The Human Type I Interferon Receptor

IDENTIFICATION OF THE INTERFERON β-SPECIFIC RECEPTOR-ASSOCIATED PHOSPHOPROTEIN*

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We used specific antibodies recognizing the receptor 1 (IFNAR1) and the recently cloned receptor 2.2 (IFNAR2.2) chains of the human type I interferon receptor complex to demonstrate that the interferon β (IFN-β)-specific receptor-associated phosphoprotein is IFNAR2.2 and not an unknown or additional receptor component. Immunoprecipitation experiments demonstrated that IFNAR2.2 is present in Daudi cells as a cell surface protein of approximately 90–100 kDa, which is tyrosine-phosphorylated and associated with IFNAR1, upon stimulation of cells with IFN-β. IFNAR2.2 was not detected associated with IFNAR1 in cells stimulated with IFN-α, suggesting differences in receptor interaction between the two type I interferons. Both IFNAR1 and IFNAR2.2 undergo tyrosine phosphorylation upon induction by either IFN-α or IFN-β. Therefore, it is unclear as to why IFNAR2.2 is not detectable in IFNAR1 immunoprecipitates in IFN-β-treated cells. These data suggest that, although IFN-α and IFN-β may utilize similar receptor chains, they interact with IFNAR1 and IFNAR2.2 in different ways.

Cytokines such as the type I interferons (IFN)1 include both IFN-α and IFN-β (1). Type I IFNs induce a variety of cellular responses including immunomodulatory, antiviral, and antiproliferative effects. These biological responses are initiated by the interaction of the type I IFN with its cell surface receptor. This interaction brings together two receptor chains, the interferon α/β receptor 1 chain (IFNAR1) (2) and receptor chain 2.2 (IFNAR2.2) (3–5). These two receptor subunits are not preassembled on the cell surface but rather are induced to associate in the presence of ligand (6). The formation of the heteromeric receptor results in the formation of a functionally active receptor which leads to the activation of cytoplasmic proteins which mediate IFN signaling (7). Ligand-induced association of IFNAR1 and IFNAR2.2 results in the phosphorylation of IFNAR1 (8, 9) and IFNAR2.2 (10) on tyrosine residues by the Janus kinases, JAK1 and Tyk2 (11). Receptor phosphorylation subsequently results in the activation of STATs (signal transducers and activators of transcription) proteins by additional phosphorylation events. Such events lead to the formation of IFN-inducible transcription factors which bind to cis-acting IFN-stimulated response elements present in IFN-inducible genes (11).

IFN-α and IFN-β appear to utilize a common receptor complex and activate similar intracellular signaling pathways (1, 12). However, a number of observations suggest that differences may occur in the ability of IFN-α or IFN-β to induce certain biological effects. These include the preferential induction of an IFN-specific gene (13), differential growth inhibitory effects (14), and erythropoietic effects (15). Furthermore, it is possible to select Tyk2-deficient cells lines unresponsive to IFN-α but responsive to IFN-β (16, 17). One possible explanation for differential signaling events between type I IFNs would be the existence of an IFN-β-specific third component of the type I receptor. A candidate for such an additional third component of the receptor has been described previously as an IFN-β-specific receptor-associated phosphoprotein (BRAP). When cells are stimulated with IFN-β or IFN-β 1b, this protein becomes tyrosine-phosphorylated and associated with IFNAR1. This protein has been detected in MOLT-4 (18), U266 (19), and Daudi (20) cells and migrates as a broad band on SDS-PAGE with an apparent molecular mass of 90–100 kDa.

In this study, we made use of specific high affinity antibodies generated against either IFNAR1 or IFNAR2.2 to demonstrate that IFNAR2.2 represents the IFN-β-specific receptor-associated phosphoprotein. These results suggest that specific interactions of IFN-β with IFNAR2.2 results in a distinct conformational assembly of the type I IFN receptor.

