to human IFN- α or to human IFN- α only when both human IFN- α 1 and human IFN- α 2 or both human IFN- α 1 and human IL-10R2 are coexpressed respectively [16;21], we can analyze similar hindrances in forming biologically active IFN- α or IFN- α receptor complexes. Because most cells coexpressing IFN- α 1 and IFN- α 2 exhibited FRET, we expected that most cells expressing IFN- α 1 and IFN- α 2 will be biologically responsive to IFN- α . Finally, because Type I IFN and IFN- α initiate similar signaling cascades; we expected that only some cells expressing IFN- α 1 and IL-10R2 are biologically responsive to IFN- α .

To visualize the expression of a single chain within a functional complex, we stably cotransfected CHO-q3 cells with one plasmid expressing a receptor chain tagged with the strawberry fluorescent protein (StFP) and a second plasmid expressing its nonfluorescent partner receptor chain (i.e., IFN- α 2/StFP and IFN- α 1, IFN- α 1/StFP and IFN- α 2, IFN- α 1/StFP and IFN- α 2c, and IFN- α 2c/StFP and IFN- α 1). We used two-color flow cytometry to correlate the expression of receptors (by StFP fluorescence) with the biological sensitivity to IFN (by increased binding of FITC-conjugated secondary antibodies). Because the detection of StFP-tagged proteins is less sensitive when FITC-conjugated antibody complexes are bound to the cell surface, we analyzed the expression of fluorescent receptors

separately, in identical cells that are not treated with antibodies. In this fashion, we can compare the fraction of cells that express receptors with the fraction of cells that induce MHC Class I surface antigen. Because we designed these experiments so that bioactive receptor complexes contain one fluorescent receptor chain, we assume that all MHC-inducible cells are a subset of the cells that expresses a fluorescent receptor chain.

3.3.1 Most cells expressing IFN- γ R1 and IFN- γ R2 respond to IFN- γ —

As expected, most cells expressing IFN- R2/StFP or IFN- R1/StFP were sensitive to IFN- (Fig. 3A-F). Of the 26.8% of the population that expressed significant levels of IFN- R2/StFP (Fig. 3A), 70% of that 26.8% ($21.3\% - 2.3\% = 19.0\%$ of the total population) induced MHC Class I in response to IFN- treatment (Fig. 3B, 3C). Similarly, 68% of the 23.6% of cells expressing IFN- R1/StFP (Fig. 3D) induced MHC Class I surface antigen in response to IFN- ($18.4\% - 2.3\% = 16.1\%$ of the total population, Fig 3E, 3F). Thus, the coexpression of IFN- receptor chains is usually sufficient to reconstitute full IFN- bioactivity in heterologous cells.

3.3.2 Few cells containing IFN- α R1 and IFN- α R2c respond to IFN- α —

Contrasting the above results with IFN- receptors, only small fractions of cells expressing IFN- R1/StFP or IFN- R2c/StFP were sensitive to IFN- 2 (Fig. 3G-L). Of the 7.1% of the population that expressed significant levels of IFN- R1/StFP (Fig. 3G), only 26% of that 7.1% ($4.7\% - 2.8\% = 1.9\%$) was sensitive to IFN- 2 (Fig. 3H, I). Similarly, of the 18.3% of cells expressing IFN- R2c/StFP (Fig. 3J), only 6.7% of that 18.3% ($4.3\% - 3.2\% = 1.1\%$ of the total population) was sensitive to IFN- 2 (Fig. 3K, L). This result implies that simply coexpressing IFN- R2c and IFN- R1 ectopically does not generate bioactivity in response to IFN- 2 in all hamster cells, even though both fluorescent receptor chains are biologically active.

3.3.3 Few cells harboring IFN- λ R1 and IL-10R2 are sensitive to IFN- λ —

To see whether coexpression of IFN- R1 and IL-10R2 (like IFN- R1 and IFN- R2c) confers a biological response to IFN- 3 in only a fraction of cells coexpressing both chains, we coexpressed IFN- R1/EGFP and IFN- R2c/StFP or coexpressed IFN- R1/EGFP and IL-10R2/StFP, and compared the fraction of cells that were sensitive to IFN- 2 or IFN- 3 respectively to the fraction of cells that express EGFP-tagged receptor and StFP-tagged receptor. In both populations, significant expression of only one chain was observed, suggesting that expression of the other chain was poor or undetectable. In the presented representative experiment, 10.9% of cells expressed observable levels of IFN- R2c/StFP, and a distinct 25.5% of the population expressed detectable levels of IFN- R1/EGFP (Fig. 3M); analogously, 10.6% of cells expressed visible relative levels of IL-10R2/StFP, and a distinct 14.9% of cells express significant levels of IFN- R1/EGFP (Fig. 3P).

As seen above with Type I IFN receptors, only a fraction of cells within each population contained biologically active receptor. IFN- 2 induced MHC Class I in only 3.3% of cells transfected with IFN- R1/EGFP and IFN- R2c/StFP ($14.7\% - 11.4\%$, Fig. 3N, 3O), while IFN- 3 induced MHC Class I antigens in only 3.7% of cells transfected with IFN- R1/EGFP and IL-10R2/StFP ($6.0\% - 2.3\%$, Fig. 3Q, 3R). These percentages are far less than the expression of any single chain. We observed stronger induction of Class I MHC by IFN- 3 than by IFN- 2.

