Cells express a variety of STAT (signal transducer and activator of transcription) transcription factors that are structurally homologous and yet function specifically in response to particular cytokines. The functions of the individual STATs are dependent on distinct protein-protein interactions. STAT1 and STAT2 are activated by tyrosine phosphorylation in response to type I interferons-α/β (IFN-α/β) and subsequently form a multimeric transcription factor designated the IFN-α-stimulated gene factor 3 (ISGF3). ISGF3 is a unique STAT complex because it also contains a non-STAT molecule, p48, which is a critical DNA-binding component. We provide evidence that STAT2 specifically interacts with p48 in vivo before and after IFN-α stimulation. The specificity of ISGF3 formation is therefore a result of the distinct nature of the STAT2 molecule. Coimmunoprecipitation assays demonstrate p48 association with STAT2 but not STAT1. Hybrid STAT2-STAT1 molecules were used to identify a region of STAT2 which specifically associates with p48. The region of STAT2 interaction spans an amino-terminal region of two predicted coiled coils. The studies demonstrate the in vivo existence of a STAT2-p48 complex and a distinct STAT2-STAT1 complex after IFN-α stimulation. Data suggest that distinct bipartite complexes STAT2-p48 and STAT2-STAT1 translocate to the nucleus and associate on the DNA target site as ISGF3.

Interferons (IFNs) play a fundamental role in immune defense, they confer resistance to viral infections, inhibit cellular proliferation, and activate a variety of cells in the immune system (1, 2). IFNs elicit these physiological responses by binding to cell surface receptors and transducing a signal to the nucleus that activates expression of a subset of genes. The discovery of a regulated signal transduction pathway in the IFN system has served as a paradigm for receptor to nucleus signal transmission by a variety of cytokines (3–6). A class of transcription factors which resides in a latent state in the cytoplasm of the cell becomes activated by tyrosine phosphorylation in response to specific stimulation. These transcription factors have been designated signal transducers and activators of transcription (STATs) (3). Seven distinct human STAT molecules have been identified that can be phosphorylated by tyrosine kinases of the Janus kinase family (6). Following phosphorylation, the STATs can associate via their SH2 domains and phosphotyrosine domains to form homodimers or heterodimers (7). The STAT dimers translocate to the nucleus and bind to specific DNA sequences in the promoters of stimulated genes. The DNA target site of the STATs was first identified for the IFN-γ-activated STAT1 as a palindromic of GAAA residues (TTCCNNNGAAA) (3–5). The STAT molecules can bind to this consensus target site as homodimers or heterodimers. Since distinct gene expression is induced by different cytokines, many studies have focused on the intricate question of specificity of STAT action.

One of the STAT factors, STAT2, is distinct in that it does not appear to recognize a DNA target site as a homomeric complex. STAT2 was first identified as a subunit of the multimeric transcription factor, IFN-α-stimulated gene factor 3 (ISGF3), which forms in response to IFN-α/β stimulation (8). ISGF3 is composed of two STATs, STAT1 and STAT2, in association with a non-STAT molecule, p48 (8–10). p48 is a member of a different family of transcription factors designated the IFN regulatory factor family and is a critical DNA binding component of ISGF3 (10). ISGF3 does not bind to a palindromic target site but rather to a consensus tandem repeat of GAAA residues (GGAAAANNGAAG) known as the IFN-α-stimulated response element (ISRE) (3–5). Since transcriptional specificity is regulated by precise protein-protein interactions acting in conjunction with a particular DNA target site, we examined the protein-protein association of the STATs with p48. Although the STAT molecules share structural similarity, the unique association with p48 has led to the identification of a particular domain in STAT2 which specifies formation of ISGF3.