MATERIALS AND METHODS

Cells and Reagents—Daudi cells were obtained from ATCC and grown in RPMI 1640 media (Life Technologies Inc.) supplemented with 20% (v/v) bovine calf serum and antibiotics. Human interferon α8 (specific activity = 2 × 106 units/ml) was provided by Ciba-Geigy AG Basel, and human IFN-β 1b (4.5 × 107 units/ml), in which cytochrome 17 was replaced by a serine, was produced at Berlex Biosciences as described previously (21, 22). The anti-phosphotyrosine (Tyr(P)) monoclonal antibody Ab-2 was obtained from Oncogene Sciences, anti-mouse IgG immunoglobulin conjugated to horseradish peroxidase and anti-rabbit IgG immunoglobulin conjugated to horseradish peroxidase were purchased from Transduction Laboratories. Nonidet P-40 was purchased from Calbiochem, all protease inhibitors were purchased from Boehringer Mannheim, and all buffers and reagents were purchased from Sigma.

Production of Antibodies to IFNAR1 and IFNAR2—IFNAR1 antibodies were produced as described previously (22). IFNAR2.2-specific polyclonal antibodies were generated against a soluble form of IFNAR2.2 (IFNAR2.2s). The plasmid phSER 97 containing a full-length cDNA coding IFNAR2.1 was isolated as described previously (22). The extracellular domain, obtained from phSER 97, ending at site Arg-242, of IFNAR2.1 was expressed using a baculovirus expression system (BacPac). A 6-histidine residue (6His) addition at the C terminus of IFNAR2.2s was included in the construct, and IFNAR2.2s was purified from the expression system media using a nickel chelate affinity resin column, as described previously (23). Purified IFNAR2.2s was then used to immunize rabbits for the production of a polyclonal antibody.

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recognizing IFNAR2.2

**Immunoprecipitations and Immunoblotting**—Cells (1.0 × 10^6) were stimulated with either IFN-α or IFN-β 1.b at 200 units/10^6 cells at 37 °C for 15 min in a CO2 incubator. After treatment, cells were quickly harvested at 4 °C by centrifugation (3000 × g, 3 min) and immediately solubilized in ice-cold lysis buffer (100 mM Tris, pH 8.0, containing 150 mM NaCl, 10% glycerol (v/v), 1% Nonidet P-40 (v/v), 1.0 mM each of orthovanadate, sodium pyrophosphate, sodium fluoride, EDTA, phenylmethylsulfonyl fluoride, 5.0 μg/ml leupeptin, and 5.0 μg/ml trypsin inhibitor. The lysate was centrifuged (16,000 × g, 30 min) at 4 °C, and the supernatant was collected. Cell lysates were immunoprecipitated using either monoclonal anti-IFNAR1 antibodies (40H2 or 4B1) as described previously (20) or IFNAR2.2 rabbit polyclonal antiserum (10 μl of antiserum/10^6 cells), followed by SDS-PAGE analysis using NovaPheos 8% Tris-glycine gels. After electrophoresis, proteins were transferred to polyvinylidenedifluoride filters (Pro-Blot) and blocked with 20 mM Tris, pH 8.0, containing 150 mM NaCl, 1.0 mM orthovanadate, 1.0 mM sodium pyrophosphate, 1.0 mM sodium fluoride, 1.0 mM phenylmethylsulfonyl fluoride, and 0.1% Tween 20 overnight, at room temperature. The filters were subsequently incubated with either anti-IFNAR1 (0.5 μg/ml) or anti-IFNAR2.2 (10 μl of antiserum/10 ml of blocking buffer) antibodies for 2–3 h at room temperature followed by 4 × 10-min washes with blocking buffer. The washed filter was then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 2–3 h at room temperature, washed 4 × 10 min, and developed using chemiluminescence (Enhanced Chemiluminescence Detection Kit, Pierce).

**Ligand Binding Assay**—Radiolabeling of IFN-α and IFN-β 1.b and ligand binding assays on Daudi cells were performed as described previously (22).

