Stat3 Recruitment by Two Distinct Ligand-induced, Tyrosine-phosphorylated Docking Sites in the Interleukin-10 Receptor Intracellular Domain*

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Recent work has shown that IL-10 induces activation of the JAK-STAT signaling pathway. To define the mechanism underlying signal transducer and activator of transcription (STAT) protein recruitment to the interleukin 10 (IL-10) receptor, the STAT proteins activated by IL-10 in different cell populations were first defined using electrophoretic mobility shift assays. In all cells tested, IL-10 activated Stat1 and Stat3 and induced the formation of three distinct DNA binding complexes that contained different combinations of these two transcription factors. IL-10 also activated Stat5 in Ba/F3 cells that stably expressed the murine IL-10 receptor. Using a structure-function mutagenesis approach, two tyrosine residues (Tyr427 and Tyr477) in the intracellular domain of the murine IL-10 receptor were found to be redundantly required for receptor function and for activation of Stat3 but not for Stat1 or Stat5. Twelve amino acid peptides encompassing either of these two tyrosine residues in phosphorylated form coprecipitated Stat3 but not Stat1 and blocked IL-10-induced Stat3 phosphorylation in a cell-free system. In contrast, tyrosine-phosphorylated peptides containing Tyr374 or Tyr396 did not interact with Stat3 or block Stat3 activation. These data demonstrate that Stat3 but not Stat1 or Stat5 is directly recruited to the ligand-activated IL-10 receptor by binding to specific but redundant receptor intracellular domain sequences containing phosphotyrosine. This study thus supports the concept that utilization of distinct STAT proteins by different cytokine receptors is dependent on the expression of particular ligand-activatable, tyrosine-containing STAT docking sites in receptor intracellular domains.

Interleukin-10 (IL-10)1 is a cytokine produced by Th0 and Th2 CD4+ T cells, CD5+ B cells, and macrophages (1–5) that inhibits inflammatory and cell-mediated immune responses while enhancing humoral immunity (6–9). IL-10 exerts its pleiotropic effects following interaction with a specific IL-10 receptor that is expressed in low numbers on IL-10-responsive cells (1, 10). IL-10 binds to its receptor in a homogeneous manner with high affinity ($K_d = 9 \times 10^9$ m$^{-1}$) (11, 12). The cDNA encoding the ligand-binding polypeptide of the IL-10 receptor was recently cloned, and the receptor polypeptide was characterized as a 110-kDa glycoprotein (1, 10). Based on sequence homology, this protein has been characterized as a member of the type II cytokine receptor family that also contains the IFN$\alpha$ and IFN$\gamma$ receptor polypeptides (13). This single chain does not reconstitute a functionally active IL-10 receptor when expressed in fibroblasts, indicating that at least one additional polypeptide is required. However, the identity of the accessory molecule(s) has not been established.

Receptor proteins belonging to the type I and type II cytokine receptor families are now known to utilize the JAK-STAT family of proteins for signal transduction (14–22). Currently, this family consists of four Janus family tyrosine kinases (JAK-1, JAK-2, JAK-3, and TYK-2) and seven latent cytosolic transcription factors (Stat1-Stat6). Recent work has indicated that each cytokine receptor uses a distinct but often overlapping combination of JAKs and STATs to effect development of cytokine-specific cellular responses. Thus, at least some of the specificity of this signal transduction pathway appears to be determined by the recruitment of particular STAT proteins to the activated receptor.

Recently, we obtained insights into the mechanism underlying STAT recruitment by cytokine receptors though experiments that focused on defining the structure and function of the IFN$\gamma$ receptor in signaling the induction of cellular responses (23, 24). That work revealed that IFN$\gamma$-IFN$\gamma$ receptor interaction leads to the rapid phosphorylation of a particular tyrosine contained within the pentameric sequence YDKPH in the intracellular domain of the human and murine IFN$\gamma$ receptor $\alpha$ chains (residues 440–444 and 420–424 in the human and murine proteins, respectively) (24). A peptide containing the phosphorylated YDKPH sequence bound to Stat1 and prevented ligand-induced Stat1 tyrosine phosphorylation and expression of DNA binding activity. This sequence thus appears to act as the IFN$\gamma$ receptor docking site for Stat1 but not for any other STAT family member. Using purified recombinant proteins, we found that Stat1 binds directly to the phosphorylated YDKPH sequence (25). The latter result thus eliminates the possibility that the interaction is mediated through an acrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; PEC, peritoneal exudate macrophage cell.

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adapter protein. Taken together, these observations suggest a mechanism whereby STAT protein recruitment by distinct cytokine receptors is dependent on the specificity of the SH2 domain in the STAT protein for a particular phosphotyrosine-containing sequence motif within the intracellular domain of the cytokine receptor. This concept has been strongly supported by subsequent work showing that the specific array of STAT proteins recruited by ligated cytokine receptors can be altered either by subsequent sequences from other receptors that recruit different STAT proteins (26) or by exchanging the SH2 domains of one STAT for another (27).