MATERIALS AND METHODS

Cell Culture—Human HeLa S3, HT1080, and HT1080 mutants (U3A and U6A, gifts of G. R. Stark, Cleveland Clinic Foundation Research Institute) were cultured in Dulbecco’s modified Eagle’s medium with 8% fetal bovine serum. Transfections were performed with calcium phosphate-DNA coprecipitates (11), and stable transformants were selected by resistance to G418 (250 μg/ml) and screened for STAT expression. Generation of hybridoma cell lines was performed by immunization of BALB/c mice with bacterially expressed p48 or STAT1 fusion proteins. Spleens from the mice were fused to NSO cells, and IgG1-producing hybridomas were selected (12–14).

Plasmid Constructs—Reverse transcription-polymerase chain reaction of HeLa cell mRNA was performed with oligonucleotides that

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STAT2 Interaction with p48—The appearance of ISGF3 in response to IFN-α can be demonstrated by an electrophoretic mobility shift assay with a radiolabeled ISRE oligonucleotide (Fig. 1A). Antibodies used in our study that specifically recognize STAT1, STAT2, or p48 reduce the appearance of the ISGF3-DNA complex when added to the DNA binding reaction, demonstrating the protein presence in the complex.

The formation of ISGF3 suggested that the STAT1 and STAT2 molecules were unique in their ability to associate with the p48 subunit. It was possible that STAT1 and STAT2 could only associate with the p48 molecule in conjunction with the DNA target. Alternatively, interaction with the p48 molecule might require an IFN-α-induced STAT1-STAT2 heterodimer or an IFN-α-induced modification of STAT1 or STAT2. Several models of protein-protein interactions were feasible including the possibility that p48 could discriminate between the STATs and specifically interact with one of the STAT molecules before and/or after IFN-α stimulation.

To determine the specificity of p48 involvement in the formation of the ISGF3 transcription factor we analyzed association of p48 with the STATs by coimmunoprecipitation of protein lysates prepared from HeLa cells untreated or treated with IFN-α and incubated with a radiolabeled ISRE oligonucleotide (ISG15).

Control antibodies (c) or antibodies to STAT1, STAT2-C, or p48 were added to the DNA binding reactions as indicated. Panel B, coimmunoprecipitation of p48 with STAT2. Protein lysates were prepared from HT1080 cells untreated (lane 1), stimulated with IFN-γ for 18 h (lanes 2–6), and stimulated with IFN-α for 45 min (lanes 3–6). Proteins were immunoprecipitated with anti-p48 antibodies (lanes 1–3), anti-STAT2-C polyclonal antibodies (lane 4), anti-STAT1 antibodies (lane 5), or control antibodies (lane 6). The immunoprecipitated proteins were separated by SDS-PAGE, transferred to membrane, and detected by immunoblot with anti-p48, anti-STAT1, and anti-STAT2-N monoclonal antibodies. Migration of STAT1 (ST1), STAT2 (ST2), and p48 is noted by arrows as is the migration of the immunoglobulin heavy chain (Ig HC). Panel C, association of STAT2 and p48 in the absence of STAT1. U3A cells (STAT1-deficient) were untreated (lane 1) or stimulated with IFN-γ for 18 h and IFN-α for 45 min (lanes 2 and 3). Protein lysates were immunoprecipitated with anti-p48 antibodies (lanes 1 and 2) or anti-STAT2-C polyclonal antibodies (lane 3). Proteins were detected by immunoblot with anti-p48 and anti-STAT2-N monoclonal antibodies.
IFN treatment (lanes 2 and 3). Extended treatment with IFN-γ is known to increase the steady-state levels of p48 protein, and for this reason there is an increase in the level of preexisting STAT2/p48 detectable in these assays. Anti-STAT2 antibody reciprocally is able to coimmunoprecipitate the p48 molecule (lane 4).