**RESULTS**

Characterization of Baculovirus-expressed Soluble IFNAR2.2 and Anti-IFNAR2.2 Antibodies—A soluble form of IFNAR2.2 was expressed in Sf9 cells infected with baculovirus encoding the extracellular domain of IFNAR2.2 (IFNAR2.2s) containing a 6-histidine (6-His) addition at the C terminus to aid in purification. IFNAR2.2s was purified from media of infected Sf9 cells using a nickel chelate affinity column (23). The purity of IFNAR2.2s isolated in this manner was greater than 90% as determined by SDS-PAGE and amino acid analysis (data not shown).

To determine if IFNAR2.2 could bind ligand, we tested the ability of IFNAR2.2s to compete for ligand binding with the native receptor present on the surface of Daudi cells. Phosphorylated IFN-α8 (278 pM, specific activity 50 μCi/μg) or IFN-β 1.b (278 pM, specific activity 60 μCi/μg) was incubated with Daudi cells in the presence of increasing concentrations of IFNAR2.2s (1.0 pM–200 nM) (Fig. 1). As shown in Fig. 1, purified IFNAR2.2s was able to compete for the binding of both IFN-α8 (closed circles) and IFN-β 1.b (open diamonds) to the human type I IFN receptor present on Daudi cells. The IC<sub>50</sub> of competition was 1.3 nM ± 1.0 nM (mean ± S.E., n = 3) indicating that IFNAR2.2s had lower ligand binding affinity than the native (IFNAR1/IFNAR2.2) receptor (24). Therefore, this form of IFNAR2.2s could bind ligand and was used to produce a specific IFNAR2.2 antisera in rabbits.

Anti-IFNAR2.2 blocked type I IFN binding to Daudi cells (data not shown) and precipitated a tyrosine-phosphorylated protein of 90–100 kDa from IFN-stimulated detergent-solubilized Daudi cell lysates (Fig. 2, lanes 2 and 3). This 90–100-kDa phosphoprotein was specifically precipitated with anti-IFNAR2.2 and had the expected size of IFNAR2.2 (10). We were unable to detect IFNAR2.1, which contains a shortened cytoplasmic domain (3–5), using anti-IFNAR2.2 antisera for immunoprecipitation followed by phosphotyrosine immunoblotting. IFNAR2.2 was tyrosine-phosphorylated in cells stimulated with either IFN-α8 or IFN-β 1.b (Fig. 2, lanes 2 and 3). The specificity of anti-IFNAR2.2 was confirmed by the observations that phosphorylated IFNAR2.2 was not detected in unstimulated cells (Fig. 2, lane 1) or when immunoprecipitations were performed with preimmune sera using Daudi cells that had been preincubated with IFN-β 1.b (Fig. 2, lane 4). Furthermore, preincubation of anti-IFNAR2.2 with purified, soluble IFNAR2.2 completely abolished its ability to immunoprecipitate phosphorylated IFNAR2.2 from IFN-treated Daudi cells (not shown). Therefore, anti-IFNAR2.2 specifically precipitated IFNAR2.2 from detergent-solubilized lysates derived from IFN-stimulated Daudi cells.

**IFNAR2.2 Is Associated with IFNAR1 Selectively in IFN-β 1.b-stimulated Daudi Cells**—When Daudi cells were stimulated with either IFN-α8 or IFN-β 1.b and immunoprecipitated using a monovalent antibody directed against IFNAR1 (4B1), a
major tyrosine-phosphorylated protein was detected, exhibiting an apparent molecular mass of 120–130 kDa (Fig. 3, lanes 2 and 3). This phosphoprotein was previously identified as IFNAR1 and does not appear to be phosphorylated in unstimulated cells (Fig. 3, lane 1). A second tyrosine-phosphorylated protein (BRAP) with an apparent molecular mass of 90–100 kDa was also observed but only in anti-IFNAR1 precipitates derived from Daudi cells that had been stimulated with IFN-β 1.b (Fig. 3, lane 3). Furthermore, IFNAR2.2 and BRAP have identical molecular masses when they are compared on the same gel (Fig. 3, lanes 3, 5, and 6). Because of the IFN-β-specific association of this phosphoprotein with IFNAR1, this protein was described previously as a possible third component of the type I IFN receptor complex and was suggested to be selectively involved in IFN-β signaling (18–20).