To explore the generality of this mechanism, we have studied signaling through the ligated IL-10 receptor. This model was selected because: (a) IL-10 receptors are known to belong to the same cytokine receptor family as the IFN receptors; (b) ligation of the IL-10 receptor results in activation of the JAK-STAT signaling pathway (28–31); and (c) IL-10 induces pleiotropic biologic responses that are distinct from those induced by IFN-γ (2). In this report, we demonstrate that IL-10 activates Stat1, Stat3, and in certain cells, Stat5, leading to the formation of three or (in certain cells) four distinct DNA binding complexes. We show that a pair of tyrosine residues residing at positions 427 and 477 within the intracellular domain of the murine IL-10 receptor are obligatorily required for Stat3 recruitment but not for Stat1 or Stat5 recruitment and for induction of an IL-10-dependent biologic response. Finally, we define the Stat3 binding sites on the IL-10 receptor and demonstrate that phosphopeptides containing these docking sites bind selectively to Stat3 but not Stat1 and inhibit ligand-dependent Stat3 activation in a cell-free system. Collectively, these results demonstrate the specific recruitment of Stat3 by the IL-10 receptor, document the functional relevance of this recruitment, and demonstrate a novel process whereby different STAT proteins are recruited by different docking sites on the same receptor. The latter results thus form the basis for a novel mechanism by which a single cytokine receptor can effect multiple STAT protein recruitment.

EXPERIMENTAL PROCEDURES

Cells

Primary murine splenocytes and resident peritoneal macrophages were obtained from naive BALB/c mice (The Jackson Laboratory, Bar Harbor, ME). Macrophages were allowed to adhere to six-well cell culture plates in 4 ml of complete Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2% L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Ba/F3 is an IL-3-dependent murine pro-B cell line that lacks IL-10 receptors and was propagated as described (10). Ba/F3 derivatives expressing wild-type (BaMR) or mutant murine IL-10 receptors were prepared by stably transfecting cells with expression plasmids encoding the appropriate receptor form followed by selection with G418 (Life Technologies, Gaithersburg, MD) as described (10, 31). Surviving cell populations were cloned, and single cell clones were expanded and maintained by culture in IL-3. IL-10 receptor expression in each cell population was determined by Scatchard analysis (10). The murine RAW264.7 macrophage cell line was obtained from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 2% L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin as described (12).

Reagents

Purified recombinant murine IL-10 (specific activity, 1.5 × 10^7 units/mg) was prepared as described previously (12). Recombinant murine IFN-γ (specific activity, 4.7 × 10^7 IU/mg) was generously provided by Genentech (South San Francisco, CA). Protein G-Sepharose was purchased from Pharmacia Biotech Inc. Protein A-Sepharose and streptavidin-Sepharose were obtained from Sigma or Pierce. Horseradish peroxidase-conjugated goat antisera specific for rabbit IgG was purchased from U. S. Biochemical Corp. The 4G10 phosphotyrosine-specific mAb (murine IgG2b) was generously provided by Dr. Brian Drucker (Division of Hematology, Oregon Health Sciences University, Portland, OR). Horseradish peroxidase-conjugated antiphosphotyrosine mAb RC-20, and anti-Stat3 and Stat5 mAbs were purchased from Transduction Laboratories (Lexington, KY). Stat5 rabbit polyclonal IgG was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Different polyclonal antisera specific for the carboxyl-terminal regions of Stat1, Stat2, Stat3, Stat4, or Stat5 were prepared as described (22). Stat1 mAbs ATO-2F5 and ATO-1D6 were prepared as described (25).

Peptides

The following peptides, based on the murine IL-10 receptor, were used in these studies: 370–381, QQLGYTHQDQQDD; 392–403, GQPKFTCAADT; and 414–421, -CTTTTCTGGTCTTCTGGAAATA (24). Peptides were synthesized on an Applied Biosystems model 432A peptide synthesizer (Foster City, CA) with or without the addition of an amino-terminal biotin group using the biotin-amidocaproate N-hydroxysuccinimide ester (Sigma), as outlined in the manufacturer’s instructions. Phosphotyrosine-containing biotinylated and nonbiotinylated peptides were synthesized using FMOC (N-(9-fluorenylethoxycarbonyl)-phosphorylated tyrosine obtained from Advanced Chemtech (Louisville, KY). Peptides were released from the resin and deprotected according to the manufacturer’s instructions. Peptides were concentrated by lyophilization and purified by reversed-phase high-performance liquid chromatography using a Vydac C18 column (Hesperia, CA) as described (24). The composition of the peptides was confirmed by electrospray mass spectrometry. A single moiety was detected displaying the appropriate molecular mass for each peptide. Amino acid composition and peptide concentrations were determined using a Beckman 6300 amino acid analyzer (Beckman Instruments).

Oligonucleotides

An 18-base pair oligonucleotide probe based on the IFN-γ response region (GRR) within the promoter of the FcγRI gene (sense strand, 5'-ATTGATTTCCCCAGAAA; antisense strand, 5'-CTTTTCTGGAAATA) was synthesized on a Beckman Oligo 1000 DNA synthesizer (Fullerton, CA). A biotinylated derivative of this probe was prepared by adding Biotin-ON-Phosphoramidite (Clontech, Palo Alto, CA) at the 5’ end of the oligonucleotide according to the manufacturer’s protocol. Oligonucleotides were annealed by heating 50 μg of the 5’ and 3’ strands at 95°C for 5 min, and double-stranded oligonucleotides were purified on a 4% low-melting agarose gel (Sigma). The sequence of the 20-base pair M67 oligonucleotide probe used in this study is: sense strand, 5'-TGACATTCTCCGATTAC; antisense strand, 5'-GTTAAAAAGCATTACAGCCT. Oligonucleotides were labeled with [32P]dATP as described (24).