What is clearly noted is that there is no association detectable between p48 and STAT1, whether anti-p48 antibodies or anti-STAT1 antibodies are used (lane 5). However, the ability of STAT1 to coimmunoprecipitate with STAT2 after IFN-α treatment is readily apparent, as is the IFN-α-induced phosphorylation indicated by a decreased STAT1 mobility (lane 4) (7). A cell line that is deficient in the STAT1 protein (U3A; Ref. 24) (Fig. 3C). These cells were transformed stably with the STAT2/1 hybrid gene. Protein lysates were prepared and tested by immunoprecipitation/immunoblot for STAT2/1 interaction with p48. The analysis clearly demonstrated the ability of the p48 antibody to coimmunoprecipitate STAT2/1 in the absence of the STAT2 molecule.

The ability of the reciprocal hybrid, STAT1/2, to bind p48 was also tested. Stable transformants were generated with a STAT1/2 chimera that contained a T7 epitope tag in the carboxyl terminus, and the specific STAT2/1 chimera was identified by reactivity with anti-T7 antibody (Fig. 3D). The STAT1/2 hybrid was able to associate with the p48 molecule by coimmunoprecipitations. Together the experiments with STAT1/2 and STAT2/1 identified an amino-terminal 324aa region of STAT2 which was required for p48 recognition in vivo.

To determine if the STAT1/2 or the STAT2/1 protein could recognize p48 directly, an in vitro association assay was performed. Radiolabeled STAT chimera proteins were synthesized in vitro and incubated with the GST-p48 fusion protein (Fig. 4). The STAT2/1 hybrid protein demonstrated a specific binding to GST-p48 (lane 3) compared with GST alone (lane 5). However, the STAT1/2 hybrid protein did not display specific binding to the p48 protein since the levels of protein associated with GST-p48 or GST were equivalent (lane 2). Although association of STAT2 with p48 does not require STAT1 (Fig. 1), it was possible that the association of the STAT2/1 chimera with p48 was dependent on the function of an endogenous STAT2 molecule. To exclude this possibility, cells were analyzed which are deficient in the endogenous STAT2 protein (U6A; Ref. 24) (Fig. 3C). These cells were transformed stably with the STAT2/1 hybrid gene. Protein lysates were prepared and tested by immunoprecipitation/immunoblot for STAT2/1 interaction with p48. The analysis clearly demonstrated the ability of the p48 antibody to coimmunoprecipitate STAT2/1 in the absence of the STAT2 molecule.

Direct Interaction of STAT2 and p48—Since the STAT2/p48 complex exists in untreated cells, the protein interaction is not dependent on protein modification induced by IFN-α. For this reason we analyzed the association of STAT2 and p48 in vitro to determine if the interaction required an unidentified bridging molecule (Fig. 2). An in vitro transcription/translation system was used to generate protein corresponding to STAT2 or STAT1 radiolabeled with [35S]methionine. Radiolabeled proteins were incubated with bacterially expressed GST-p48 protein or GST protein immobilized on glutathione-agarose beads. Bound proteins were eluted, separated by SDS-PAGE, and detected by autoradiography. The protein-protein associations mimicked those seen in vivo. A specific in vitro interaction of STAT2 with p48 was readily detectable, whereas the STAT1 protein did not interact with p48. These results demonstrate a direct interaction of STAT2 with p48 that does not require a bridging or docking molecule and does not require an IFN-induced modification.

Hybrid STAT Molecules Map p48 Recognition Site on STAT2—To identify the region of STAT2 that specifically interacts with p48 in vivo we generated hybrid molecules of STAT1 and STAT2. Since the STAT molecules have similarity in domain organization and amino acid sequence this approach should maintain integrity of the STAT structure. The STAT chimeras used in the assays are fusions at 324aa/325aa, and therefore the carboxyl-terminal region of the proteins contain the DNA binding specificity region, the SH2 domain, and the phosphoryrosine domain of one specific STAT molecule (21–23) (Fig. 3A). The ability of p48 to interact with these chimeric proteins was tested in vitro and in vivo.

