Identification of an Interferon-γ Receptor α Chain Sequence Required for JAK-1 Binding*

(Received for publication, October 11, 1995)

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We have shown previously that a four-amino acid block residing at positions 266–269 (LPKS) in the intracellular domain of the human interferon-γ (IFN-γ) receptor α chain is critical for IFN-γ-dependent tyrosine kinase activation and biologic response induction. Herein we show that this sequence is required for the constitutive attachment of the tyrosine kinase JAK-1. Using a vaccinia expression system, a receptor α chain-specific monoclonal antibody coprecipitated J AK-1 from cells coexpressing J AK-1 and either (a) wild type IFN-γ receptor α chain, (b) a receptor α chain truncation mutant containing only the first 59 intracellular domain amino acids, or (c) a receptor mutant containing alanine substitutions for the functionally irrelevant residues 272–275. In contrast, J AK-1 was not coprecipitated when coexpressed with a receptor α chain mutant containing alanine substitutions for the functionally critical residues 266–269 (LPKS). Mutagenesis of the LPKS sequence revealed that Pro–267 is the only residue obligatorily required for receptor function. In addition, Pro–267 is required for JAK-1 binding. These results thus identify a site in the IFN-γ receptor α chain required for constitutive JAK-1 association and establish that this association is critical for IFN-γ signal transduction.

Interferon-γ (IFN-γ) is a potent immunomodulatory cytokine that exerts its pleiotropic biologic effects by interacting with a single high affinity receptor expressed on the surface of nearly all host-derived cells (1–5). IFN-γ receptors consist of two species of matched polypeptides: a 90-kDa α chain (6–10) that is responsible for ligand binding, ligand trafficking through the cell, and signal transduction and a 62-kDa β chain (11, 12) that plays only a minor role in ligand binding (13) but which is obligatorily required for function (5). IFN-γ signal transduction is known to require at least three other components in addition to the receptor polypeptides. Two of these are the tyrosine kinases, JAK-1 and JAK-2 (14–18), which become rapidly activated following IFN-γ receptor ligation and effect the tyrosine phosphorylation of the third component, a latent cytosolic transcription factor, Stat1, which subsequently forms an activated homodimer, translocates to the nucleus, and initiates transcription of IFN-γ-inducible genes (19–25).

We have previously described a series of structure function analyses performed on the human IFN-γ receptor α chain intracellular domain. These studies involved replacing specific IFN-γ receptor amino acids with alanine residues and examining the capacity of the resulting mutant human receptor α chains to confer human IFN-γ responsiveness to a murine cell line containing the human receptor β chain (SCC16–5). Using this approach we and others identified two topographically distinct regions in the receptor α chain intracellular domain that were required for receptor signaling (26–28). The first was a block of four amino acids (LPKS) situated 13 residues from the transmembrane domain at positions 266–269 that was required for ligand-induced activation of intracellular tyrosine kinase activity, phosphorylation of the receptor α chain intracellular domain, and induction of IFN-γ-mediated cellular responses. The second was a membrane distal sequence located at positions 440–444 consisting of the residues YDKPH. The tyrosine residue within this sequence was found to be a physiologically important substrate site for the IFN-γ-activated tyrosine kinase activity. Ligand-induced phosphorylation of Tyr–440 was shown to form a Stat1 docking site on the receptor α chain, which also contained the residues Asp–441 and His–444. Binding of Stat1 to this site was shown to be a prerequisite for IFN-γ-dependent Stat1 tyrosine phosphorylation and subsequent signal transduction events (25).

A key question that remains unanswered is the precise function of the membrane proximal LPKS sequence in the IFN-γ receptor intracellular domain. Recent coprecipitation experiments performed by Igarashi et al. (29) suggest that JAK-1 associates with the IFN-γ receptor α chain. Therefore it is possible that the LPKS sequence may play a role in mediating receptor interaction with JAK-1.

