Application of Genomic DNA Affinity Chromatography Identifies Multiple Interferon-α-regulated Stat2 Complexes*

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Interferon-α (IFN-α)-induced signal transduction is mediated by the phosphorylation-activation of the signal transducer and activator of transcription (STAT) proteins Stat1, Stat2, and Stat3. Previous studies have shown that these activated STATs dimerize to form four distinct STAT complexes which translocate to the nucleus and activates transcription by binding to specific promoter elements. The interferon-stimulated gene factor-3 (ISGF3) consists of Stat2 and Stat1 heterodimers in association with a DNA-binding protein, p48, that binds to the interferon stimulated response element. Homo- and heterodimers of Stat1 and Stat3 bind to the palindromic interferon response element (pIRE). In this report we demonstrate the utility of a biochemical procedure that we have developed, based on genomic DNA affinity chromatography, for the identification of IFN-α-induced STAT complexes. Using this approach, we identified ISGF3-independent Stat2-containing STAT complexes. Results from the analysis of Stat2 complexes in the electrophoretic mobility shift assay were consistent with genomic DNA affinity chromatography results and identified a Stat2:1 complex that binds with low affinity to the pRE of the interferon regulatory factor-1 gene. Immunoprecipitation studies of Stat2 revealed an IFN-α-dependent co-precipitation of both Stat1 and Stat3. Taken together, our results suggest that IFN-α activates, in addition to ISGF3, other Stat2-containing STAT complexes, one of which binds to an element related to the interferon regulatory factor-1 pRE.

Interferons (IFNs) are secreted proteins engaged in many diverse biological activities, including regulation of cell growth and differentiation, inhibition of viral replication, and modulation of the immune system (1). IFNs target responsive cells by interacting with distinct cell surface receptors. The high affinity interaction between IFN-α/β and its specific cell surface receptor leads to the activation of the receptor-associated cytoplasmic tyrosine kinases of the Jak family, Jak1 and Tyk2 (2). This membrane proximal event correlates with the activation of effector molecules leading to gene activation (4). These effectors are members of a family of latent cytoplasmic transcription factors of the signal transducer and activator of transcription (STAT) family (5, 6), implicated in cytokine signal transduction (7). STAT proteins possess both Src homology-2 (SH2) and Src homology-3 (SH3) domains (6). STATs associate via their SH2 domains with specific phosphotyrosine residues in the cytoplasmic domains of the activated receptor components, leading to a Jak-dependent phosphorylation/activation of receptor associated STATs (8–10). The STAT proteins then dimerize via SH2-phosphotyrosyl interactions (11) and translocate to the nucleus where they bind specific promoter sequences, thereby regulating gene expression.

IFN-α induces the phosphorylation of the STAT proteins Stat1, Stat2, and Stat3 (12, 13). To date, these activated STATs have been shown to form four distinct STAT complexes. A STAT complex designated the interferon-stimulated gene factor 3 (ISGF3) is formed between Stat2 and Stat1 (Stat2:1) in association with a DNA-binding adapter protein, p48, of the interferon regulatory factor (IRF) family of proteins (5, 14–18). ISGF3 transcriptionally activates a subset of interferon-stimulated genes (ISGs) that contain an interferon-stimulated response element (ISRE), with a consensus sequence AGTTTC-NNTTTCN/C/T (19). Additionally, homo- and heterodimers of Stat1 and Stat3 (Stat1:1, Stat1:3, and Stat3:3) recognize an interferon-stimulated response element (pRE), with a consensus sequence TTC/ANNNG/TAA, regulating expression of a distinct subset of ISGs (20, 21). Studies with mutant cell lines that are deficient in response to IFN-α indicate that alternative pathways to regulate gene expression, via elements distinct from the previously described ISRE or pRE, may exist (22).