Nevertheless, we isolated clones expressing both IFN- R1/EGFP and IFN- R2c/StFP or expressing both IFN- R1/EGFP and IL-10R2/StFP that exhibited anti-EMCV activity in response to IFN- 2 or IFN- 3 respectively (data not shown). Thus Type III IFN receptors, like Type I IFN receptors, are only conditionally active.

3.4 Other interactions between Type I IFN receptor chains

We also investigated interactions between IFN- R1 and IFN- R2b, a primate-specific splice variant of IFN- R2c that uses an alternate nonfunctional intracellular domain that does not bind Jak1 or Stat2. We present this data in Supplementary Text 4 and Supp. Fig. 6. Summarily, IFN- R1 interacts slightly stronger with IFN- R2c than with IFN- R2b.

We also investigated interactions between IFN- R1 chains and between IFN- R2c chains, based on the observations of FRET between IFN- R2 chains and between IFN- R1 chains, and our supposition that they are largely nonspecific due to their low affinity [11]. We found that while homodimeric interactions among the IFN- and IFN- receptor chains are weaker than is the heterodimeric interaction between IFN- R1 and IL-10R2, the affinity of homodimeric interactions between Type I IFN receptor chains is similar to that between IFN- R2c and IFN- R1, suggesting that the observed FRET between IFN- R1 and IFN- R2c arises largely from nonspecific interactions in most cells; these data are explained in more detail in Supplementary Text 5 and Supp. Figs. 7 and 8.

3.5 Intracellular proteins binding IFN- α R1 and IFN- α R2c

Because the interaction between IFN- R1 and IFN- R2c is very weak, we surmised that, similar to what was observed within the IFN- receptor complex, intracellular proteins (like RACK-1 and the Janus kinases that bind IFN- R1 and IFN- R2c) promote the specific association and permit signaling to propagate from IFN- R1 and IFN- R2c in cells.

3.5.1 Interactions between receptor chains and Janus kinases—We not only investigated FRET between IFN- R1 and Tyk2 or between IFN- R2c and Jak1, pairs known to coimmunoprecipitate *in vitro* [22–27], but also between IFN- R1 and Jak1 and IFN- R2c and Tyk2 to see whether cross-interactions occur in cells that have not been observed upon extraction from cells. To accomplish this, we coexpressed either IFN- R1/StFP or IFN- R2c/StFP and either Jak1/CiFPm or Tyk2/CiFPm in 293T cells, and measured FRET efficiencies between the protein pairs in various representative cells within the four populations.

As shown in Figs 4A and 4B, significant FRET was seen with all four combinations of Type I IFN receptor and Janus kinase. IFN- R1 displays similar cellular affinity (~ 100 relative complexes/cell) for both Jak1 (Fig. 4A, grey circles) and Tyk2 (Fig. 4A, black circles). With the exception of a few cells possessing higher FRET efficiency between Tyk2 and IFN- R1 (the interaction observed *in vitro*, black circles), the two datasets overlapped completely. Although IFN- R2c exhibited FRET with both Jak1 (Fig. 4B, black circles) and Tyk2 (Fig. 4B, grey circles), IFN- R2c interacted somewhat stronger with Tyk2 (~ 30 relative complexes/cell) than with Jak1 (the interaction observed *in vitro*, ~ 50 relative complexes/cell). The optimal FRET efficiency for each pair in most cells in all four populations is about 0.30 (or 0.60 for a few cells expressing IFN- R1 and Tyk2).

3.5.2 RACK-1 influences the interaction between IFN- α R1 and IFN- α R2c—

RACK-1 is a scaffolding protein that couples the Type I IFN receptor chains to their signaling components by directly interacting with IFN- R2c, Tyk2 and Jak1 as well as Stat1 [28;29]. Hypothesizing that RACK-1 promotes an association between IFN- R1 and IFN- R2c, we coexpressed IFN- R1/CiFP and IFN- R2c/StFP in the absence of RACK-1 and in the presence of either RACK-1 or RACK-1/TFP and searched for differences among the three transiently transfected populations.

With the exception of one cell in which a high FRET efficiency was seen with relatively few numbers of IFN- R1:IFN- R2c pairs, the absence of RACK-1 (Fig. 4C, black circles)

resulted primarily in low-affinity receptors possessing an average affinity of about 500 pairs/cell; under similar conditions, the interaction between IFN- R1 and IFN- R2 was half-optimal at 50–100 pairs/cell [11]. In the presence of RACK-1, whether expressed without a fluorescent protein (Fig. 4C, red circles) or with TFP attached to the COOH-terminus of RACK-1 (Fig. 4C, green circles), the affinity of IFN- R1 and IFN- R2c increased by about 3-fold to about 100–200 pairs/cell. The optimal FRET efficiency between IFN- R1/CiFP and IFN- R2c/StFP increased slightly, if one again ignores cells possessing high FRET efficiencies at lower numbers of IFN- R1:IFN- R2c pairs. As a negative control, we coexpressed IFN- R2/CiFP with IFN- R2c/StFP (Fig. 4C, unfilled black circles); significant FRET between the mismatched receptor chains was never observed, and resembled the FRET seen between most cells expressing IFN- R1/EGFP and IFN- R2c/OFP.