In the current study we document that JAK-1 indeed binds to the IFN-γ receptor α chain in a constitutive manner and demonstrate that the LPKS sequence of the receptor plays a key role in mediating the association. Furthermore, we report the fine mapping of the LPKS sequence and show that the proline residue at position 267 is required for JAK-1 receptor α chain binding and receptor function. These results thus establish the physiologic relevance of the molecular interactions that occur between the IFN-γ receptor and one of its two required tyrosine kinase components.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Purified recombinant human and murine IFN-γ (specific activities, 3.8 × 10⁵ IU/mg and 4.7 × 10⁵ IU/mg, respectively) were generously provided by Genentech, Inc. (South San Francisco, CA). Recombinant human IFN-α2 (specific activity, 1.7 × 10⁶ IU/mg), a species-specific form of human IFN-α, was generously provided by Dr. Satwant Narula (Scherin-Plough Research Institute, Kenilworth, NJ). GIR-208 and GIR-94 are murine monoclonal antibodies specific for distinct epitopes on the human IFN-γ receptor α chain (30). 4G10, a murine IgG2b anti-phosphotyrosine-specific monoclonal antibody (31), was generously provided by Dr. Brian Drucker (Oregon Health Sciences University, Portland, OR). Purified antibodies were biotinylated as described previously (30). The monoclonal antibody 11–4.1 recognizes murine H-2Kk and was purified as described previously (26). A cDNA encoding human JAK-1 was kindly provided by Dr. S. Nagata of Osaka Bioscience Institute (Osaka, Japan). Protein A-agarose and Brij 96 detergent were obtained from Sigma. Streptavidin-phycocerythrin was purchased from ChromoProbe (Redwood, CA) and streptavidin-horseradish peroxidase from Zymed (South San Francisco, CA). 9E3, a mAb specific for the Type I human IFN receptor, was
generously provided by Dr. D. Goeddel, Tularik (South San Francisco, CA) (32). JAK-1 antisera was purchased from Upstate Biotechnology Inc. (Lake Placid, NY).

Cells and Cell Culture—SCC16–5, a murine fibroblast cell line that contains a single copy of human chromosome 21 (33, and Colo-205, a human adenocarcinoma cell line, were cultured as described previously (26, 34).

Plasmid Construction and DNA Transfection—Construction of expression plasmids encoding the wild type human IFN-γ receptor α chain or a human IFN-γ receptor α chain mutant in which residues L<sup>266</sup>PKS<sup>269</sup> were mutated in a block to four alanine residues has been previously described (26, 27). Plasmids encoding human IFN-γ receptor α chain mutants, which contain alanine substitutions for either Leu-266, Pro-267, or Lys-268 or multiple alanine substitutions for all the serine and threonine residues within the intracellular domain region from residues 256–303 were generated in a similar manner using primers based on the nucleotide sequence of the human IFN-γ receptor α chain sequence (6). The accuracy of all polymerase chain reaction-generated DNA was confirmed by dye-dideoxy sequencing (Sequenase, U. S. Biochemical Corp.). Cells were transfected using the calcium phosphate precipitation method, selected by culture in G418, and cloned by limiting dilution as described (26).

Analysis of Transfected Murine Cells for Responsiveness to Human IFN-γ—Responsiveness of transfected cells to human IFN-γ was assessed following culture of the cells in the presence of either buffer or human IFN-γ (1000 U/ml) for 72 h followed by quantitation by fluorescence-activated cell sorter of MHC class I antigen expression as described previously (26). As a control to ensure competency of the downstream events in the MHC class I induction pathway, all cultures were stimulated in a similar manner with homologous murine IFN-γ and analyzed by flow cytometry.

Immunoprecipitations and Western Blotting—Immunoprecipitations and Western blotting were performed as described previously (25) except that 5 × 10<sup>6</sup> Colo-205 cells were lysed in a lysis buffer consisting of 25 mM Tris-HCl, pH 8.0, 1% Brij-96, 150 mM NaCl, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, 10 μM each of leupeptin and aprotinin. Western blotting was performed using biotinylated GIR-94 (1 μM), biotinylated 4G10 (1 μM), or JAK-1 antisera (1:1000).

Protein Expression by Vaccinia Infection and Transient Transfection—HeLa cells were infected with recombinant vaccinia virus expressing T7 RNA polymerase and then transfected 45 min later as described previously (35, 36) with Bluescript expression vectors containing the cDNAs for wild type or mutant IFN-γ receptor α chains and/or JAK-1. The cDNAs were inserted into the vector downstream from the T7 promoter. Fourteen hours after infection, cells were washed once with PBS and lysed in 1 ml of lysis buffer. After incubation at 4 °C for 20 min, nuclei were pelleted in a microcentrifuge for 10 min at 10,000 × g. One hundred μl of the supernatant was kept as total cell lysate for Western blotting. The remainder was subjected to immunoprecipitation as described above.