Electrophoretic Mobility Shift Assay

Five million primary murine splenocytes or murine macrophages were plated in six-well cell culture plates. The macrophages were allowed to adhere to the cell culture plates for 4 h prior to stimulation. When IL-3-dependent derivatives of the Ba/F3 cell line were used, 5 × 10^6 cells were plated in the absence of IL-3, cultured for 4 h, and then stimulated with either PBS, 400 ng of IL-10, or 4 ng of IFN-γ in a total volume of 4 ml. Cells were harvested after various periods of time, and nuclear extracts were prepared as described previously (12). In brief, cells were removed from the plates either by gentle scraping or washing, washed in ice-cold PBS, and resuspended in 1 ml of cell lysis buffer consisting of 10 mM HEPES, pH 7.9, containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 10 μg/ml each of leupeptin and aprotinin, and 0.05% Nonidet P-40. After 30 min at 4°C, nuclei were pelleted by centrifugation at 1000 × g for 5 min at 4°C, and nuclear proteins were extracted by incubation for 30 min at 4°C with 50 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, containing 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin and aprotinin). Nuclear membranes were then pelleted for 5 min at 10,000 × g, and protein concentrations of the nuclear extracts were determined using the BCA assay (Pierce).

Quantitation of DNA binding activity in 3 μg of nuclear extracts was performed using the [32P]-labeled GRR probe as described (12, 24). Gels were dried and visualized by autoradiography using Kodak XAR film (Eastman Kodak Co., Rochester, NY). To determine the identity of transcription factors present in the DNA binding complexes, supershift experiments were performed using STAT-specific antisera and control normal rabbit serum at a dilution of 1:100.
STAT Protein Precipitation

Use of Biotinylated Double-stranded GRR Oligonucleotide Probes—One hundred ng of biotinylated forms of the double-stranded GRR probe was incubated for 30 min at 4 °C with 100 μl of streptavidin-Sepharose. The mixture was washed in PBS and combined with 1 ml of nuclear extracts derived from 5 × 10⁷ BaMR cells that had been treated for 7 min at 37 °C with 100 ng/ml murine IL-10. After incubation for 2 h at 4 °C, the Sepharose was pelleted at 1000 × g for 5 min and washed by repeated centrifugation and resuspension in 800 μl of lysis buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 0.5% Nonidet P-40, 10 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 750 μM dithiobisreitol, 1 mM phenylmethylsulfonfluoride, and 10 μg/ml each of leupeptin and aprotonin). The Sepharose was resuspended in 50 μl of 2 × Laemmlibuffer containing 180 mM 2-mercaptoethanol (Sigma), and the suspension was heated to 70 °C for 5 min. Samples were divided in half and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, CA), and membranes were blocked for 18 h at 4 °C with 50 mM Tris buffered physiologic saline (TBS), pH 8.0, containing 2% bovine serum albumin and 0.5% Tween 20 (Sigma). Membranes were washed in TBS containing 0.5% Tween 20 and incubated with Stat1- or Stat3-specific antisera at a 1:3000 final dilution, then washed again and incubated with peroxidase-conjugated goat-anti-rabbit-IgG at a dilution of 1:7500 for 20 min. Antibody binding was detected by chemiluminescence using the ECL detection reagent (Amersham Corp.).

Use of anti-STAT Antibodies—BaMR, RAW264.7, and PEC were washed once in PBS/10% fetal calf serum, resuspended in 1 ml of lysis buffer, and then incubated at 4 °C for 30 min. The nuclei were pelleted at 10,000 × g for 10 min at 4 °C. The protein concentration of the lysate was normalized to 1.1 mg of total protein in 1 ml of lysis buffer. The lysate was precleared by sequential treatment with either 2 μl of normal hamster sera (Stat1 IP) or 2 μl of normal rabbit sera (Stat3 IP), followed by the addition of 40 μl of protein G-Sepharose (Stat1) or protein A-Sepharose (Stat3). After a 45-min incubation at 4 °C, the Sepharose was pelleted, and 10 μg of ATO-2F5 (anti-Stat1), 10 μg of anti-Stat5 affinity-purified rabbit polyclonal antibody, or 2 μl of anti-Stat3 polyclonal sera were added to the lysates and incubated for 45 min at 4 °C. Forty-five μl of the appropriate Sepharose were added to each tube and incubated for 45 min at 4 °C. The Sepharose was pelleted and washed five times; then the proteins contained in the precipitate were analyzed for the presence of Stat1, Stat3, or Stat5 by SDS-PAGE and Western blotting (utilizing the corresponding mAb at a concentration of 5 μg/ml).

Use of Biotinylated Wild-type or Phosphorylated IL-10 Receptor Peptides—One hundred μl of streptavidin-Sepharose was incubated for 30 min at 4 °C with a final concentration of 100 μM phosphorylated or nonphosphorylated biotinylated peptides in a final volume of 500 μl. Beads were pelleted by centrifugation (1000 × g for 5 min) and washed once with 800 μl of PBS. Fifty million unstirminated BaMR cells were lysed in 1 ml of 20 mM HEPES buffered physiologic saline, pH 7.4, containing 0.5% Nonidet P-40, 5% glycerol, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF, 750 μM dithiobisreitol, 1 mM phenylmethylsulfonfluoride, and 10 μg/ml each of leupeptin and aprotonin for 30 min at 4 °C, and protein concentration was determined by BCA. The protein concentration of the lysate was adjusted to 1 mg/ml, and 2 μl of lysate were incubated for 2 h at 4 °C with each of the peptide-Sepharose conjugates with constant mixing. The Sepharose was pelleted and washed five times; then the proteins contained in the precipitates were analyzed for the presence of Stat1 by SDS-PAGE and Western blotting as described above.

