Interaction between the Components of the Interferon $\gamma$ Receptor Complex*

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Interferon $\gamma$ (IFN-$\gamma$) signals through a multimeric receptor complex consisting of two different chains: the IFN-$\gamma$ receptor binding subunit (IFN-$\gamma$R, IFN-$\gamma$R1), and a transmembrane accessory factor (AF-1, IFN-$\gamma$R2) necessary for signal transduction. Using cell lines expressing different cloned components of the IFN-$\gamma$ receptor complex, we examined the function of the receptor components in signal transduction upon IFN-$\gamma$ treatment. A specific IFN-$\gamma$R2:IFN-$\gamma$ cross-linked complex was observed in cells expressing both IFN-$\gamma$R1 and IFN-$\gamma$R2 indicating that IFN-$\gamma$R2 (AF-1) interacts with IFN-$\gamma$ and is closely associated with IFN-$\gamma$R1. We show that the intracellular domain of IFN-$\gamma$R2 is necessary for signaling. Cells coexpressing IFN-$\gamma$R1 and truncated IFN-$\gamma$R2, lacking the COOH-terminal 51 amino acids (residues 286-337), or cells expressing IFN-$\gamma$R1 alone were unresponsive to IFN-$\gamma$ treatment as measured by MHC class I antigen induction. Jak1, Jak2, and Stat1α were activated, and IFN-$\gamma$R1 was phosphorylated only in cells expressing both IFN-$\gamma$R1 and IFN-$\gamma$R2. Jak2 kinase was shown to associate with the intracellular domain of the IFN-$\gamma$R2.

It has been shown that the active receptors for several cytokines consist of at least two subunits (Jung et al., 1987; Kishimoto et al., 1992; Miyajima et al., 1992; Akira et al., 1993; Taniguchi and Minami, 1993). The multicomponent structure of the interferon $\gamma$ receptor complex was proposed based upon studies with human-rodent and mouse-hamster somatic cell hybrids. Interferon $\gamma$ (IFN-$\gamma$) binds to the IFN-$\gamma$ receptor binding subunit (IFN-$\gamma$R1), a species-specific cell-surface receptor encoded on human chromosome 21 (Jung et al., 1987) and mouse chromosome 16 (Hibino et al., 1991) was found to be important for signal transduction (Cook et al., 1992; Farrar et al., 1991, 1992). The first, proximal to the transmembrane region, is necessary for both receptor-ligand internalization and biological responses (Farrar et al., 1991). The second region, near the carboxyl terminus, includes Tyr-457 (Tyr-440, starting at the putative first amino acid of the mature chain), Asp-458, and His-461, which are required for biological responsiveness (Cook et al., 1992; Farrar et al., 1992). After phosphorylation of Tyr-457, Stat1α (Schindler et al., 1992) binds to this region (Stat1α recruitment site) due to specific interaction between the SH2 domain of Stat1α and phosphorylated Tyr-457 of IFN-$\gamma$R1 (Greenlund et al., 1994), with resultant phosphorylation of Tyr-701 of Stat1α (Shuai et al., 1993a). This phosphorylation is probably caused by the IFN-$\gamma$-activated tyrosine kinases Jak1 and/or Jak2, members of the Jak family of cytoplasmic protein tyrosine kinases (for review, see Ziemiecki et al., 1994; Ihle et al., 1994, 1995; Ihle and Kerr, 1995). Jak1 and Jak2 tyrosine kinases participate in signal transduction initiated by IFN-$\gamma$ as shown in mutant cell lines defective in the IFN-$\gamma$ signal transduction pathway suggesting that the IFN-$\gamma$ receptor components and the Jak kinases interact (Müller et al., 1993; Silvennoinen et al., 1993; Watling et al., 1993). In this report, we elucidate the structure of the IFN-$\gamma$ receptor complex and the role of the receptor components in the activation of the IFN-$\gamma$ signal transduction pathway.