Protein lysates were prepared from cells stably transformed with the STAT2/1 chimera or the STAT1/2 chimera and analyzed for association with p48 by coimmunoprecipitation/immunoblot. The STAT2/1 chimera encodes a protein of approximately 91 kDa similar to STAT1. It is identified by immunoprecipitation with an antibody that recognizes the carboxyl terminus of STAT1 and by immunoblot with an antibody that recognizes the amino terminus of STAT2. The STAT2/1 chimera was found to interact specifically with p48 by coimmunoprecipitation with anti-p48 antibodies (Fig. 3B, lanes 1 and 2). Immunoprecipitation of STAT2/1 with anti-STAT1 antibodies demonstrates a detectable signal for p48 (lane 3).

The middle panel shows a longer exposure of the p48 section of the film. The expression levels of STAT2/1 are lower than the endogenous STAT2 and may be responsible for the lower levels of detectable p48. It is also possible that the carboxyl terminus of STAT2 can contribute to the stability of associated p48. It can also be noted that a doublet of STAT2/1 appears after IFN-α treatment. The slower migrating band corresponds to tyrosine-phosphorylated STAT2/1 as can be shown by reaction with anti-phosphotyrosine antibody (lower panel).

Although association of STAT2 with p48 does not require STAT1 (Fig. 1), it was possible that the association of the STAT2/1 chimera with p48 was dependent on the function of an endogenous STAT2 molecule. To exclude this possibility, cells were analyzed which are deficient in the endogenous STAT2 protein (U6A; Ref. 24) (Fig. 3C). These cells were transformed stably with the STAT2/1 hybrid gene. Protein lysates were prepared and tested by immunoprecipitation/immunoblot for STAT2/1 interaction with p48. The analysis clearly demonstrated the ability of the p48 antibody to coimmunoprecipitate STAT2/1 in the absence of the STAT2 molecule.

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Predicted Coiled Coil Domains of STAT2—The amino acid sequences of the STAT molecules were examined for predicted structural features that might allow a better understanding of specific protein-protein interactions. A structural feature that was readily identified by a statistical analysis is that of a coiled coil in the domain of STAT2 interaction with p48 (Fig. 5) (25–27). Coiled coils are superhelices of two or more α-helices wrapped around each other and were first recognized in many fibrous structural proteins. The first identification of a coiled coil structure in a transcription factor led to its functional demonstration in dimerization (leucine zipper) (28). Two computer-based methods to detect the repetitive hydrophobic and hydrophilic amino acids in coiled coils were used to analyze the structure of STAT2 (Coils, 26; Paircoil, 27) (Fig. 5). Both methods predicted two coiled coils within the region necessary to bind p48: Coils 138–173 and 192–231 amino acids; Paircoil 138–173 and 191–229. An analysis of the STAT1 molecule also revealed predicted coiled coils within this region: Coils 134–186 and 255–297 amino acids; Paircoil 131–171 and 257–287.
Since only the STAT2 molecule appears to bind p48, the sequence and/or the spacing of the STAT2 coils may be critical.