3.5.3 Jak1 and Tyk2 influences the interaction between IFN-αR1 and IFN-αR2c

—Because interactions between either IFN- R1 or IFN- R2c and either Jak1 or Tyk2 are visible spectroscopically, we hypothesized that Jak1 and Tyk2 influences the interaction between IFN- R1 and IFN- R2c. Thus we replotted the data from Figs. 2F and 4C (except for cells expressing nonfluorescent RACK-1), and included data in which IFN- R1/OFP and IFN- R2c/ChFP was coexpressed with Jak1/TFP and Tyk2/CiFP [30]. The calculated FRET efficiencies of the original data between IFN- R1 and IFN- R2c were converted to the predicted FRET efficiency between EGFP and OFP to make the datasets more comparable [11].

We found that the coexpression of both Jak1 and Tyk2 increased the FRET efficiency between IFN- R1 and IFN- R2c and significantly enhanced their affinity of interaction to at most 40–80 relative pairs/cell (Fig. 4D, purple circles). These data overlapped the two datapoints in Fig. 4C where high FRET efficiencies are seen with relatively few IFN-

R1:IFN- R2c pairs, suggesting that in those two cells, endogenous Jak1 and Tyk2 may be present in many of these complexes.

To assess whether Tyk2 or Jak1 promotes stronger FRET between IFN- R1 and IFN- R2c by themselves, we coexpressed either nonfluorescent Jak1 or Tyk2 with IFN- R1/EGFP and IFN- R2c/OFP. Although the coexpression of Tyk2 alone did not influence FRET between IFN- R1 and IFN- R2c (data not shown), the coexpression of Jak1 alone increased the FRET efficiency of a few cells expressing IFN- R1 and IFN- R2c (Fig. 4D, grey circles with blue borders); we speculate that in those few cells, the levels of Jak1 approximated those of IFN- R1:IFN- R2c complexes.

3.6 Summary of results

Overall, we found that overexpression of IFN- R1 disrupts FRET between IFN- R1 and IFN- R2c. Expression of IFN- R1 and IFN- R2c alone results only in a low-affinity interaction or colocalization and is not sufficient to restore biological activity — IFN- R1 and IFN- R2c must be able to juxtapose in the absence of ligand in order to exhibit biological activity in the presence of ligand. Coexpression of RACK-1 or Jak1 allows IFN-

R1 and IFN- R2c to interact; Tyk2 further improves the interaction between IFN- R1 and IFN- R2c. Similar requirements may hold in order for IFN- R1 and IL-10R2 to interact and respond to IFN- 3. The requirement of transmembrane receptor chains to interact in the absence of ligand in order to initiate signaling in the presence of ligand may apply to many cytokine receptor complexes.

4. Discussion

4.1 The Type I IFN receptor is conditionally preassociated

Studies analyzed *in vitro* conclude that IFN- β and IFN- γ interact only indirectly and only in the presence of Type I IFN [3–6]. Our data studying IFN- β and IFN- γ in their native environment gave conflicting results. Only in a few cells were IFN- β and IFN- γ juxtaposed; in most cells (especially when levels of IFN- β exceeded those of IFN- γ ; Fig. 1B, 1D), IFN- β and IFN- γ exhibited FRET only at high levels of both chains (Fig. 2F, 4C, 4D).

4.2 Activation requires ligand-independent receptor chain interaction

Importantly, a ligand-independent juxtaposition of IFN- β and IFN- γ is required for a biological response to ligand to be observed. In CHO-q3 cells where IFN- β and IFN- γ are coexpressed but did not exhibit FRET, treatment with IFN- α did not elicit significant FRET nor did it result in biological activity; however, when IFN- β and IFN- γ exhibited FRET, IFN- α initiates biological activity (Fig. 2). Analysis of populations of stably transfected CHO-q3 cells paralleled our interaction data: only a fraction of cells expressing IFN- β and IFN- γ induced MHC Class I surface antigens.

Because Type I receptor chains that cannot juxtapose in the absence of IFN- α will not interact nor initiate signaling in the presence of IFN- α , our data argue against structural models in which ligand assembles a receptor complex from dissociated receptor chains and initiates signaling from an active receptor complex simply by nucleating receptor chains. Because Type I IFN binds both IFN- β and IFN- γ in cells that are insensitive to IFN- α with kinetics similar to those found in cells that are sensitive to IFN- α [31], we hypothesize that a component that helps to maintain association of the Type I IFN receptor complex plays an obligate role in permitting signal transduction.

Ectopically expressed IFN- β and IFN- γ are biologically active, even when tagged with fluorescent proteins (Figs. 2, 3), implying that under the right conditions, biologically active fluorescent Type I IFN receptor complexes can be formed. We thus sought to understand which cellular conditions to avoid and which to promote in order to encourage ligand-independent receptor chain interactions and to observe biological activity.

4.2.1 Stoichiometry-based receptor activation—A stoichiometric excess of IFN-

β should be avoided. A stoichiometric excess of IFN- β (at least three-fold) inhibited FRET observed between IFN- β and IFN- γ (Figs. 1B, 1D, 2E). In contrast, a five-fold higher stoichiometric excess of IFN- γ (homologous to IFN- β) did not inhibit FRET between IFN- γ and IFN- β (Figs. 1A, 1C). It would be interesting if the rapid turnover of IFN- β in cells when Tyk2 is not bound [32], when the receptor is not signaling [33;34] or when Type I IFN signaling is initiated [35] exists to prevent inhibition of the Type I IFN receptor complexes by excessive levels of endogenous IFN- β .