EXPERIMENTAL PROCEDURES

Reagents, Restriction Endonucleases, and Other Enzymes—All restriction endonucleases were from Boehringer Mannheim Biochemicals or New England Biolabs; Sequenase 2.0 and T4 DNA ligase were from United States Biochemical Corp. The [α-32P]JATP and [γ-32P]JATP were from DuPont NEN. The cross-linker bis(sulfosuccinimidyl)suberate (BS3) was from Pierce Chemical Co. All other chemical reagents were analytical grade and purchased from United States Biochemical Corp.

Plasmid Construction and Site-specific Mutagenesis—The full-length Hu-IFN-$\gamma$R2 cDNA from plasmid pSK1 (Soh et al., 1994) was released by digestion with Sall restriction endonuclease and cloned into the Sall site of the vector M13mp18. The resultant clone was designated M13-IFN-$\gamma$R2. The oligonucleotides 5'-GGCTTTTATGATATTGTGCT-3', 5'-GATAGAAGATTTAAAAGAC-3', and 5'-CTCCACCATAG-
CATCCCAT-3' were used to change amino acid codon TGT to AGT (Cys-174 to Ser-174), to mutate Tyr-294 to Phe-294 (codon TAT to TTT), and to introduce a stop codon TAG after Pro-285 of IFN-γR1 as antigen.

Immunoprecipitations and Blotting—Cells were starved overnight in serum-free media and subsequently stimulated with IFN-γ (1000 units/ml) for 15 min at 37°C. Cells were solubilized in a lysis buffer consisting of 0.1% Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 0.15 mM NaCl, 10 mM NaF, 5 mM sodium pyrophosphate, 1 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, leupeptin (1 μg/ml), and pepstatin (1 μg/ml) for 1 h at 4°C. Following a 1-h incubation at 4°C, the samples were centrifuged at 10,000 × g for 10 min. The supernatants were cleared with a suspension of rabbit serum-agarose (Sigma, catalog No. R-6755) for 1 h at 4°C. After separation of the agarose by centrifugation, the eluted fractions were incubated with the indicated antibodies for 2–16 h at 4°C. Protein A-agarose was then added for 1 h at 4°C to bind the immune complexes. The protein A-agarose was washed three times in the lysis buffer and boiled in SDS sample buffer to remove bound components. The samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred with the Trans-Blot SD. Semi-Dry Transfer Cell (Bio-Rad, catalog No. 170-3910) according to the manufacturer’s instructions to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were then blocked with 3% (w/v) blocking agent (Amersham, catalog No. NIP551) and 1% bovine serum albumin in PBS with 0.5% Tween-20 (PBST), 10% glycerol, 1 μl of glucose in 1% at 22°C. The membranes were washed in PBST twice for 15 min and probed with the indicated primary antibodies for 1–4 h at room temperature. After washing in PBS and 1% bovine serum albumin with donkey anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies and developed with the enhanced chemiluminescence method (Amerham, ECL™ Western blotting analysis system, catalog No. RPN2108), according to the manufacturer’s protocol.

Glutathione S-Transferase Hu-IFN-γR2 Intracellular Domain Fusion Protein (GST/IFN-γR2) for Affinity Binding of Proteins—Six insect cells were infected with baculovirus encoding j ak1 or j ak2 kinases, and cell lysates were prepared and incubated with the GST/IFN-γR2 fusion protein or with GST alone immobilized on glutathione-Sepharose as described previously (Withthuhn et al., 1993). After extensive washing, the proteins associated with the fusion proteins were eluted in SDS sample buffer and resolved by SDS-PAGE and transferred to nitrocellulose, and probed with antisera to j ak1 and j ak2 kinases as described (Withthuhn et al., 1993).

j ak1 and j ak2 Activation Assay—Cells were starved overnight in serum-free media and subsequently stimulated with IFN-γ (200 units/ml) for 10 min at 37°C. The cells were lysed, and j ak1 and j ak2 proteins were immunoprecipitated as described above. Activation of j ak1 and j ak2 was determined by an in vitro kinase assay with a peptide corresponding to the putative phosphorylation site of j ak2 as a substrate. Specific phosphorylation of the j ak2 peptide (VLPQD-KEYYKVKEPEGE) by activated j ak1 and j ak2 was performed as previously described (Hoffmann et al., 1993). Cross-linking and trans-