To date, the procedure to identify STAT complexes induced by IFN-α was dependent on the prior identification of the target DNA elements involved (23, 24). However, other IFN-α-induced STAT complexes may form that target yet unidentified DNA elements. In this report we describe a novel biochemical approach utilizing genomic DNA affinity chromatography (GDAC) for the identification of IFN-α-inducible STAT complexes. This approach permits the identification of STAT complexes irrespective of the DNA recognition elements involved. We show that this technique can be used to identify the previously characterized IFN-α-inducible STAT complexes that recognize both the ISRE of the 2′-5′ oligoadenylate synthetase (OAS) gene and the pRE of the interferon regulatory factor 1 (IRF-1) gene. Our studies also identified ISGF3-independent Stat2-containing STAT complexes. Since we are able to demonstrate that an IFN-α-inducible Stat2:1 complex binds with low affinity to the IRF-1 pRE, in the absence of p48, and Stat3 will co-precipitate with Stat2 following IFN-α treatment of cells, we infer that multiple Stat2-containing complexes are induced upon IFN-α treatment of responsive cells.
The supernatant (cytoplasmic fraction) was supplemented to 60 mM Na₄P₂O₇, 1 mM NaF, 250 mM Hepes (pH 7.9), 4 mM Tris (pH 7.9), 0.6 mM EDTA, 10 mM KCl, 5 mM NaF. Cells were concentrated to 2 × 10⁸ cells/ml and centrifuged at 7,000 g. The pellet (crude nuclei) was suspended in hypotonic buffer, layered over a 30% sucrose solution, and washed twice with PBS and incubated in lysis buffer for 30 min. The cell lysate was clarified by centrifugation at 12,000 g for 5 min, and the supernatant was adjusted to 150 mM NaCl. Lysate from the equivalent of 10⁹ cells was incubated with 1 μg of preimmunee rabbit IgG, and 50 μl of a 10% suspension of Staphylococcus aureus protein A (Sigma), for 1 h. Following centrifugation, the supernatant was incubated with 1 μg of anti-Stat2 polyclonal antiserum or preimmunee rabbit IgG for 1 h and then with a 1:100 dilution of protein A-Sepharose (Pharmacia Biotech Inc.) was added for an additional 1 h. The Sepharose beads were washed three times with 1 ml of immunoprecipitation buffer, and bound proteins were eluted by boiling with reducing SDS-PAGE sample buffer. Lysis buffer contains: 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 300 mM NaCl, 50 mM Tris (pH 7.5), 0.5 mM Na₃VO₄, 0.5 mM phenylmethylsulfonfluoride, 3 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A. This buffer adjusted to 150 mM NaCl constitutes the immunoprecipitation buffer.

Mobility Shift Assay—10 μg of cytoplasmic extract from untreated or IFN-α-treated cells were analyzed using the electrophoretic mobility shift assay (EMSA), by a modification of the procedure described previously (28). Briefly, extracts were incubated with or without double-stranded oligodeoxynucleotides corresponding to the IRF-1 pRE, the 2′-5′ OAS ISRE, or a mutant ISRE, in the presence of 1 μg poly(dI-dC)poly(dI-dC), in EMSA buffer for 30 min at room temperature (final volume: 21 μl). Protein-DNA complexes were resolved on a 4.5% polyacrylamide gel using 0.2× TBE (final concentration 18 mM Tris borate, 1.5 mM EDTA, pH 8.0) as running buffer. For supershift experiments, 0.5 μl of polyclonal antisera to Stat1, Stat2, Stat3, or preimmune sera were incubated with protein extracts for 30 min at 4°C prior to the addition of DNA. EMSA buffers contained: 12 mM Hepes (pH 7.9), 40 mM KCl, 5 μM MgCl₂, 0.12 mM EDTA (pH 8.0), 0.06 mM EGTA (pH 8.0), 0.5 mM dithiothreitol, and 10% glycerol.