Others have studied how variations in relative levels of IFN- β and IFN- γ influence Type I IFN bioactivity. In one study [36], it was revealed that relatively high levels of IFN-

β compared to that of IFN- γ are required for IFN- α to have specific activity resembling that of IFN- α in 2fTGH cells. However, ectopic expression of IFN- γ was more effective than ectopic expression of IFN- β even though both were transcribed from the same promoter. The obtained IFN- α -sensitive clones with ectopically expressed IFN-receptor chains had either balanced levels of IFN- β and IFN- γ (both low or both high), or had high levels of IFN- γ relative to that of IFN- β . Although no clones with a high level of IFN- β and a low level of IFN- γ were described in that study, the same

research group previously isolated a WISH clonal cell line in which IFN- R1 was overexpressed that was sensitive to IFN- 2 and IFN- [37]. In a second study [38], strong expression of IFN- R2c in hepatic carcinoma cell lines was required to observe strong specific activity of IFN- 2 or IFN- 8; expression of IFN- R1 protein was consistently poor in these cell lines.

Realizing that the direct determination of the levels or the ratio of label-free receptor chains is difficult, we suspect that cell lines expressing a definitive excess of label-free IFN- R1 were not obtained, especially if obtaining clones sensitive to IFN- or IFN- were the end goal. We obtained a stoichiometric excess of IFN- R1 by coexpressing IFN- R1/EGFP with IFN- R2c/OFP; the expression of the latter is most likely hindered by the slow folding of the OFP relative to the EGFP [11;39].

We do not understand how an excess of IFN- R1 prevented association of IFN- R2c with stoichiometric levels of IFN- R1 in the cell. Perhaps there is a limited amount of Tyk2 (or other IFN- R1 associated protein) under our conditions, and removal of this protein from IFN- R2c by mass action hinders the interaction between IFN- R1 and IFN- R2c. Other than Tyk2, PRMT1 is known to bind IFN- R1 in the absence of signaling [40],

Interestingly, extreme overexpression of IFN- R2 relative to IFN- R1 reduced the magnitude of MHC Class I induction. MHC induction was stronger in cells expressing lower relative levels of IFN- R2/StFP than in cells expressing higher levels of IFN- R2/StFP (Fig. 3C). To contrast this difference, a tenfold difference in IFN- R1/StFP expression did not alter the magnitude of MHC Class I induction (Fig. 3F). We found this decrease in response by comparing the “slope” of cells that did not induce MHC Class I antigens to those that did induce these antigens. Supplementary Figure 7 (left and right) contains an enlargement of panels C and F from Figure 3 respectively with illustrative diagonal lines to emphasize these “slopes”. However, we acknowledge that the decrease in biological response when IFN- R2 levels were higher can be explained by lower levels of IFN- R1:IFN- R2 complexes if IFN- R1 levels are reduced to accommodate ultra-high levels of IFN- R2.

4.2.2 Inclusion of cytosolic proteins in the preassembled Type I IFN receptor complex—Even in cells in which IFN- R1 was not overexpressed, the coexpression of IFN- R1 and IFN- R2c did not guarantee a biological response. Analysis of the FRET between IFN- R1 and IFN- R2c as a function of the number of IFN- R1:IFN- R2c pairs revealed that in most cells, IFN- R1 and IFN- R2c exhibit FRET with an affinity resembling completely nonspecific interactions (Fig. 4, Supp. Fig. 10). The affinity of the interaction between IFN- R1 and IFN- R2c in q3 cells that harbored active receptors was considerably lower (Fig. 2F), implying that the interaction between IFN- R1 and IFN- R2c somehow acquired specificity, most likely as a consequence of the interaction of IFN- R1 and IFN- R2c with endogenous components.

One endogenous protein that we tested was RACK-1, a scaffolding protein that juxtaposes the Jak/Stat components with IFN- R1 and IFN- R2c [28;29]. RACK-1 improved the apparent affinity between the two chains by at least five-fold (Fig. 4C). Separate experiments confirmed that RACK-1/TFP, IFN- R1/CiFP and IFN- R2c/StFP were all present in comparable levels (not shown).

Jak1 is thought to play both an enzymatic role and a structural role in the IFN- receptor complex [41]. Supporting this, we previously showed that Jak1 partially mediates the interaction between IFN- R1 and IFN- R2 [9], and may enhance the affinity of the interaction between IFN- R1 and IFN- R2 [11]. Because the affinity between IFN- R1 and IFN- R2c was slightly higher than that between IFN- R1 and IFN- R2b (Supp. Fig. 6), we

hypothesized that Jak1 supports an interaction between IFN- R1 and IFN- R2c; we confirmed this by coexpressing Jak1, IFN- R1/EGFP and IFN- R2c/OFP, and observing significant FRET between IFN- R1/EGFP and IFN- R2c/OFP with an apparent affinity of about 100 relative pairs/cell (Fig. 4D). Thus Jak1 allows IFN- R1 and IFN- R2c to maintain an interaction.