Electrophoretic Mobility Shift Assays (EMSA)—EMSA were performed with a 22-base pair sequence containing a Stat-1 binding site corresponding to the GAS element in the promoter region of the human IRF-1 gene (5′-GATCGATTTCCCGAAGATCATG-3′) (Yuan et al., 1994). Two oligonucleotides, 5′-GATCGATTTCCCGAAGATCATG-3′ and 5′-CATGATTTCCCGAAGATCATG-3′, were annealed by incubation for 10 min at 65°C, 10 min at 37°C, and 10 min at 22°C and labeled with 32P-dATP by filling in with the Klenow fragment of DNA polymerase I in the presence of the other three dNTPs (Sambrook et al., 1989). Extracts were prepared by modification of the method of Dignam et al. (1983). Cells were grown to confluence in 100-mm dishes, treated with Hu-IFN-γ (1000 units/ml) for 15 min at 37°C, washed with ice-cold PBS, and harvested by scraping in ice-cold PBS. Cells were pelleted and resuspended in 100 μl of buffer RSB (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 6 μM MgCl2, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, pepstatin (1 μg/ml), leupeptin (1 μg/ml), and pepstatin (1 μg/ml) for 1 h at 4°C. After washing with PBS, the cells were resuspended in 0.5 ml of PBS containing 5 mM MgCl2, and cross-linked by adding 0.2 μl bis(sulfosuccinimidyl) suberate (BS3), freshly prepared in PBS, to a final concentration of 2 mM. The cross-linking reaction was terminated after 60 min at 4°C by the addition of 10 μl of 1 M Tris-HCl, pH 7.4. The cells were recovered by centrifugation and then extracted in 25 μl of PBS containing 1% Nonidet P-40, 0.5 mM NaCl, 5 mM EDTA, and 1 μM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM antipain, 10 μM benzamidine-HCl, 3 μM aprotinin, 1 μM chymostatin, and 1 μM pepstatin. The extracts were analyzed by SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide vertical slab gels (Laemmli, 1970). Gel-separated proteins were transferred to Kodak XAR-5 film with an intensifying screen for 12 h at 80°C.

Antibodies—Rabbit anti-j ak1 and anti-j ak2 antibodies were developed against synthetic peptides (KTLIEKERFEYSRKPRVCVTSP and DSRQKLFQYEDKHQPAPKC) respectively, corresponding to the end of the second kinase-like domains of murine j ak1 and j ak2, respectively. Rabbit anti-Stat1α antibody, raised against the carboxyl terminus of Stat1α and purified from j ak1 antisera, detected the phospho-tyrosine antibody was from Sigma (catalog No. P-3300). Rabbit anti-IFN-γR1 antibody was prepared with the extracellular domain of Hu-
 extracts were used directly for EMSAs or stored at –80 °C until use. EMSA reactions contained 2.5 µM of nuclear extract, 1 ng of 32P-labeled probe (specific activity 106 cpm/µg), bovine serum albumin (24 µg/ml), poly(dI:dC) (160 µg/ml), 20 mM HEPEs, pH 7.9, 1 mM MgCl2, 4.0% Ficoll (Pharmacia), 40 mM KCl, 0.1 mM EGTA, and 0.5 mM dithiothreitol in a total volume of 12.5 µl. For the supershift assay, 1 µl of a 1:10 dilution (equivalent to 0.1 µl of the undiluted antibody) of anti-HLA class I, or anti-HLA-B7 monoclonal antibodies (W6/32) followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The mouse anti-HLA monoclonal antibodies (W6/32) followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The cells were then analyzed by cytolytography. The dotted lines represent cells not treated with IFN, and solid lines represent cells treated with 1000 units/ml of the indicated IFNs. FIG. 1. Induction of HLA-B7 surface antigen. Induction of HLA-B7 surface antigen by IFN-γ of the parental 16-9 cells, expressing only the IFN-γ-α chain (A, B, C); 16-9/IFN-γ-R2, 16-9 cells expressing both IFN-γ-R1 and IFN-γ-R2 chains (D, E); and 16-9/IFN-γ-R2t, 16-9 cells expressing the IFN-γ-R1 chain and the truncated IFN-γ-R2 chain (E and F). HLA-B7 antigen was detected by treatment of cells with mouse anti-HLA monoclonal antibodies (W6/32) followed by treatment with fluorescein isothiocyanate-conjugated goat anti-mouse IgG. The cells were then analyzed by cytolytography. The dotted lines represent cells not treated with IFN, and solid lines represent cells treated with 1000 units/ml of the indicated IFNs. A, C, and F show results after Hu-IFN-γ treatment of cells, and B, D, and E, results after treatment with Hu-IFN-α/A/D. Relative fluorescence values are shown on a log scale as described (Hibino et al., 1992).