A STAT2 Domain That Associates with p48—The functional domain of STAT2 which interacts with p48 was further defined by constructing a STAT1 chimera encoding a substitution of STAT2 148–324aa for the homologous region of STAT1 (Fig. 6A). The ability of this STAT1/2 chimera to interact with p48 was seen following transfection into U3A cells.2 The ability of the STAT1/2 protein to bind p48 was also demonstrated in vitro (Fig. 6B). Radiolabeled STAT1/2 was synthesized in vitro and incubated with the GST-p48 fusion protein or the GST protein. The STAT1/2/1 molecule specifically binds p48. Panel A, diagram of hybrid constructs of STAT1 (ST1) and STAT2 (ST2). The SH2 domains and the specific phosphotyrosines are indicated. A T7 epitope tag was introduced in the carboxyl terminus of the STAT1/2 chimera. Panel B, immunoprecipitation of STAT2/1 with p48. Stable transfectants of HT1080 with the STAT2/1 chimera were treated with IFN-γ for 18 h (lanes 1–4) and with IFN-α for 45 min (lanes 2–4). Proteins were extracted and immunoprecipitated with p48 antibody (lanes 1 and 2), STAT1-C monoclonal antibody (lane 3), or control antibody (lane 4). The top panel represents an immunoblot with p48 antibody and STAT2-N monoclonal antibody. The middle panel displays a longer exposure of the p48 section of the top panel. The bottom panel represents the same membrane rebotted with anti-phosphotyrosine antibody. The STAT2/1 (ST2/1) chimera and p48 are indicated with arrows. Panel C, STAT2/1 interaction with p48 is independent of STAT2. U6A cells (STAT2-deficient) were transformed stably with the STAT2/1 chimera gene and were either untreated (lane 1) or treated with IFN-γ (lanes 2–4) and IFN-α (lanes 3 and 4). Protein lysates were analyzed by immunoprecipitation/immunoblot as indicated. Panel D, p48 does not associate with STAT1/2. Stable transfectants of HT1080 with the STAT1/2 chimera were treated with IFN-γ and treated with IFN-α for 45 min (lanes 3 and 4). Immunoprecipitations were performed with p48 antibody (lanes 2 and 4), anti-T7 antibody (lanes 1 and 3), or control antibody (lane 5). Immunoblots were performed with anti-T7 antibody (top panel) or anti-p48 antibody (bottom panel).

* M. Martinez-Moczygemba and N. C. Reich, unpublished observations.
The analyses with hybrid STAT molecules served to minimize disruption of the STAT molecule integrity, and results with the chimeras mapped a region of STAT2 interaction with p48 requiring STAT2 148aa to 324aa. Although chimeras were used, it was possible that an amino-terminal fragment of STAT2 was sufficient to interact directly with p48. To test this possibility several fragments of the STAT2 protein were assayed for binding to p48 in vitro. The in vitro transcription/translation system was used to generate radiolabeled STAT2 protein fragments from the native STAT2 gene or from STAT2 deletion mutants. The DNAs were linearized with different restriction enzymes prior to transcription to produce the different proteins that were tested for binding to GST-p48 (Fig. 6A).

The results of these binding assays demonstrated that a STAT2 protein fragment containing 1–324 amino acids (Sph1), as is present in the STAT2/1 chimera, was sufficient to bind p48 (Fig. 6B). However, a STAT2 protein fragment of 1–125 amino
acids (ApaI) did not bind p48. These analyses confirmed the in vivo binding results.

To define further the carboxyl-terminal border of STAT2 necessary to bind p48, a series of deletion constructs was generated with Bal-31 exonuclease initiating at the Bsu36I site (270aa). These constructs were used to generate STAT2 protein fragments for the in vitro binding assay corresponding to 1–190, 1–217, 1–230, and 1–250 amino acids (Fig. 6A). The STAT2 proteins encoding 1–250 and 1–230 amino acids contain both of the predicted coiled coils and specifically bind p48 (Fig. 6C). However, deletion mutations that interrupt or remove the second predicted coiled coil and possess only the first coiled coil do not recognize the p48 molecule (1–217 and 1–190 amino acids). These results show a requirement for the second predicted coiled coil of STAT2 for binding to p48.