However, the coexpression of Tyk2 and Jak1 with IFN- R1 and IFN- R2c dramatically increased the apparent affinity and the observed FRET efficiency between IFN- R1 and IFN- R2c (Fig. 4D). We hypothesize that not only the transmembrane receptor chains but also Jak1, Tyk2 and also RACK-1 need be present in comparable levels for full receptor activity to be observed. Notably, it has been shown that Janus kinases such as Tyk2 and Jak1 interact independently of receptor activation [42]. A structural model encompassing these requirements is shown in Fig. 5. In summary, IFN- R1 and IFN- R2 do not directly interact strongly (Fig. 5A, green and red, respectively) unless other proteins like RACK-1 (Fig. 5B, purple), Jak1 and Tyk2 (Fig. 5C, green-yellow and blue, respectively) are present to anchor the intracellular domains together.

4.3 Structural model of the activation of the Type I IFN receptor complex

We now combine our data here with that from others published and recently reviewed [2;43], allows us to forward the following model for how we believe the Type I IFN receptor complex is assembled and activated. IFN- R1, and its constitutively bound Tyk2 kinase, reversibly interacts with IFN- R2c and its constitutively bound kinase Jak1 (Fig. 5D). We place RACK-1 with IFN- R2c based on immunoprecipitation experiments [28].

Because (1) many receptors bind Jak1 or Tyk2, (2) many proteins are known to bind RACK-1, and (3) the receptor complex must be preassembled to respond to Type I IFN, specificity must be involved to form the Type I IFN receptor complex in the first place. We believe that, as in the IFN- receptor complex, elements in the extracellular domains of IFN- R1 and IFN- R2c participate in the initial interaction between the IFN- R1:Tyk2 complex and the IFN- R2c:Jak1 complex (Fig. 5E). We hypothesize native specificity between IFN- R1 and IFN- R2c exists even though the interaction is very weak because when we use ECFP and EYFP (that apparently interact with each other while attached to IFN- R1 and to IFN- R2c), IFN- R1 and IFN- R2c interact with higher affinity than do two IFN- R1 chains or two IFN- R2c chains (Supp. Fig. 7A, 8A).

The interactions among Jak1 and Tyk2 and the intracellular domains of IFN- R2c and IFN- R1 are of higher affinity than are interactions between IFN- R2c and IFN- R1 (Table 1). We therefore believe that once the initial complex in Fig. 5E is formed, interactions among the intracellular domains occur that stabilize the preassembled complex (Fig. 5F). Because Jak1 alone increased FRET between IFN- R1/EGFP and IFN- R2c/OFP (Fig. 4D), we propose that the initial intracellular interaction is mediated by Jak1, that contacts IFN- R1 and/or Tyk2. Tyk2 and RACK-1 then participate by an unknown molecular mechanism.

These intracellular interactions are necessary to keep the receptor preassociated because the extracellular domains readily dissociate (Fig. 5G). There is firm evidence to support this: (1) interactions between soluble Type I IFN receptors or membrane-tethered extracellular domains is not observed in the absence of ligand [6;44–46], (2) neither crosslinking nor co-immunoprecipitation of IFN- R1 to IFN- R2c is observed in the absence of Type I IFN [4], and (3) interactions between IFN- R1/CiFP and IFN- R2c/StFP were not significantly greater than the presumably nonspecific interactions seen between IFN- R1 chains or between IFN- R2c chains (Supp. Figs. 7B, 8B).

We hypothesize that dissociated extracellular domains allow Type I IFN (light blue) to interact directly with IFN- R1 and IFN- R2c (Fig. 5H). Kinetic studies have revealed that the principal and most stable contact of Type I IFN is with IFN- R2 because the K_a of IFN- 2 with soluble, antibody-immobilized, surface-immobilized, or cell-associated IFN- R2 is 3–10 nM [44;47;48] while the K_a of IFN- 2 with surface-immobilized IFN- R1 (5 μ M) is a thousand-fold higher; contact of IFN with IFN- R1 likely occurs as an IFN- R2:IFN complex [44;45].

Activation of the tyrosine kinases Jak1 and Tyk2, phosphorylation of the intracellular domains and of the tyrosine kinases (Fig 5H, black circles), and initiation of signaling cascades likely occur immediately after Type I IFN binds to the extracellular domains of the receptor complex; rigidification of the extracellular domain of IFN- R1 and other conformational changes in the extracellular domains of both IFN- R1 and IFN- R2 by Type I IFN [49–51] likely propagate a conformation that promotes the immediate activation of the intracellular domains.

4.4 Conditional induction of antiviral activity

We found that antiviral activity was not consistently observed in CHO-q3 cell clones when MHC Class I induction was observed. Evidence is mounting that paths other than the Jak/Stat pathway (that is required for MHC Class I induction) need to be activated for antiviral activity to be observed. For example, the activation and tyrosine phosphorylation of Jak1, known to vary among Type I IFN subtypes, must be formidable to observe antiviral activity [52]; knockout of Akt1 and Akt2 (that phosphorylates mTOR that then phosphorylates S6 kinase) inhibited antiviral activity but did not alter MHC induction [53]. In correlation, a subset of monoclonal antibodies raised against IFN- R2 were raised that inhibited neither binding of Type I IFN nor Jak/Stat signaling, but inhibited antiviral and antiproliferative activities induced by Type I IFNs [54].