RESULTS

Biological Assay—We measured class I MHC antigen induction to evaluate the ability of the cloned components of the human IFN-γ receptor complex to transduce a signal upon Hu-IFN-γ treatment in hamster cells. The 16-9 cells, expressing only the IFN-γ-α chain of the human IFN-γ receptor complex, exhibit little or no response to Hu-IFN-γ (Fig. 1A; see also Soh et al., 1994). The 16-9/IFN-γ-R2 cells, 16-9 cells stably transfected with an expression vector encoding the intact IFN-γ-R2 under the control of the cytomegalovirus promoter, exhibited a substantial response to Hu-IFN-γ (Fig. 1C). To test if the intracellular domain of IFN-γ-R2 is necessary for signal transduction, we introduced a stop codon after amino acid 285 of IFN-γ-R2 resulting in a deletion of the COOH-terminal 51 amino acids. Hu-IFN-γ did not induce MHC class I antigens in 16-9 cells stably transfected with the expression vector encoding the truncated IFN-γ-R2t (16-9/IFN-γ-R2t) (Fig. 1E). As a control, it was shown that all cells responded to Hu-IFN-α/A/D demonstrating that the MHC class I antigen could be induced in all cell lines (Fig. 1, B, D, and F). We therefore conclude that the intracellular domain of the IFN-γ-R2 plays an important role in IFN-γ signal transduction. The portion of the intracellular domain of IFN-γ-R2, which was removed by a premature termination codon, had only one Tyr residue at position 294. Because phosphorylation of tyrosine residues in various receptors (including IFN-γ-R1) is shown to be important for signal transduction, we mutated this Tyr residue to Phe. Cells expressing this mutated IFN-γ-R2-Y294F were active in MHC class I antigen induction (data not shown), indicating that this Tyr is not necessary for the activity of the IFN-γ-R2 chain.

Cross-linking—To investigate the interaction between the chains of the IFN-γ receptor complex and IFN-γ, we carried out cross-linking experiments with cell lines expressing different components of the human IFN-γ receptor complex. The following cell lines were used: CHO-B7/IFN-γ-R2 cells, CHO-B7 cells stably expressing Hu-IFN-γ-R2 but not Hu-IFN-γ-R1, and 16-9, 16-9/IFN-γ-R2, and 16-9/IFN-γ-R2t cells defined above. A cross-linked band of IFN-γ-HuIFN-γ-R1 of approximately 120 kDa was observed in cell lines expressing the IFN-γ-R1 chain. However, in cells also expressing IFN-γ-R2, we observed the appearance of a lower molecular weight cross-linked product (IFN-γ: IFN-γ-R2) of approximately 60 kDa in size (Fig. 2). This band migrated faster when truncated IFN-γ-R2t in 16-9 cells (16-9/IFN-γ-R2t) was substituted for the intact IFN-γ-R2 chain (16-9/IFN-γ-R2) (Fig. 2). We therefore conclude that the 60-kDa band is a complex composed of IFN-γ-R2 and IFN-γ. We did not observe any cross-linked product in the CHO-B7/IFN-γ-R2 cells expressing only the IFN-γ-R2 chain.