IL-10-dependent STAT Protein Phosphorylation in a Cell-free System

One hundred million BaMR cells were resuspended in 2 ml of reaction buffer (10 mM HEPES, pH 7.5, containing 100 mM NaCl, 20 mM MgCl₂, 200 μM ascorbic acid, 4 mM ATP, 2 mM EGTA, 1 mM phenylmethylsulfonfluoride, and 10 μg/ml each of leupeptin and aprotonin) and disrupted in a steel Dounce (32). The extent of cell disruption was monitored by trypan blue exclusion. Homogenates were diluted to a protein concentration of 40 mg/ml in reaction buffer, and 450 μl (18 mg total protein) were incubated in the absence or presence of 100 μM phosphorylated or nonphosphorylated peptides for 45 min at 4 °C. Subsequently, the homogenates were treated for 15 min at 37 °C with 500 ng of IL-10. The reaction was stopped by the addition of 1.5 ml of lysis buffer. Nuclei were removed by centrifugation at 10,000 × g for 10 min, and lysates were precleared by incubation with 4 μl of normal rabbit serum and 80 μl of protein A-Sepharose. STAT proteins were precipitated using 1 μl of Stat3-specific antisera and 100 μl of protein A-Sepharose. Immunoprecipitates were washed five times and analyzed by SDS-PAGE and Western blotting using the corresponding mAb at a concentration of 5 μg/ml.

RESULTS

IL-10 Treatment of Cells Leads to the Generation of Multiple DNA Binding Species—IL-10 treatment of IL-10 receptor-expressing cultured and primary cells (BaMR, RAW264.7, PEC, and splenocytes) resulted in the generation of at least three electrophoretically distinct DNA binding complexes (denoted as bands A, B, and C) that can be detected in nuclear extracts within minutes after ligand addition (Fig. 1). These complexes were visualized using an electrophoretic mobility shift assay (EMSA) that used an oligonucleotide probe (GRR probe) previously shown to be recognized by several different activated STAT complexes. The EMSA complexes induced by IL-10 were more complex than those induced following IFNγ treatment of the same cells. None of the nuclear extracts derived from either unstimulated receptor-bearing cells or IL-10-treated cells that lack the IL-10 receptor (Ba/F3) showed binding to the GRR probe. The appearance of the IL-10-induced DNA containing complexes in cell nuclei was dependent on the time of stimulation and the amount of IL-10 used. IL-10-induced gel shift complexes were first observed in the nucleus after 1–3 min, were maximal after 7–15 min, and disappeared 60 min after IL-10 addition (data not shown). These kinetics were distinct from those of the IFNγ-induced complexes that appeared more rapidly (i.e., were detectable in the nucleus after only 1 min of treatment) and that were still detectable in the nucleus 2–3 h after stimulation. Thus, IL-10 induces the activation of multiple DNA binding complexes with STAT-like DNA binding specificity in both cultured and primary IL-10-responsive cells.

Stat1 and Stat3 Are Activated in All IL-10-treated Cells, and Stat5 Is Additionally Activated in IL-10-treated Pro-B CellsExpressing the IL-10 Receptor—Supershift experiments performed on all IL-10-treated cells revealed that ligand induced
the activation of both Stat1 and Stat3. Using peritoneal macrophages as an example (Fig. 2A), bands B and C were supershifted by Stat1-specific antiserum (lane 3), and bands A and B were supershifted by Stat3-specific antiserum (lane 5). Moreover, a combination of Stat1 and Stat3 antisera completely supershifted all three IL-10-induced EMSA complexes (data not shown). Stat2 (lane 4), Stat4 (lane 6), and Stat5 (lane 7) antisera did not influence the mobility of the EMSA complexes. These results indicate that the IL-10-induced bands A, B, and C represent a Stat3 homodimer, Stat1/Stat3 heterodimer, and a Stat1 homodimer, respectively, in these cells. Similar results were obtained using RAW264.7 cells and splenocytes (data not shown). The validity of these observations was confirmed by the demonstration that phosphorylated forms of Stat1 and Stat3 could be precipitated with Sepharose-associated biotinylated double-stranded GRR probe from IL-10-treated cells (data not shown). Recently, Horvath et al. (33) demonstrated that an oligonucleotide probe (M67) derived from the serum-inducible element of c-fos binds to activated Stat3 with higher affinity than activated Stat1. When IL-10 EMSAs were performed using the M67 probe, the resulting gel shift pattern showed a much higher proportion of band A compared to GRR gel shifts. The band A observed with the M67 probe was completely supershifted by Stat3-specific antiserum (data not shown). Thus, IL-10 induces activation of Stat1 and Stat3 in all cells expressing functionally active IL-10 receptors.