To further characterize BRAP, we immunoprecipitated lysates prepared from Daudi cells stimulated with either IFN-α8 or IFN-β 1.b using the 4B1 (anti-IFNAR1) monoclonal antibody. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride filters, and the resultant filters were immunoblotted with either anti-IFNAR1, anti-IFNAR2.2, or Tyr(P) antibodies (Fig. 4). Equal amounts of IFNAR1 were immunoprecipitated from either stimulated or unstimulated Daudi cells as determined by IFNAR1 immunoblots (Fig. 4, lanes 1–3). However, when filters from IFNAR1 immunoprecipitates were probed with our IFNAR2.2 antibody, we observed the presence of a 90–100-kDa protein corresponding to the expected molecular mass of IFNAR2.2 only in Daudi cells that had been stimulated previously with IFN-β 1.b (Fig. 4, lane 6). IFNAR2.2 was not detected in IFNAR1 precipitates from Daudi cells stimulated with IFN-α8 (Fig. 4, lane 5) or in unstimulated cells (Fig. 4, lane 4). Stripping and reprobing the same immunoblot with an anti-phosphotyrosine monoclonal antibody revealed the presence of an equal amount of phosphorylated IFNAR1 in Daudi cells stimulated with either IFN-α8 or IFN-β 1.b (Fig. 4, lanes 8 and 9). IFNAR1 did not become tyrosine-phosphorylated in unstimulated Daudi cells (Fig. 4, lane 7). As expected, one observed the presence of the 90–100-kDa BRAP in IFN-β 1.b (Fig. 4, lane 9) but not IFN-α8 (Fig. 4, lane 8)-treated Daudi cells. The phosphoprotein observed with slower mobility than IFNAR1 in Fig. 4, lane 9, was not reproducible and therefore was considered nonspecific. Finally, we also observed the presence of IFNAR1 in IFNAR2.2 immunoprecipitates using Daudi cell lysates derived from IFN-β 1.b-stimulated Daudi cells (Fig. 5, lane 3), but not in IFN-α8-stimulated or unstimulated Daudi cells (Fig. 5, lanes 1 and 2).

**DISCUSSION**

Once a functional type I IFN receptor is assembled, the associated kinases carry out specific phosphorylation events which lead to intracellular signaling events and IFN-induced gene activation (25). The transcriptional activation of IFN-inducible genes requires a number of previously identified and perhaps still unidentified cellular proteins. One such protein whose identity and role in IFN signaling remains unclear is a phosphoprotein that co-precipitated with IFNAR1 and becomes tyrosine-phosphorylated in response to IFN-β but not to other type I IFNs tested (18–20). This phosphoprotein has been proposed to represent an additional component of the type I receptor which is selectively involved in IFN-β signaling. Because of the potential importance of this protein in IFN signaling, we have focused our efforts on its characterization and identification. Thus, we produced specific antibodies directed against IFNAR2.2 to determine if IFNAR2.2 and the IFN-β-specific receptor-associated phosphoprotein represent the same protein. Initially we have shown that IFNAR1 and IFNAR2 are phosphorylated on tyrosine residues in Daudi cells stimulated with either IFN-α8 or IFN-β 1.b. We then observed that stimulation of Daudi cells with IFN-β 1.b induced the formation of a stable complex between IFNAR1 and IFNAR2.2 in which both proteins are tyrosine-phosphorylated. In contrast, while both proteins are tyrosine-phosphorylated when Daudi cells are stimulated with IFN-α8, the formation of a stable complex between IFNAR2.2 and IFNAR1 could not be detected using identical immunoprecipitation techniques. Furthermore, we found that IFNAR2.2 represents the previously described IFN-β-specific receptor-associated phosphoprotein. It is still unclear what role, if any, phosphorylation events play in mediating the interaction between IFNAR1 and IFNAR2.2.