The formation of an intracellular disulfide bridge between a Cys residue at position 174 of the human IFN-γ-R2 and the corresponding Cys residue at position 167 of the human IFN-γ-R1 (Soh et al., 1994) could account for the interaction between these two subunits of the IFN-γ receptor complex. To test this hypothesis, we mutated IFN-γ-R2 at position 174 from Cys to Ser. However, the mutated IFN-γ-R2-C174S remained fully active in cell lines expressing IFN-γ-R2. We did not observe any cross-linked product in the CHO-B7/IFN-γ-R2 cells expressing only the IFN-γ-R2 chain.

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IFN-γ-R1 and IFN-γ-R2 Chains Are Required for Jak1, Jak2, and Stat1α Activation—Phosphorylation and activation of Jak1 and Jak2 tyrosine kinases after IFN-γ treatment of human cells, as well as phosphorylation of Stat1α, have been demonstrated (Silvennoinen et al., 1993; Shuai et al., 1993b). To determine the contribution of each chain of the IFN-γ receptor complex in activation of intracellular signaling, we examined Jak1 and Jak2 activation and activation of Stat1α in cells expressing various components of the receptor.
In cells expressing both chains of the human IFN-γ receptor complex (16-9/IFN-γR2), IFN-γ activated Jak1 and Jak2 as measured by the in vitro phosphorylation of Jak1 and Jak2 substrate peptide by immunoprecipitated Jak1 and Jak2 kinases, respectively (Fig. 3). However, we did not observe activation of Jak1 and Jak2 in the 16-9 cells expressing only one chain of the human IFN-γ receptor complex (Fig. 3). The 16-9/IFN-γR2 cells, expressing the IFN-γR1 and the truncated IFN-γR2, failed to show activation of the Jak kinases (Fig. 3). In addition, we observed ligand-induced phosphorylation of Jak1 and Jak2 only in the cell line 16-9/IFN-γR2 expressing both chains of the human IFN-γ receptor complex (Fig. 4). Phosphorylation of Jak1 and Jak2 upon IFN-γ treatment was not detected in 16-9 and 16-9/IFN-γR2 cells (Fig. 4).

Analogous results were obtained for activation of Stat1α. Only in 16-9/IFN-γR2 cells expressing both receptor chains did IFN-γ produce an active Stat1α as measured by the electrophoretic mobility shift assay (Fig. 5A). The formation of the Stat1α DNA-binding complex upon IFN-γ treatment was suppressed after addition of an excess of unlabeled oligonucleotides as a competitor (Fig. 5A). To show that this complex was formed by Stat1α proteins, anti-Stat1α antibodies were added to the nuclear extracts from IFN-γ-treated 16-9/IFN-γR2 cells, and the extracts were incubated with the same radiolabeled probe. The specific DNA-binding complex was supershifted after addition of anti-Stat1α antibodies (Fig. 5B).

It was shown that overexpression of Jak1 and Jak2 by transient transfection leads to tyrosine autophosphorylation of Jak1 and Jak2 and activates Stat1α, as measured by DNA binding (Silvennoinen et al., 1993). However, overexpression of Jak1 and/or Jak2 by stable transfection of 16-9 cells, expressing only HuIFN-γR1, did not permit IFN-γ to induce MHC class I antigens. Furthermore, the activation of Stat1α was not detected in these cells. Thus, both receptor chains are necessary and sufficient for activation of Jak1, Jak2, and

Fig. 3. Activation of Jak1 and Jak2 tyrosine kinases upon IFN-γ treatment. Top panel, the activation of Jak1 and Jak2 was determined with an in vitro kinase assay as described under “Experimental Procedures” with a peptide substrate corresponding to the putative phosphorylation site of Jak2. The cell lines were 16-9, 16-9/IFN-γR2, and 16-9/IFN-γR2t as defined in the legend to Fig. 1. Bottom two panels, equal loading of Jak proteins in the assay was demonstrated by using one-third of the samples in Western blotting with Jak1 (middle panel) and Jak2 (bottom panel) antibodies.