In contrast, supershift analysis performed on nuclear extracts of IL-10-treated Ba/F3 pro-B cells that stably express the murine IL-10 receptor (BaMR cells) revealed the presence of an additional DNA binding component that displayed a mobility that was identical to the Stat3 homodimer but which was not recognized by anti-Stat3 (Fig. 2B). This analysis revealed that band A was only partially supershifted with Stat3-specific antiserum (lane 5). However, the band was also partially supershifted with antiserum specific for Stat5 (lane 7). The combination of Stat3- and Stat5-specific antisera completely supershifted band A (data not shown). Moreover, a mixture of Stat1-, Stat3-, and Stat5-specific antiserum was able to completely supershift all EMSA complexes observed in IL-10-treated BaMR cells (lane 9). Thus, BaMR cells respond to IL-10 by activating Stat5 in addition to Stat1 and Stat3. These results thus demonstrate that IL-10 activates the transcription factors Stat1 and Stat3 in a wide variety of IL-10 receptor-bearing cell types and, in addition, can activate Stat5 in a cell-restricted manner.

To examine whether the difference in EMSA complex formation observed in BaMR cells compared to other cells was attributable to differences in cellular expression of Stat1, Stat3, or Stat5, we performed immunoprecipitation/Western blot analyses on each cell type using STAT protein-specific antibodies (Fig. 3). Stat1 (top panel), Stat3 (middle panel), and Stat5 (bottom panel) were expressed at roughly equivalent levels in BaMR, RAW264.7, and PEC cells. Thus, the differential IL-10-dependent activation of Stat5 seen in BaMR cells is not due to the enhanced expression of this particular STAT protein.

Two Tyrosine Residues at Positions 427 and 477 in the Intracellular Domain of the Murine IL-10 Receptor Are Critical for Ligand-dependent Stat3 Activation—We next investigated whether Stat1, Stat3, and Stat5 were recruited to the IL-10 receptor in a manner analogous to that used in IFNγ signaling, i.e. through interaction of specific STAT proteins with a specific phosphoryrosine-containing sequence within the intracellular domain of the receptor. The ligand binding subunit of the murine IL-10 receptor intracellular domain contains four tyrosine residues at positions 374, 396, 427, and 477. Two of these, Tyr427 and Tyr477, are conserved in human and murine IL-10 receptors (1). We, therefore, generated a family of IL-10 receptor deletion or point mutants that lacked different combinations of intracellular domain tyrosine residues and stably expressed each mutant or wild-type receptor in murine Ba/F3 cells.}

**Fig. 2.** Stat1 and Stat3 are activated in all IL-10-treated cells, and Stat5 is additionally activated in IL-10-treated pro-B cells expressing the IL-10 receptor. Primary murine peritoneal macrophages (PEC) (A) or cultured IL-10 receptor-positive BaMR cells (B) were treated either with PBS or recombinant murine IL-10 (100 ng/ml for 7 min), and nuclear extracts were prepared. Three μg of each extract were assayed for DNA binding activity in the presence of antisera specific for Stat1, Stat2, Stat3, Stat4, or Stat5 at final concentrations of 1:100 using the electrophoretic mobility shift assay as described in Fig. 1.

**Fig. 3.** Cellular levels of Stat1, Stat3, and Stat5 expression are roughly equivalent in all cell types tested. BaMR, Raw264.7, and PEC cells were lysed, and the protein concentration of the lysate was adjusted to 1.1 mg/ml. One milliliter of lysate was incubated with the appropriate normal sera (NS) or antibodies specific for Stat1 (top panel), Stat3 (middle panel), or Stat5 (bottom panel). Following immunoprecipitation, the precipitates were analyzed for the presence of Stat1, Stat3, or Stat5 by SDS-PAGE and Western blotting utilizing the corresponding monoclonal antibody at a concentration of 1 μg/ml.
cells. As determined by Scatchard analysis, the resulting cell lines expressed comparable levels of IL-10 receptor that displayed identical ligand binding affinities in the range of 33–39 pm (10). Whereas unstimulated receptor binding cells did not induce DNA binding activity, the appropriate gel shift complexes were induced in IL-10-stimulated Ba/F3 cells that had been stable transfected with the wild-type IL-10 receptor cDNA and which expressed 6300 receptors/cell, i.e. BaMR cells (Fig. 4A, lanes 1 and 2, respectively). Ba/F3 cells expressing a mutant IL-10 receptor lacking residues 282–414 that thereby deletes tyrosines 374 and 396 (6300 receptors/cell) also proliferated when exposed to IL-10 (29). In contrast, cells bearing IL-10 receptor mutants that were unable to activate Stat3 in response to ligand (Δ402–559 and Y427F/Y477F) failed to mount a proliferative response to IL-10. Thus, we established a direct correlation between the IL-10 receptor tyrosine residues needed for Stat3 activation and biologic response induction.