RESULTS

JAK-1 Associates with the IFN-γ Receptor α Chain in Colo-205 Cells—Based on the previous report that JAK-1 constitutively associates with the IFN-γ receptor α chain in human HeLa cells (29) we investigated whether a similar association could be detected in the IFN-γ-responsive human adenocarcinoma cell line Colo-205 used in our laboratory. For these studies the human IFN-γ receptor α chain was immunoprecipitated from unstimulated or IFN-γ-treated Colo-205 cells, and the precipitates were analyzed for the presence of JAK-1 by Western blotting (Fig. 1, left panel, lanes 1 and 2). JAK-1 was present in precipitates derived from either unstimulated cells or cells treated with IFN-γ for 5 min. The association of JAK-1 with the receptor α chain was specific since α chain precipitates did not contain JAK-2 (data not shown) and precipitates generated with antibodies against an irrelevant Colo-205 surface receptor (i.e. the Type I TNF receptor) did not contain JAK-1 (Fig. 1, left panel, lanes 3 and 4). Western blot analysis of the IFN-γ receptor α chain immunoprecipitates using a phospho-tyrosine-specific mAb revealed that precipitates derived from IFN-γ-treated cells contained a 130-kDa tyrosine-phosphorylated band corresponding in size to JAK-1 and a 90-kDa tyrosine-phosphorylated band corresponding in size to the IFN-γ receptor α chain (Fig. 1, right panel). Neither phosphorylated product was observed in precipitates from unstimulated cells. Thus, JAK-1 associates with the IFN-γ receptor α chain in a constitutive manner, and its phosphorylation is ligand-dependent.

JAK-1 Binds to the Membrane Proximal 59 Amino Acids of the IFN-γ Receptor α Chain Intracellular Domain and Binding Is Dependent on the Presence of Receptor Residues Leu-266 to Ser-269—Since we showed previously that IFN-γ-dependent tyrosine kinase activation in cells requires the presence of the L<sup>266</sup>P<sup>268</sup>K<sup>269</sup> block of residues in the membrane proximal region of the IFN-γ receptor intracellular domain, we investigated whether this sequence was involved in mediating JAK-1-IFN-γ receptor α chain association. This possibility was examined by performing coprecipitation/Western blot analysis on cells that coexpressed JAK-1 and different forms of the human IFN-γ receptor α chain. JAK-1 was detected in IFN-γ receptor α chain precipitates derived from cells that coexpressed JAK-1 and either wild type IFN-γ receptor α chain or a truncated α chain that contained only the first 59 intracellular domain amino acids (hγR<sub>Δ51</sub>) (Fig. 2). In contrast, JAK-1 was not present in receptor immunoprecipitates derived from cells that only expressed recombinant JAK-1. Thus the JAK-1 binding site on the IFN-γ receptor is largely formed within the membrane proximal quarter of the receptor α chain intracellular domain.

Since the LP KS sequence resides in this region, we repeated the coprecipitations using two IFN-γ receptor α chain block mutants in which alanines were substituted for either the functionally important LP KS residues at positions 266–269 or the functionally unimportant SV VR residues at positions 272–275. Replacement of residues Ser-272 through Arg-275 with alanine did not diminish the ability of JAK-1 to associate with the receptor α chain. In contrast, alanine replacement of the LP KS sequence resulted in almost total abrogation of the interaction between JAK-1 and the receptor. When analyzed by receptor α chain-specific Western blotting, all receptor α chain...
Fig. 2. **JAK-1 associates with the first 59 amino acids of the IFN-γ receptor α chain and requires residues L266-PKS269.** One-hundred thousand HeLa cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase for 45 min at room temperature. The cells were then transfected by lipofection with cDNA constructs coding for JAK-1 and the full-length human IFN-γ receptor α chain (hgR, lane 1), the IFN-γ receptor α chain with residues L266-PKS269 replaced by alanines (L266-S269A, lane 2), the IFN-γ receptor α chain with S272-V275 replaced by alanines (S272-R275A, lane 3), or an IFN-γ receptor α chain truncation expressing only the first 59 amino acids of the intracellular domain (hgR-A311, lane 4). Control cells were also transfected with the wild type IFN-γ receptor α chain alone (hgR, lane 5) and JAK-1 alone (lane 6). The cells were incubated for 14 h at 37 °C, lysed, immunoprecipitated with a mAb to the IFN-γ receptor (IFN-R) α chain and Western blotted for the presence of JAK-1, as described under “Experimental Procedures” (upper panel). Total lysates were Western blotted for the IFN-γ receptor α chain (middle panel) and JAK-1 (lower panel) to control for levels of expression. IP, immunoprecipitate.