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Fig. 4. Tyrosine phosphorylation of Jak1 and Jak2 upon IFN-γ treatment. Untreated and IFN-γ-treated cells were lysed and immunoprecipitated with anti-Jak1 (first and second panels) or anti-Jak2 (third and fourth panels) antibodies as described under “Experimental Procedures.” The cell lines were: 16-9, lanes 1 and 2; 16-9/IFN-γR2, lanes 3 and 4; 16-9/IFN-γR2t, lanes 5 and 6. The cell lines are defined in the legend to Fig. 1. Immunoprecipitates were resolved on SDS-PAGE, transferred to PVDF membranes, and Western blots were probed with anti-phosphotyrosine antibodies, first and third panels; with anti-Jak1 antibodies, second panel; and with anti-Jak2 antibodies, fourth panel.

Fig. 5. Electrophoretic mobility shift assay (EMSA). A, EMSAs were performed as described under “Experimental Procedures” with the 22-base-pair labeled sequence containing the Stat1α binding site corresponding to the GAS element in the promoter region of the human IRF-1 gene (Yuan et al., 1994) with nuclear extracts from following cells: 16-9, 16-9/IFN-γR2, 16-9/IFN-γR2t, CHO-B7, and CHO-B7/IFN-γR2 cells as defined in the legends to Figs. 1 and 2. In addition, HEp-2 cells, a human epidermoid larynx carcinoma cell line, were used as a positive control. B, the supershift assays were performed as described under “Experimental Procedures” with the 16-9/IFN-γR2 cell line. The position of the Stat1α DNA-binding complex and supershifted complex are indicated by the arrows. The same unlabeled oligonucleotides were used as a competitor in 100-fold excess.

Stat1α upon IFN-γ induction.

Phosphorylation of the IFN-γR1 Chain Requires the IFN-γR2 Chain—IFN-γ induces tyrosine phosphorylation of the IFN-γR1 chain (Greenlund et al., 1994). To determine the requirements for this phosphorylation, we performed immunoprecipitation with anti-IFN-γR1 antibodies on lysates of the 16-9, 16-9/IFN-γR2, and 16-9/IFN-γR2t cells, expressing different chains of the human IFN-γ receptor complex (Fig. 6). After immunoprecipitation, the samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a PVDF membrane, and the blot was probed with anti-phosphotyrosine antibodies. The IFN-γR1 chain was not phosphorylated upon IFN-γ treatment in the 16-9 cells. Tyrosine phosphorylation of IFN-γR1 occurred upon IFN-γ treatment only in

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cells expressing both chains of the IFN-γ receptor complex (Fig. 6). The truncated IFN-γR2 did not support the ability of IFN-γ to induce tyrosine phosphorylation of the IFN-γR1 chain. This demonstrates that the IFN-γR2 chain is necessary for tyrosine phosphorylation of the IFN-γR1 chain.

Association of Jak2 kinase with the IFN-γ Receptor Complex—Ligand-independent association of Jak1 to the IFN-γR1 chain and recruitment of Jak2 to the IFN-γ receptor complex after IFN-γ treatment have been demonstrated (Igarashi et al., 1994). To investigate the role of the IFN-γR2 chain in recruitment of Jak2 into the IFN-γ receptor complex, we performed immunoprecipitation of IFN-γR1 from lysates of various cells with anti-IFN-γR1 antibodies. After immunoprecipitation, the samples were resolved by SDS-PAGE, transferred to PVDF membrane, and the blot was probed with anti-Jak2 antibodies. The band recognizable by anti-Jak2 antibodies was observed in immunoprecipitates from cells expressing both receptor subunits (16-9/IFN-γR2) and to a lesser extent in 16-9 cells expressing only the IFN-γR1 and in both cases only after IFN-γ treatment (Fig. 6). The coprecipitation of Jak2 with antibodies to IFN-γR1 after IFN-γ induction in the cells expressing only IFN-γR1 may be explained by weak association of Jak2 with dimerized IFN-γR1 formed upon IFN-γ binding or by ligand-induced association with Jak1 which is bound to the IFN-γR1 chain.