Demonstration of an Interaction between Stat3 and IL-10 Receptor Peptides Containing Phosphorylated Forms of Tyr^{427} and Tyr^{477}—To investigate whether Stat1 and/or Stat3 interacted with IL-10 receptor sequences containing Tyr^{427} or Tyr^{477}, we performed coprecipitation analyses using biotinylated 12 amino acid peptides based on IL-10 receptor sequences surrounding Tyr^{427} (residues 423–434) and Tyr^{477} (residues 473–484, respectively). The peptides contained either tyrosine or phosphotyrosine residues. Stat3 was precipitated by phosphotyrosine-containing peptides encompassing the amino acids around Tyr^{427} (residues 423–434) or Tyr^{477} (residues 473–484, respectively). The specificity of the interaction was confirmed by the observation that Stat3 was not precipitated by biotinylated forms of: (a) nonphosphorylated Tyr^{427}, or Tyr^{477},containing peptides (upper panel, lanes 1 and 2, respectively); (b) phosphorylated Tyr^{374}, or Tyr^{399},containing peptides (upper panel, lanes 1 and 2, respectively); or (c) the Stat1 binding phosphopeptide derived from the human IFNγ receptor α chain (residues 436–447 in the intracellular domain). In contrast, none of the IL-10 receptor-derived peptides were able to coprecipitate Stat1 (Fig. 5, lower panel, lanes 1–6), although Stat1 could be precipitated by the IFNγ receptor phosphopeptide (lower panel, lane 7). These results thus showed that Stat3 but not Stat1 has the ability to interact with intracellular domain sequences of anti-Stat5 serum (lane 7) and not by antisera specific for any other STAT protein (lanes 3–6), including Stat3 (lane 5). In addition, a combination of antisera specific for Stat1 and Stat5 completely supershifted all EMSA complexes present in the lane. Thus, IL-10-dependent Stat1 and Stat5 activation is promoted by IL-10 receptor sequences that are distinct from those required for activation of Stat3.

To explore the specific roles of the tyrosine residues within the 402–559 region of the IL-10 receptor, we used Ba/F3 cells that stably expressed receptor point mutants that either lacked one or both tyrosines at positions 427 and 477. Wild-type receptor-induced DNA binding activity was observed in IL-10-treated Ba/F3 cells that expressed either IL-10 receptor single point mutant (Fig. 4A, Y427F or Y477F, lanes 5 and 6, respectively). These results suggested that Stat3 activation was either not dependent on receptor intracellular domain tyrosine residues or that IL-10 receptor tyrosines Tyr^{427} and Tyr^{477} could serve a redundant STAT recruitment function. To explore this possibility, cells bearing an IL-10 receptor mutant with phenylalanine substitutions for both Tyr^{427} and Tyr^{477} were prepared (2500 receptors/cell). These cells failed to produce significant amounts of Stat3-dependent DNA binding complexes when exposed to IL-10 (Fig. 4A, lane 8). Thus, Stat3 activation requires the presence of either Y427 or Y477 in the intracellular domain of the ligand binding component of the IL-10 receptor, and at least some of the IL-10-dependent activation of Stat1 and Stat5 occurs at a site independent of these two tyrosine residues.

To validate the physiologic relevance of these results, the cell lines expressing the various IL-10 receptor mutants were monitored for the capacity to manifest IL-10-dependent proliferative responses. Ba/F3 cells lacking IL-10 receptors did not proliferate in response to IL-10 (data not shown). However, cells expressing IL-10 receptors that supported the generation of an IL-10-dependent, Stat3 containing gel shift complex (BaMR, Δ282–414, Y427F, and Y477F) proliferated when exposed to IL-10 (29). In contrast, cells bearing IL-10 receptor mutants that were unable to activate Stat3 in response to ligand (Δ402–559 and Y427F/Y477F) failed to mount a proliferative response to IL-10. Thus, we demonstrated an IL-10 receptor tyrosine residues needed for Stat3 activation and biologic response induction.

![Fig. 4. Tyrosine residues 427 and 477 in the murine IL-10 receptor intracellular domain are redundant for IL-10-dependent Stat3 activation.](image)
The activation of STAT proteins by interferons as well as other cytokines and growth factors is now known to link ligand-receptor interaction at the cell surface to specific gene activation and translocation to the nucleus of stimulated cells (34, 35). Different cytokines can stimulate the activation of different STAT proteins in differing amounts and with different kinetics. This leads to the generation of distinct patterns of homo- or heterodimeric STAT complexes that are at least partly responsible for inducing the specific pattern of gene transcripts activated by each particular cytokine. Based on our study of IFNγ receptor signaling, we recently proposed that the specific recruitment of latent STAT proteins by distinct cytokine receptors is determined by the ability of individual STAT family members to interact with distinct phosphotyrosine-containing sequences within the intracellular domain of the receptor (24). This concept was initially supported in part by the

**DISCUSSION**

The activation of STAT proteins by interferons as well as other cytokines and growth factors is now known to link ligand-receptor interaction at the cell surface to specific gene activa-

**FIG. 5. Coprecipitation of Stat3 with biotinylated tyrosine-phosphorylated IL-10 receptor peptides containing Tyr427 or Tyr477.** BaMR cell homogenates were incubated with biotinylated 12-amino acid peptides that were based on the murine IL-10 receptor sequence surrounding the four intracellular domain tyrosine residues at positions 374, 396, 427, and 477 or a 12-amino acid phosphopeptide containing the Stat1 binding site derived from the human IFNγ receptor a chain intracellular domain (440). Biotinylated peptides were precipitated from the incubation mixture using streptavidin-Sepharose, washed, and subjected to Western blot analyses that employed antisera specific for either Stat3 (upper panel) or Stat1 (lower panel).