However, the deficient antiviral activity in our system may arise from another phenomenon. First, anticytopathic protection was never complete (Fig. 2B), implying that some cells in each isolated clone are not sensitive to Type I IFN. An effective inhibition of the cytopathic effect requires that all cells be fully responsive to Type I IFN; nonresponsive cells would permit more expansion of the virus within each subpopulation than would be expected. This may also explain why only very high doses of IFN- 2 or IFN- were required to observe viable cells (over 150 nM, Fig. 2B). Consistent with a nonuniform biological response, not all cells within the bioresponsive CHO-q3 clone 6.wo exhibited FRET between the receptor chains (Fig. 2C–F). To corroborate this, there should be evidence that the Type I IFN receptor complex can form one of two different structures from cell to cell in a population. We present data supporting this prediction elsewhere [30].

4.5 The Type III IFN receptor complex

Similar to that observed with IFN- R1 and IFN- R2c, only some cells expressing both IFN- R1 and IL-10R2 exhibit biological activity in response to human IFN- 3 (Fig. 3M–R). Both the IFN- receptor complex and the Type I IFN receptor complex induce antiviral states within cells, and both receptor complexes use Tyk2, Jak1, and Stat2 to propagate signaling [1]. We therefore tested whether the assembly of the IFN- receptor complex matches that of the Type I IFN receptor complex. Although IFN- R1 and IL-10R2 exhibit FRET with an apparent affinity greater than that found between IFN- R1 chains or between IL-10R2 chains (Supp. Fig. 7C, 8C), the affinity was lower than that found between IFN- R1 and IFN- R2, approximating that of other membrane proteins that should not interact (Supp. Fig. 10). We propose that IFN- R1 and IL-10R2 must be expressed with other

intracellular proteins like Jak1 and Tyk2 in order to consistently and stably interact in the absence of IFN- and observe signaling upon IFN- treatment.

It must be stated that although the Type I IFN and IFN- receptor complexes have structural, molecular, and signaling similarities, the genetic induction differs considerably between the two receptor complexes [55]. Supporting the latter, we observed that Class I MHC induction is considerably stronger with IFN- 3 than with IFN- 2, and is comparable to the induction seen with IFN- (Figs. 3C, 3F, 3O, 3R).

4.6 Class 2 Cytokine receptor complexes are preassociated

We present data demonstrating that the biologically active Type I IFN receptor complex is a preassembled entity of at least IFN- R1, IFN- R2c, RACK-1, Jak1 and Jak2 in its resting state. The IFN- receptor complex also appears to be preassociated (Supp. Fig. 7C, 8C). Preassembly was also observed in the IFN- and IL-10 receptor complexes [8–10]. Overall, these data support our hypothesis that Class 2 cytokine receptor complexes are preassociated entities [1;56;57].

A visual display of the various relative binding affinities of the components of the three IFN receptor complexes from Table 1 is shown in Supplementary Figure 10 (red, light blue and green bars, respectively) reveals a mechanism by which Class II cytokine receptor complexes are preassembled. The weakest interactions among the receptor complex are between the receptor chains themselves (purple labels). Although the interaction between a receptor chain and a Janus kinase (orange labels) is stronger than that between two receptor chains, the interaction between the two chains is much stronger when both Janus kinases are present (blue labels), implying that interactions involving the Janus kinases maintain much of the preassociation of each receptor complex.

Nonspecific interactions (yellow-green bars) and homodimeric interactions are of similar affinity, and both sets of interactions (black labels) have lower apparent affinity than those found within most receptor complexes. We therefore believe that homodimeric receptor interactions are almost completely nonspecific.

4.7 Biological activity of receptor complexes requires preassociation

We have observed a correlation between receptor complex preassociation and biological activity. Mutations to either IFN- R1 or IFN- R2 that inhibited the preassociation of the two chains also disrupted biological activity [9]. We isolated two CHO-q3 cells lines expressing comparable levels of IFN- R1/EGFP and IFN- R2c/OFP. The clone exhibiting stronger FRET efficiency had more IFN- R2 expressed than IFN- R1 and was biologically active (Fig. 2), while the clone that was biologically inactive had poor FRET efficiency and a stoichiometric excess of IFN- R1. Preassociation of IFN- R1 and IFN- R2c is disrupted by excessive relative levels of IFN- R1; this disruption also prevented biological activity. We now hypothesize that a ligand-independent interaction or juxtaposition of Class 2 Cytokine receptor chains is a requirement for biological activity to be initiated by the receptor complex when bound by ligand. The correlation of preassociation and biological activity suggests that new opportunities to inhibit signaling by IFNs and IL-10-family cytokines can be sought by inhibiting the interaction between their receptor chains.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Used

CiFP	enhanced citrine fluorescent protein
ECFP	enhanced cyan fluorescent protein
EGFP	enhanced green fluorescent protein
EYFP	enhanced yellow fluorescent protein
FRET	fluorescence resonance energy transfer
IFN	interferon
IFN-	interferon-alpha
IFN-	interferon-gamma
IFN-	interferon-lambda
OFP	orange fluorescent protein
RACK-1	receptor for activated protein kinase-1
StFP	strawberry fluorescent protein

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HIGHLIGHTS

1. Most cells expressing IFN- R1 and IFN- R2c from cDNAs have poor FRET.
2. Most cells expressing IFN- R1 and IFN- R2c from cDNAs do not respond to IFN- .
3. Stable clones exhibiting FRET between IFN- R1 and IFN- R2c are biologically active.
4. RACK-1 and Jak-1 promote the interaction between IFN- R1 and IFN- R2c.