From this observation we hypothesized that the intracellular domain of IFN-γR2 associates with Jak2 and is responsible for recruitment of Jak2 to the IFN-γ receptor complex after IFN-γ binding. To examine the interaction between Jak2 and the intracellular domain of IFN-γR2, we prepared a glutathione S-transferase (GST)/IFN-γR2 fusion protein (GST/IFN-γR2c). Lysates from SF9 insect cells infected with baculovirus producing Jak1 and Jak2 were incubated with the GST/IFN-γR2c or GST proteins immobilized on glutathione-Sepharose. The material bound to the glutathione-Sepharose was eluted, analyzed by SDS-PAGE, blotted, and then probed with anti-Jak1 and anti-Jak2 antibodies (Fig. 7). The Jak1 and Jak2 protein concentrations were equalized and monitored from total cellular baculovirus lysates (Withthuhn et al., 1993). The GST/IFN-γR2c fusion protein was found to bind Jak2 kinase (Fig. 7), demonstrating that the intracellular domain of the IFN-γR2 chain of the IFN-γ receptor complex associates directly with Jak2.
signaling pathway upon treatment with human and mouse IFN-γ, respectively, for MHC class I induction (Soh et al., 1994; Hemmi et al., 1994) suggests that the IFN-γR2 intracellular domain interacts with the hamster and mouse signal transduction components.

The participation of the protein kinases Jak1 and Jak2 as well as Stat1α in the IFN-γ signal activation pathway has been described (Darnell et al., 1994; Ihle et al., 1994; Ziemiecki et al., 1994). It has been shown that, upon IFN-γ treatment, four participants of the IFN-γ signaling pathway (the IFN-γR1, Jak1, Jak2, and Stat1α) are phosphorylated on tyrosine and that the tyrosine phosphorylation occurs rapidly (less than 1 min) after IFN-γ treatment (Igarashi et al., 1994; Greenlund et al., 1994). However, the sequence of events leading to activation of all components and the particular role of each participant in the IFN-γ signaling pathway is still unknown. Interaction between intracellular domains of the subunits of the IFN-γ receptor complex and components of the signaling pathway was proposed (Müller et al., 1993; Watling et al., 1993). Indeed, association of Jak1 tyrosine kinase with IFN-γR1 prior to IFN-γ treatment and recruitment of Jak2 upon IFN-γ binding has been shown by communoprecipitation experiments (Igarashi et al., 1994). We elucidated the role of the second receptor chain IFN-γR2 in signal transduction with cell lines expressing different components of the human IFN-γ receptor complex.

The IFN-γR2 chain renders cells expressing the IFN-γR1 chain responsive to IFN-γ. None of the components of the signal transduction machinery (Jak1, Jak2, Stat1α) were activated upon IFN-γ treatment in cells expressing only one chain of the IFN-γ receptor complex or IFN-γR1 and the truncated IFN-γR2 (Figs. 3–5). No phosphorylation of the IFN-γR1, Jak1, or Jak2 was observed in these cells even after a longer film exposure (Figs. 4 and 6). However, we observed communoprecipitation of Jak2 with antibodies to IFN-γR1 in cells expressing both chains of the IFN-γ receptor complex after IFN-γ treatment. To a lesser extent, we observed coprecipitation of Jak2 with antibodies to the IFN-γR1 in cells expressing only IFN-γR1 and only after IFN-γ treatment (Fig. 6).