The phosphorylation of Stat3 was observed (lanes 1) in the absence of ligand, little or no activated phosphorylated Stat3 was observed (lane 1). In contrast, the addition of IL-10 to the homogenates resulted in the generation of tyrosine-phosphorylated Stat3 (lane 2). Pretreatment of BaMR homogenates with phosphopeptides containing Tyr374 (lane 3) or Tyr396 (lane 4) had no effect on the IL-10-induced phosphorylation of Stat3. In contrast, preincubation of the homogenates with the phosphorylated Tyr427-containing peptide (lane 5) completely inhibited ligand-induced Stat3 phosphorylation. The phosphorylated Tyr477 peptide (lane 6) also significantly inhibited ligand-dependent phosphorylation of Stat3. Peptides containing nonphosphorylated tyrosine at positions 427 (lane 7) or 477 (lane 8) failed to inhibit IL-10-induced Stat3 phosphorylation.

**FIG. 6. IL-10 receptor peptides containing Tyr427 or Tyr477 inhibit ligand-dependent Stat3 phosphorylation.** BaMR homogenates from 10^6 cells were incubated with either buffer (lanes 1 and 2) or the following IL-10 receptor peptides at a final concentration of 100 μM: phosphorylated 370–381 peptide (374-P), phosphorylated 392–403 peptide (396-P), phosphorylated 423–434 peptide (427-P), phosphorylated 473–483 peptide (477-P), or the nonphosphorylated control peptides 423–434 (427) and 473–483 (477). Incubation was performed for 45 min at 4°C, and lysates were subsequently extracted with buffer (lane 1) or 1 μg/ml recombinant murine IL-10 for 15 min at 37°C (lanes 2–8). After stimulation, homogenates were solubilized and cleared by centrifugation. Stat3 was precipitated using specific immune rabbit serum and protein A-Sepharose. Immunoprecipitates were subjected to SDS-PAGE and phosphotyrosine-specific Western blotting using the RC-20 mAb (upper panel). Subsequently, the membranes were stripped and blotted for Stat3 (lower panel).

**FIG. 7. Dose-dependent inhibition of Stat3 by phosphorylated IL-10 receptor peptides containing Tyr427 or Tyr477.** BaMR homogenates were incubated as described in Fig. 6 with: no peptide (lanes 1 and 2), 100 μM nonphosphorylated 423–434 peptide (427), phosphorylated 423–434 peptide in decreasing micromolar concentrations as indicated (427-P), 100 μM nonphosphorylated 473–483 peptide (477), or phosphorylated 473–483 peptide in decreasing micromolar concentrations as indicated (477-P). Reaction mixtures were incubated for 45 min prior to addition of either buffer (lane 1) or 1 μg/ml recombinant murine IL-10 (lanes 2–12) and Stat3 immunoprecipitated as in Fig. 6. Precipitates were analyzed by SDS-PAGE and Western blotting using the phosphotyrosine-specific mAb RC-20 (upper panel). Blots were stripped and reprobed with Stat3-specific antiserum (lower panel).
demonstrations that: (a) highly purified Stat1 binds to its highly purified phosphorylated IFNγ receptor docking site with an affinity of 70–137 nM (16, 25); and (b) certain phosphotyrosine-containing peptides derived from the IL-4 receptor can bind to highly purified latent Stat6 (Stat6 is the STAT protein utilized in IL-4 signaling) (36) and dissociate a preformed purified, activated Stat6 homodimer (16). More recently, similar experiments have been performed for STAT activation by certain other cytokine receptors including IL-2, IL-4, IL-6, and IFNα/β (26, 37–39). In the present report, we now extend this concept to IL-10 receptor signaling.

As shown herein, IL-10 induces the activation of two specific STAT family members in all IL-10 receptor-expressing cells tested, Stat1 and Stat3. Moreover, IL-10 activates a third STAT protein (Stat5) in pro-B cells that have been stably transfected with the wild-type murine IL-10 receptor. The levels of Stat5 expression in BaMR, Raw264.7, and PEC cells are roughly equivalent; therefore, the ability of the IL-10 receptor to activate Stat5 in Ba/F3 cells is apparently not due to high expression levels of Stat5 in these cells. The utilization of Stat3 by the IL-10 receptor was found to be the result of the interaction of Stat3 with either one of two phosphotyrosine-containing sequences within the membrane distal portion of the intracellular domain of the IL-10 receptor. The sequences encompassing the Stat3 binding sites on the IL-10 receptor were distinct from the sequence from the Stat1 binding site defined previously on the IFNγ receptor and did not react with Stat1. Thus, our results support the concept that at least some cytokine receptors mediate their specific biologic responses through a direct interaction of the intracellular domain of the receptor with specific STAT family members and that the specific array of STAT proteins utilized by distinct cytokine receptors is determined by binding site specificity of specific STAT proteins. The mechanism of recruitment of Stat1 and Stat5 to the ligated IL-10 receptor is left unanswered by these binding experiments. However, this report documents a relatively unique feature of the IL-10 receptor in its ability to recruit different STAT proteins via distinct docking sites.