Figure 1.

Inhibition of FRET by moderate overexpression of IFN- R1 relative to IFN- R2c. (A) 293T cells were transiently transfected with a plasmid coexpressing IFN- R2/OFP and IFN- R1/EGFP, and the FRET efficiency between the two receptor chains was measured in a selection of cells and plotted as a function of the molar ratio of IFN- R2 to IFN- R1. (B) 293T cells were transiently transfected with a plasmid coexpressing IFN- R1/EGFP and IFN- R2c/OFP, and the FRET efficiency between the two receptor chains was measured in selected cells and plotted as a function of the IFN- R1:IFN- R2c molar ratio. Open circles are data obtained from cells whose spectra are indistinguishable from those containing only OFP. The data from (A) and (B) were pooled from multiple experiments. Either IFN- R2 and IFN- R1 (C) or IFN- R1 and IFN- R2c (D) were tagged with TFP and CiFP (red), OFP and EGFP (black), or StFP and EGFP (green), and FRET efficiency (proportional to the diameter of the open circle) was plotted as a function of the calculated relative IFN- R2 and IFN- R1 levels or relative IFN- R1 and IFN- R2c levels. The diagonal lines in (C) and (D) denote balanced expression of IFN- R1 and IFN- R2 or of IFN- R1 and IFN- R2c.

Figure 2.

Bioactivity and interaction between IFN- R1/EGFP and IFN- R2c/OFP. (A) Induction of MHC Class I surface antigens in CHO-q3 clone 5.g+o (left, in light blue) and in clone 6.wo (right, in purple). The filled histograms with thin black borders are from untreated cells, while the unfilled histograms with the thick black border come from cells treated with 1000 units/ml human IFN- α 2 for three days. (B) Antiviral activity of human Type I IFNs in CHO-q3 cell clones expressing IFN- R1/EGFP and IFN- R2c/OFP. Clones 5.g+o (top), 5.wo (middle) and 5.2nf (bottom) were treated with either serially two-fold diluted (left to right) 200 ng/mL IFN- α 2 (first row), serially two-fold diluted 200 ng/mL IFN- β (second row) or no IFN and either no virus (third row, left six wells) or no IFN and VSV (third row, right six wells). Cells surviving viral infection are stained with crystal violet. For (C–E), the data from CHO-q3 clone 5.g+o (light blue) and clone 6.wo (purple) are coplotted with data from 293T cells transiently transfected with plasmids expressing IFN- R1/EGFP and IFN- R2c/OFP (black open circles). FRET efficiencies are plotted as a function of (C) IFN- R1/EGFP relative levels, (D) IFN- R2c/OFP relative levels, (E) the molar ratio of IFN- R2c/OFP to IFN- R1/EGFP, and (F) the relative number of IFN- R1/EGFP:IFN- R2c/OFP pairs formed.

Figure 3.

MHC induction in CHO-q3 cells stably transfected with plasmids encoding cDNA's of various interferon receptors. Populations of CHO-q3 cells were obtained that stably expressed the following receptor pairs: (A–C) IFN- R1 and IFN- R2/StFP, (D–F) IFN- R1/StFP and IFN- R2, (G–I) IFN- R1/StFP and IFN- R2c, (J–L) IFN- R1 and IFN- R2c/StFP, (M–O) IFN- R1/EGFP and IFN- R2c/StFP, and (P–R) IFN- R1/EGFP and IL-10R2/StFP. These cells were either untreated (A, D, G, J, M, and P) or were treated with primary anti-MHC antibody and FITC-conjugated secondary anti-murine IgG antibody after three days treatment with either no ligand (B, E, H, K, N, and Q) or with 1000 U/mL IFN- (C, F), 1000 U/mL IFN- 2 (I, L, and O), or 4 U/mL IFN- 3 (R). The percentage of total cells that were within each gate is indicated near each gate. To calculate the percentage of cells that demonstrate induced MHC Class I surface antigen, the percentage gated without ligand treatment was subtracted from the percentage gated after ligand treatment. Note that the fluorescence of GFP-tagged receptors is obscured by the presence of high levels of FITC-conjugated antibodies bound to constitutively expressed MHC Class I surface antigen.

Figure 4.

Interactions between Type I IFN receptors and cytosolic proteins. All data are presented as obtained FRET efficiencies as a function of the number of donor:acceptor pairs formed. (A) A plasmid encoding IFN- R1/StFP was coexpressed with a plasmid encoding either Jak1/CiFP (grey circles) or Tyk2/CiFP (black circles). (B) A plasmid encoding IFN- R2c/StFP was coexpressed with a plasmid encoding either Jak1/CiFP (black circles) or Tyk2/CiFP (grey circles). Note that for (A) and (B) black circles derive from pairs known to coimmunoprecipitate *in vitro*. (C) FRET between IFN- R1/CiFP and IFN- R2c/StFP was measured in the absence of transfected RACK1 (black circles), in the presence of ectopically expressed RACK-1 (red circles) and in the presence of RACK-1/TFP (green circles). IFN- R2c/StFP was also coexpressed with a mismatched receptor chain, IFN- R2/CiFP (black unfilled circles). (D) Cytosolic proteins enhance the interaction between IFN- R1 and IFN- R2c. FRET between IFN- R1 and IFN- R2c was measured and if necessary, converted to an equivalent FRET efficiency between EGFP and OFP. Unfilled blue circles are replotted from Figure 2F, and are from cells in which IFN- R1/EGFP and IFN- R2c/OFP were coexpressed. Grey-filled blue circles are replotted from Fig. 2F, and are from cells in which IFN- R1/EGFP and IFN- R2c/OFP were coexpressed with Jak1. Black circles are replotted from Fig. 4C and are from cells expressing only IFN- R1/CiFP and IFN- R2c/StFP. Green circles are replotted from Fig. 4C and are from cells expressing IFN- R1/CiFP, IFN- R2c/StFP and RACK-1/TFP. Purple circles are from cells expressing IFN- R1/OFP, IFN- R2c/ChFP, Jak1/TFP and Tyk2/CiFP.