We proposed that IFN-γR2 may associate directly with Jak2 and that the major IFN-γ-induced recruitment of Jak2 to the IFN-γ receptor complex is the result of association of IFN-γR2 with IFN-γR1 after IFN-γ binding. To investigate the possibility of association of IFN-γR2 with Jak2, we used the GST/IFN-γR2 intracellular domain fusion protein. The specific association of Jak2 with GST/IFN-γR2C was observed (Fig. 7) indicating that Jak2 associates with the intracellular domain of the IFN-γR2 directly in the absence of the IFN-γ ligand. The coprecipitation of Jak2 with antibodies to IFN-γR1 after IFN-γ induction in cells expressing only IFN-γR1 may be explained by the existence of a low affinity Jak2 binding site on dimerized IFN-γR1 chains upon IFN-γ binding or by the ligand-induced interaction between Jak2 and Jak1, which is bound to the IFN-γR1 chain. We propose that one region of Jak2 interacts with IFN-γR2 and a second region of Jak2 with the IFN-γR1 dimer or with Jak1 attached to the IFN-γR1. An analogous situation has been observed with the IL-2-induced association of the IL-2Rβ chain with Jak3: it was shown that Jak3 primarily associates with the γ, chain of the IL-2 receptor complex, but after IL-2 stimulation is weakly coprecipitated with the IL-2Rβ chain which primarily associates with Jak1 (Russell et al., 1994). In the presence of the truncated IFN-γR2t chain, IFN-γ-dependent coprecipitation of Jak2 with IFN-γR1 chain was not seen (Fig. 6). This may be due to different conformations of the IFN-γ receptor complex formed upon IFN-γ binding with and without the IFN-γR2 chain. Another explanation may be that the endogenous hamster IFN-γR2 in the cell lines expressing the human IFN-γR1 chain can still perform some function, such as bringing Jak2 to the complex, but is not sufficient for signal transduction. To test this possibility, cells with a deletion of the IFN-γR2 gene will be required. It should be noted, however, that we have not been able to detect binding of Jak2 in vitro to a GST/Hu-IFN-γR1 cytoplasmic domain fusion protein (data not shown).

Based on the results, we propose a model for the cascade of events in IFN-γ signaling (Fig. 8). There are at least two receptor subunits: IFN-γR1, the primary ligand binding subunit, and IFN-γR2, the second chain of the IFN-γ receptor complex. Both are required for signal transduction. Upon primary binding of the IFN-γ dimer to the IFN-γR1, all the components of the receptor signaling complex are brought together due to association of IFN-γ with the IFN-γR1 and the association of IFN-γR1 with IFN-γR2 (Hibino et al., 1992). The most likely stoichiometry of this complex is two molecules of IFN-γR1, two of IFN-γR2, and one of the IFN-γ dimer. Jak1 is associated with IFN-γR1 before IFN-γ treatment (Igarashi et al., 1994). IFN-γR2 associates primarily with Jak2 and brings Jak2 kinase to the IFN-γ receptor complex upon IFN-γ treatment. After oligomerization of the receptor chains, Jak1 and Jak2 tyrosine kinases associated with the intracellular domains of IFN-γR1 and IFN-γR2, respectively, are brought together and reciprocally activated by phosphorylation. This interaction of Jak1 and Jak2 kinases results in their activation, probably via heterodimerization of the kinases, and consequently in phosphorylation of Tyr-457 of the IFN-γR1 chain, which comprises the Stat1α recruitment site (Greenlund et al., 1994). Recruitment of Stat1α to the receptor complex due to the specific interaction between the Stat1α SH2 domain and the phosphorylated Tyr-457 of the IFN-γR1 chain (Greenlund et al., 1994) results in phosphorylation of Tyr-701 of Stat1α (Shuai et al., 1993a) and in subsequent Stat1α homodimerization (Shuai et al., 1994). Most likely, the proximity of two recruitment sites after IFN-γR1 dimerization facilitates Stat1α dimerization and dissociation of the dimer from the receptor complex. Existence of two recruitment sites for Stat IL-4 in close proximity on one receptor chain of the IL-4 receptor complex substitutes for the necessity to bring two separate chains, such as IFN-γR1, together to form a STAT dimer (Hou et al., 1994). The Stat1α dimer formed in response to IFN-γ then translocates to the nucleus and interacts with the GAS element in the promoter.
regions of IFN-γ-inducible genes, the process that begins the induction of this family of genes.

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