The functionally important regions of the intracellular domain of the murine IL-10 receptor were identified using deletion and substitution mutagenesis approaches. These experiments were possible because expression of wild-type murine IL-10 receptors in Ba/F3 cells leads to the generation of a functionally active receptor that signals for IL-10-dependent proliferative responses (1, 10). This approach showed that two tyrosine residues at positions 427 and 477 could each function in the IL-10 signal transduction process. Although individual substitution of either Tyr427 or Tyr477 with phenylalanine did not ablate IL-10-dependent proliferative responses, substitution of both residues led to the generation of a functionally inactive receptor. Similar effects were observed using an EMSA that monitored IL-10-dependent STAT activation. Moreover, cells bearing the IL-10 receptor deletion mutant Δ402–559 activated Stat1 and Stat5 upon ligand binding, whereas Stat3 activation was completely ablated. Thus, the biochemical of Stat3 activation is placed in contrast to that of Stat1 or Stat5. Using phosphopeptides in a manner similar to that used to identify the Stat1 binding site on the IFNγ receptor (24), the interaction of Stat3 was demonstrated with either of the two sequences derived from a membrane distal portion of the intracellular domain of the IL-10 receptor encompassing phosphophorylated forms of Tyr427 and Tyr477. The biological relevance of this interaction was demonstrated by showing that phosphotyrosine-containing peptides corresponding to the amino acids 423–434 and 473–484 of the IL-10 receptor inhibited IL-10-dependent Stat3 activation, whereas peptides encompassing the other two potential phosphotyrosine-containing receptor sequences did not.

The apparent overlapping actions of Tyr427 and Tyr477 in the IL-10 receptor are in contrast with the single functionally important tyrosine residue in the intracellular domain of the IFNγ receptor α chain (24). In the latter case, ligand-induced IFNγ receptor α chain dimerization is thought to bring into close proximity two receptor-bound Stat1 molecules that results in the efficient generation of an activated phosphorylated Stat1 homodimer (24, 34, 40–42). This concept is supported by the observation that murine IFNγ receptor α chains either containing a point mutation at the functionally critical Tyr420 position or lacking all but three intracellular domain amino acids act as dominant-negative mutants (43). In contrast, we have not been able to detect a dominant-negative effect by a cytoplasmically truncated form of the IL-10 receptor. This result suggests that either IL-10 receptor dimerization may not be important in the signaling process or that the two redundant tyrosine residues within the native IL-10 receptor intracellular domain provides a backup mechanism that insures effective Stat3 dimerization.

The intracellular domains of the human and murine IL-10 receptor proteins are only 60% identical at the amino acid level (1, 10). Moreover, the human protein contains only two conserved intracellular domain tyrosine residues (Tyr446 and Tyr496) that correspond to the two functionally important tyrosines we identified in the intracellular domain of the murine IL-10 receptor (i.e. Tyr427 and Tyr477). Perhaps of importance, the sequences surrounding each of the two conserved tyrosine residues are conserved. Specifically, the murine and human sequences containing the equivalent of the murine Tyr427 residue are FQGYQKQTR and FQGYLRQTR, respectively, and the murine and human sequences containing the murine Tyr477 equivalent are AAGYLKQES and AKGYLKQD, respectively. Thus, the functional importance of these two intracellular domain regions is also suggested by the conservation of sequence around the two membrane distal tyrosine residues. In addition, the two sets of human and murine intracellular domain sequences share remarkable sequence similarities to one another, especially at positions −1 to +4 relative to the tyrosine residue. The consensus sequence appears to be GYLKQHy, where Hy is an amino acid with a terminal hydrophilic group. If this sequence represents an authentic Stat3 binding site (which becomes activated upon phosphorylation of the tyrosine residue), it would be expected to occur in the polypeptides of receptors for other cytokines that utilize Stat3 for signaling. It is, therefore, of interest that four similar sequences occur within the intracellular domain of gp130, which is known to induce Stat3 activation. These include the GYRHKQV, KYFKQN, SYLPLQT, and GYMPQ sequences found at various positions in the intracellular domains of both murine and human gp130. The latter two sequences have recently been implicated in Stat3 binding to gp130 by transplanting them into the erythropoietin receptor intracellular domain, thereby conferring on that receptor the ability to activate Stat3 (26).

The putative Stat3 binding motif is distinct from the Stat1 binding site that we identified previously within the IFNγ receptor α chain intracellular domain (GYDKPH). On the basis of IFNγ receptor structure-function analyses and Stat1 binding assays, we know that the IFNγ receptor residues at positions +1 and +4 relative to the phosphotyrosine are critical for mediating high affinity Stat1 interaction with the phosphorylated IFNγ receptor (23–25). It is of importance that the IFNγ receptor phosphopeptide did not coprecipitate Stat3, thus con-
firming that the Stat3 binding site is distinct from that of Stat1. We have not yet established the identity of the key residues within the motif that mediates Stat3 attachment, nor have we defined whether these residues will be in the same +1 and +4 orientation relative to the phosphotyrosine as was observed for Stat1. These experiments are currently ongoing in the laboratory.

The experiments described in this report have focused entirely on the importance of specific tyrosine-containing receptor sequences responsible for STAT recruitment. An important issue not addressed in this study is the region of the STAT proteins responsible for binding to the receptor. Since Stat3, like the other STAT proteins, contains an SH2 domain, it is logical that these regions are responsible for docking to the phosphotyrosine-containing sequence in the activated IL-10 receptor. This idea is supported by two recent observations logical that these regions are responsible for binding to the receptor. Since Stat3, like the other STAT proteins, contains an SH2 domain, it is known that these regions are responsible for docking to the phosphotyrosine-containing sequence in the activated IL-10 receptor. This idea is supported by two recent observations logical that these regions are responsible for binding to the receptor. Since Stat3, like the other STAT proteins, contains an SH2 domain, it is known that these regions are responsible for docking to the phosphotyrosine-containing sequence in the activated IL-10 receptor.

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