Figure 5.

Assembly and activation of the Type I IFN receptor complex. (A) The two transmembrane receptor chains IFN- R1 (green) and IFN- R2c (red) interact very weakly by themselves; the observed FRET is essentially mediated by nonspecific juxtapositions brought about by diffusion through the plasma membrane (brown and white bar). (B) RACK-1 (purple) immunoprecipitates with IFN- R2c, and promotes the interaction between IFN- R1 and IFN- R2c, possibly by slowing their dissociation. (C) A complex of IFN- R1 and Tyk2 (blue) can interact reversibly with a complex of IFN- R2c and Jak1 (green-yellow) within the plasma membrane to form the Type I IFN receptor complex. This tetrameric complex is considerably more stable than that stabilized by RACK-1 alone. (D) We combine these scenarios so that the physiological Type I IFN receptor complex forms when a complex of IFN- R1 and Tyk2 interacts with a complex of IFN- R2c, Jak1, and RACK-1. The initial complex formed (E) is unstable until the intracellular proteins interact (likely initiated by Jak1). The resulting complex (F) is stable enough to allow dissociation of the extracellular domains (G). (H) Type I IFN (light blue) activates the Type I IFN receptor complex by stably contacting IFN- R2c, and then contacting IFN- R1 (stably or reversibly, depending on the subtype of Type I IFN bound). The resulting conformational change allows the enzymatic activation of Tyk2 and Jak1 and results in tyrosine phosphorylations (black dots) at two sites on IFN- R1, two sites on IFN- R2c, and on Jak1 and Tyk2.

Table 1

Affinities of various interactions among the components of the Type I and Type III IFN receptor complexes. These data are summarized from Figs. 2F, 4, Supp. Figs. 2B, 4B, 5, 6, and experiments not displayed in this manuscript. The asterisk above EC₅₀ denotes relative values. If the apparent affinity is diffuse, a range is given; if subpopulations with different affinities are present, individual constants are delimited by commas; a value in parentheses indicates rarely seen data. The data are separated by types of interacting proteins.

Donor	Acceptor	extra proteins	cell line	exc.	EC ₅₀ * (pairs/cell)
interactions between accessory chains					
IL-10R2/CiFP	IL-10R2/SiFP		293T	488	(300),1000–2000
IFN- R1/ECFP	IFN- R1/EYFP		293T	442	300–2000
IFN- R1/CiFP	IFN- R1/SiFP		293T	514	(80),300–1000
interactions between ligand-binding chains					
IFN- R1/CiFP	IFN- R1/SiFP		293T	488	(100),1000
IFN- R2c/ECFP	IFN- R2c/EYFP		293T	442	300–2000
IFN- R2c/CiFP	IFN- R2c/SiFP		293T	514	40–500
IFN- R2c/ECFP	IFN- R2b/EYFP		293T	442	300–2000
IFN- R2b/ECFP	IFN- R2b/EYFP		293T	442	300–2000
interactions between ligand-binding chain and accessory chain					
IFN- R1/EGFP	IL-10R2/SiFP		CHO-q3	488	3–10
IFN- R1/CiFP	IL-10R2/SiFP		293T	488	(70), 200
IFN- R1/ECFP	IFN- R2b/EYFP		293T	442	200–2000
IFN- R1/ECFP	IFN- R2c/EYFP		293T	442	70–800
IFN- R1/EYFP	IFN- R2c/ECFP		293T	442	(4),30–300
IFN- R1/EGFP	IFN- R2c/OFP		293T	442	500–1000
IFN- R1/EGFP	IFN- R2c/SiFP		CHO-q3	488	10–50
IFN- R1/CiFP	IFN- R2c/SiFP		293T	514	500–1000
IFN- R1/CiFP	IFN- R2c/SiFP	RACK-1	293T	514	60–300
IFN- R1/CiFP	IFN- R2c/SiFP	RACK-1/TFP	293T	442	50–400
IFN- R1/OFP	IFN- R2c/ChFP	Jak1/TFP, Tyk2/CiFP	293T	442	20–100
interactions between Janus kinase and receptor chains					
Jak1/CiFP	IFN- R1/SiFP		293T	514	(30),300

Donor	Acceptor	extra proteins	cell line	exc.	EC ₅₀ * (pairs/cell)
Jak1/CiFP	IFN- R2c/StFP		293T	514	50–200
Tyk2/CiFP	IFN- R1/StFP		293T	514	30–300
Tyk2/CiFP	IFN- R2c/StFP		293T	514	20–80