Derivatives were expressed at similar levels and displayed the appropriate molecular mass indicating that the alanine substitutions were not causing an abnormal truncation of the mutant proteins. Thus, the JAK-1 binding site in the intracellular domain of the IFN-γ receptor α chain is critically dependent on the functionally important LPKS sequence residing near the membrane.

Proline 267 Is the Only Residue Required for Receptor Function in the Membrane Proximal Region of the IFN-γ Receptor Intracellular Domain—Our previous functional analyses of the IFN-γ receptor α chain also revealed that the L266-PKS269 amino acid block contained the only residues within the membrane proximal region of the receptor’s intracellular domain that were required for induction of IFN-γ-dependent biologic responses. To determine which of the amino acids in this sequence were required, we generated a set of human receptor α chain alanine point mutants. Each mutant or wild type human IFN-γ receptor α chain was then stably expressed in the murine SCC16-5 fibroblast cell line that constitutively expressed the human IFN-γ receptor β chain. The resulting clones were assayed for the presence of a functional human IFN-γ receptor by quantitating the ability of human IFN-γ to enhance MHC Class I expression. As determined by fluorescence-activated cell sorter analysis all transfected cells expressed comparable levels of the human IFN-γ receptor α chain on their surface (Table I).

As expected, SCC16-5 cells transfected with empty vector did not respond to human IFN-γ while SCC16-5 cells expressing the wild type human IFN-γ receptor showed significant enhancement of MHC class I expression following incubation with human IFN-γ (Table I). Cells expressing a mutant receptor α chain, which contained a block replacement of four alanines for the entire LPKS sequence (LPKS-A1.2), were unresponsive to human IFN-γ. Cells expressing receptor α chain mutants that contained alanine substitutions for Leu-266 (LA266.11), Lys-268 (KA268.5), and all the serine and threonine residues within the first 40 intracellular domain amino acids including the serine residue at position 269 (S/T.4) responded to human IFN-γ. Based on dose-response experiments, the response to human IFN-γ of cells expressing these three mutant receptors was indistinguishable from that of cells expressing the wild type human IFN-γ receptor α chain (data not shown). In contrast, SCC16-5 cells expressing the receptor α chain with an alanine substitution for Pro-267 (PA267.7) were unable to respond to human IFN-γ. This observation was validated by two controls. First, all of the cell lines responded to homologous murine IFN-γ indicating that expression of the human receptor α chain derivatives did not affect the general ability of the cell to regulate MHC class I expression. Second, identical results were obtained when the cell lines were tested for the ability of human IFN-γ (in combination with lipopolysaccharide) to induce nitric oxide production from the cells (data not shown). Thus Pro-267 is the only amino acid located within the membrane proximal region of the α chain intracellular domain that is required for induction of an IFN-γ-dependent biologic response.

Pro-267 Is Required for JAK-1 Association with the IFN-γ Receptor α Chain—Since Pro-267 was the only residue within the L266-PKS269 block that is required for biologic function, we investigated whether Pro-267 was also critical in mediating JAK-1-IFN-γ receptor α chain association. This was examined by coexpressing JAK-1 with specific IFN-γ receptor α chain mutants in cells, immunoprecipitating the receptor, and analyzing the precipitates for the presence of JAK-1 by Western blotting. Cells coexpressing JAK-1 and either the wild type receptor or a receptor with the functionally unimportant residue Lys-268 mutated to alanine showed equivalent amounts of associated JAK-1 (Fig. 3). Thus, substituting an alanine for a residue that is not critical for the induction of biologic responses does not affect the ability of JAK-1 to associate with the receptor. In contrast, alanine replacement of the functionally critical Pro-267 resulted in a receptor mutant that was essentially unable to bind JAK-1. Pro-267 is therefore required both for the induction of biologic responses and for JAK-1-receptor association.