**DISCUSSION**

This study provides evidence that a molecular interaction preexists between STAT2 and p48 before and after IFN-α stimulation. Although the STAT molecules share a general structural similarity and significant amino acid identity, they appear to possess distinct elements that allow selective interactions with diverse transcription factors (29, 30). The multimeric ISGF3 transcription factor formed in response to IFN-α contains STAT1 (91- or 84-kDa splice products), STAT2 (113 kDa), and p48. All three protein subunits are required for specific ISRE DNA binding and transcriptional activation. The formation of ISGF3 was shown previously to require STAT1 and STAT2 tyrosine phosphorylation, dimerization, and translocation to the nucleus (3). It was proposed that the STAT1-STAT2 dimer subsequently associated with the p48 subunit on the ISRE DNA target to form ISGF3 (31, 32). We show here that a specific interaction of the STAT2 molecule with p48 exists in the absence of the DNA target and appears to determine formation of ISGF3. Association of p48 with STAT1 was not demonstrable by coimmunoprecipitation of cell lysates or by in vitro binding assays. Although the IFN-α-induced STAT1-STAT2 heterodimer was detected readily by coimmunoprecipitation with anti-STAT1 antibody, the dimer did not contain p48 (Fig. 1B, lane 5). These results are interpreted to indicate the existence of two distinct complexes following IFN-α stimulation: STAT2-p48 and STAT2-STAT1. Association of the bipartite complexes appears to occur on the DNA ISRE target to form the multimeric ISGF3 transcription factor. The exact stoichiometry of components in ISGF3 is as yet unresolved. The estimation of relative subunit levels following purification of ISGF3 by DNA affinity (9, 33) and the ability of p48 and STAT1 in ISGF3 to contact the ISRE (32) are compatible with our results and model. The configuration of direct half-sites in the ISRE may allow STAT2-p48 to bind to one half-site and STAT2-STAT1 to bind to the other half-site. Alternative models have also been envisioned (32), but in the absence of additional structural information the models remain to be tested.

The amino-terminal domain of STAT2 that associates with p48 appears to be defined by a structure containing two predicted coiled coils at 138–173 and 191–211aa (Fig. 5) (25–27). Although STAT1 also has predicted coiled coils in this region, the specific amino acid sequence or domain structure does not specify interaction with p48. Previous studies had suggested an association of STAT1 and p48 (31, 34). A directed yeast two-hybrid transcription system detected interaction of STAT1 with p48 (31), and in vitro mixing of crude lysates from cells overexpressing STAT1 and p48 showed apparent complex binding to DNA (38). In contrast, our studies performed in human cells at physiological levels of proteins only detect STAT2-p48 complexes. Our studies in vitro demonstrate a specific interaction of p48 with a STAT2 domain encompassing the two predicted coiled coils between 138 and 230 amino acids. The STAT2-p48 interaction is resistant to 0.5% Nonidet P-40 since this detergent was included in the lysis buffer. Even if lysates are prepared in the absence of detergent the STAT2-p48 complex is apparent, but there is no detectable p48 association with STAT1. A previous report demonstrated in vitro binding of p48 with both STAT2 and STAT1 using a similar GST interaction assay (31). The data clearly showed a p48 interaction with STAT2 which was many fold greater than that detectable with STAT1. Certainly there are inherent differences in amounts of [35S]-radiolabeled proteins synthesized in vitro that could affect the results, as well as differences in binding/washing conditions.

Cells express many different STAT factors, and accurate STAT activation and function in response to cytokines depend on precise protein-protein interactions. Recruitment by specific cytokine receptors and Janus kinases can dictate distinct STAT activation. Association of STATs with heterologous transcription factors can determine transcriptional induction of particular genes. The specificity of STAT2 interaction with p48 appears to dictate recognition of the tandem GAAA repeat of the ISRE by the ISGF3 complex. A region of p48 which interacts with the STATs to form ISGF3 was originally identified by in vitro reconstitution experiments to be located in the carboxy-terminal half of the protein, 217–377aa (35). The p48 protein does not contain regions of predicted coiled coil, but it does contain pockets of hydrophobic stretches in the carboxyl terminus which may participate in interaction with STAT2. Transcriptional specificity is regulated by precise protein-protein interactions acting in conjunction with a particular DNA target site, and it is the precision of the molecular interactions that confers specificity of a rapid and specific biological response to distinct extracellular stimuli. Elucidation of the precise amino acid interactions that drive STAT2-p48 complex formation can contribute to our understanding of other protein-protein associations and offer a target of activation/intervention of receptor to nucleus signaling.

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