Receptor cross-linking studies have previously demonstrated that IFNAR1 and IFNAR2.2 are present in IFN-α and IFN-β-induced type I receptor complexes (4, 6). Recent data have demonstrated that type I IFNs bind to IFNAR2.2 and form a high affinity ligand binding site in the presence of IFNAR1 (4,
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5.22. It is clear that a ligand-induced interaction between the type I IFN receptor chains occurs in both IFN-α- and IFN-β-stimulated cells. However, it is unclear why IFNAR2.2 is not detected in IFNAR1 immunoprecipitates in Daudi cells stimulated with IFN-α.

One possible explanation for the association of IFNAR2.2 with IFNAR1 in IFN-β-stimulated cells is that antibodies used for immunoprecipitation differentially recognize or disrupt a heterodimeric receptor complex formed in IFN-α-stimulated cells. This is unlikely in the IFN-β-specific association of IFNAR2.2 with IFNAR1 observed using a number of different polyclonal and monoclonal antibodies recognizing IFNAR1. It is also possible to detect the association of IFNAR1 in IFNAR2.2 immunoprecipitates in IFN-β-stimulated cells using polyclonal antibodies recognizing IFNAR2.2. Therefore, it is unlikely that the subsequent interaction of antibody with the receptor complex formed in IFN-α-stimulated Daudi cells causes dissociation of the complex. It may also be possible that at the concentrations of IFN-α used there is simply much less IFNAR2.2 associated with IFNAR1 in IFN-α compared to IFN-β-stimulated cells. To address this issue, we determined the amount of receptor complex formed in Daudi cells stimulated with a 100-fold excess of IFN-α or IFN-β (data not shown). We could not detect any association of IFNAR2.2 with IFNAR1 at these high concentrations of IFN-α suggesting that the concentration of IFN-α used did not limit our ability to detect the IFN-β-specific heterodimeric receptor complex. It is likely that the association of IFNAR2.2 with IFNAR1 is indeed different between IFN-α- and IFN-β-stimulated cells and that the IFN-α-mediated receptor complex is much less stable. Experiments are currently ongoing to address this issue and to relate the IFN-β-induced association of IFNAR2.2 with IFNAR1 to IFN-β-specific responses.

Little is known about the specific interaction between the type I IFN receptor chains and different IFN subtypes, during or after formation of a functionally active receptor complex. A similar ligand-induced association of receptor chains has been described for the α and β chains of the type II IFN-γ receptor (26). As with the type I IFN receptor, the α and β chains of the IFN-γ receptor are not preassociated with each other on the cell surface but are induced to associate in a ligand-dependent manner. Once this association occurs, the β chain of the receptor can be detected in α chain immunoprecipitates. In addition to the associations between the ligand and its receptor chains, interactions may exist between the receptor chains themselves (27) or between the proteins associated with the cytoplasmic domain of each receptor chain. Indeed, such an interaction has been proposed to take place between STAT 2 bound to the cytoplasmic domain of IFNAR2.2 and phosphotyrosine 466 located within the cytoplasmic domain of IFNAR1 (28, 29).

It remains to be determined what the specific nature of the interaction is between the type I IFN receptor subunits and what role ligand plays in the formation the receptor complex. An intriguing possibility is that the presence of IFNAR2.2 in the IFNAR1 immunoprecipitates derived from IFN-β 1.b-stimulated Daudi cell lysates suggests that somewhat different structural forms of the receptor exist, and IFN-α or IFN-β utilize similar receptor chains but may assemble them differently. Such ligand-induced variations in receptor structure may result in differential signal transduction and biological effects.

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Addendum—At the time of preparation of this manuscript we became aware that a manuscript describing similar observations using different reagents and cell types was submitted by Platianis et al. (30) (Dr. O. R. Colamonici, personal communication).

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