### Table I

| IFN-γ Receptor α Chain | Sequence (residues 250-268) | MHC Class I Human IFN-γ α chain |
|------------------------|-----------------------------|--------------------------------|
| Wild Type              | INPLK EK511 LPKS L1S5YRS ATLET KPE5K |
| LPSK-A1.2              | AAAA                        | 35 45 52                       |
| LA266.11              | A                           | 0 59 75                       |
| PA267.7               | A                           | 33 36 38                      |
| KA268.5               | A                           | 0 54 55                       |
| SIT.4                 | A A A A A A A               | 37 38 92                      |

**Discussion**

Following receptor ligation, two temporally distinct events must occur to achieve coupling of the IFN-γ receptor to its signal transduction system: activation of the JAK-1 and JAK-2 tyrosine kinases and recruitment of Stat1 to the receptor (25, 37). Our previous studies revealed that these events are mediated by topographically distinct regions within the IFN-γ receptor α chain intracellular domain (25–27). It is now clear that the membrane distal receptor sequence serves as the Stat1 docking site following tyrosine phosphorylation. However, the precise role of the membrane proximal residues required for kinase activation remains unclear. The studies presented herein document that the functionally important membrane proximal L266-PKS269 sequence identified in our previous studies plays a critical role in mediating the constitutive bind-
Fig. 3. JAK-1/IFN-γ receptor α chain association requires residue Pro-267. One-hundred-thousand HeLa cells were infected with a recombinant vaccinia virus expressing T7 RNA polymerase for 45 min at room temperature. The cells were then transfected with cDNA constructs coding for JAK-1 and the full-length human IFN-γ receptor α chain (hγR, lane 1), the IFN-γ receptor α chain (IFN-γRα) with residues Pro-267 replaced by alanine (PA267, lane 2), and the IFN-γ receptor α chain with Lys-268 replaced by alanine (PA267, lane 3). Control cells were also transfected with either the wild type IFN-γ receptor α chain (hγR, lane 5) or JAK-1 alone (lane 4). The cells were incubated for 14 h at 37 °C, lysed, immunoprecipitated with a mAb to the IFN-γ receptor α chain, and Western blotted for the presence of IFN-γ receptor α chain (middle panel) and JAK-1 (lower panel) to control for levels of expression. IP, immunoprecipitate.

ing of JAK-1 to the receptor α chain. Moreover, we show that within this sequence, the proline residue at position 267 plays a dominant role in effecting both JAK-1 binding and receptor function. These results thereby demonstrate that JAK-1-receptor α chain association is a prerequisite for induction of IFN-γ-dependent biological responses in cells.

The observation that JAK-1 attachment to the IFN-γ receptor α chain intracellular domain requires the LPK sequence suggests that these residues function as a box 1-like motif. In Type I cytokine receptors the box 1 motif contains a conserved proline required for JAK-1 attachment and receptor function. Importantly, a similar sequence containing a single proline (LPKS) occurs in the membrane proximal region of the IFNα receptor subunit recently cloned by Novick et al. (42). This receptor subunit also associates with JAK-1. Thus, it is possible that formation of a JAK-1 binding site on IFN receptor family members is critically dependent on this modified box 1 motif.

It is also of interest that JAK-2 was not found to associate with the receptor α chain. Recent studies by Sakatsume et al. (43) and Kotenko et al. (44) show that JAK-2 associates with the IFN-γ receptor β chain. Moreover, JAK-2/β chain association has been mapped to a 12-amino acid proline-rich sequence within the β chain intracellular domain.2 Finally, recent studies by Ashkenazi and co-workers (13) and Bach and Schreiber2 clearly demonstrate that the IFN-γ receptor α and β subunits do not constitutively associate with one another but rather become associated upon exposure to ligand. Taken together these results demonstrate that each receptor subunit associates with a specific JAK kinase. Thus ligand-dependent association of the IFN-γ receptor subunits (45, 46)2 brings into close juxtaposition inactive forms of receptor subunit-associated JAK-1 and JAK-2, which transactivate one another to initiate the IFN-γ signaling response.

REFERENCES

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