Western Blotting (Immunoblotting)—Western blotting was performed by a modification of the procedure described (29). Immunoprecipitated proteins from the equivalent of 5 × 10⁶ cells and GDAC-eluted fractions from the equivalent of 10⁹ cells (cytoplasmic) or 3 × 10⁹ cells (nuclear) were resolved on SDS-PAGE. Both continuous (7%) and gradient (4%-12%) SDS-PAGE gels were used as indicated in the figure legends. Proteins were transferred electrothermically to nitrocellulose (Schleicher & Schuell) for 1 h at 100 V. For supershift experiments, 0.5 μl of the specific antibodies was added to the elution buffer for 30 min prior to electrophoretic transfer to nitrocellulose. The conditions for Western blotting with specific antibodies were performed as described by the manufacturer. Briefly, the nitrocellulose was first incubated in blocking buffer, followed by incubation with primary antibody for 1 h at room temperature. Monoclonal antibodies to Stat1, Stat3, and Tyr(P) were used at a dilution of 1:1,200. Antibody to Stat2 was used at 1:1,000, and polyclonal antisera to p48 was used at 1:1,000. The nitrocellulose was then washed six times for 5 min each in wash buffer, followed by incubation with secondary antibody for 1 h at room temperature. Horseradish peroxidase-conjugated sheep anti-mouse (Amersham, Bucks, United Kingdom) and horseradish peroxidase-conjugated goat anti-rabbit (Sigma) were used at a dilution of 1:2,000. Immunoreactive proteins were visualized using an epichemiluminescence system (Amersham) according to the manufacturer’s instructions. Transfer buffer contains: 25 mM Tris, 192 mM glycine, 0.1% SDS, and 20% methanol. Wash buffer contains: 10 mM Tris (pH 7.5), 200 mM NaCl, and 0.1% Tween 20. Wash buffer containing 1% BSA constitutes blocking buffer.

RESULTS

Identification of IFN-α-induced STAT Complexes by GDAC—Human myeloma U266 cells were either left untreated, or treated with IFN-α at 5 ng/ml for 15 min in the presence of NaCl, which inhibits nuclear translocation of IFN-α activated STAT complexes (30). We found that the cytoplasmic accumulation of ISGF3 was maximal following a 15-min treatment with IFN-α (data not shown). The preparation of cytoplasmic extracts from IFN-α-treated cells in the presence of NaCl yields a fraction containing DNA-binding STAT complexes with relatively few other DNA-binding proteins. These cytoplasmic extracts were then analyzed using GDAC and the high salt eluates resolved by SDS-PAGE and transferred to nitrocellulose. We found that STAT complexes can be eluted from genomic DNA at KCl concentrations as low as 300 mM. At this concentration many DNA-binding proteins present in the cytoplasm remain associated with the DNA, further enhancing the identification of STATs (data not shown). Anti-Tyr(P) immunoblotting
IFN-α induces an ISGF3-independent Stat2 Complex(es) with Low Affinity for the IRF-1 pRE—The data in Fig. 1 suggest that IFN-α induces a novel Stat2 DNA-binding complex(es) with a recognition element(s) distinct from the ISRE. To confirm that an IFN-α-induced Stat2 containing complex other than ISGF3 is induced in U266 cells, the following experiments were conducted. Both cytoplasmic and nuclear extracts of IFN-α-treated U266 cells were analyzed by GDAC in the presence of ISRE oligodeoxynucleotides. The high salt eluates were examined for the presence of an ISGF3-independent Stat2 complex in immunoblotting experiments with antibodies to both Stat2 and p48. The addition of ISRE oligodeoxynucleotides to both cytoplasmic and nuclear extracts of IFN-α-treated cells had the effect of completely competing ISGF3, as evidenced by the total depletion of p48, yet residual Stat2 DNA binding activity was detected (Fig. 2A). The addition of mutant ISRE oligodeoxynucleotides had no effect on either Stat2 or p48 binding to genomic DNA.

Earlier studies suggested that Stat2 does not bind the pRE (32). However, we found that the presence of excess IRF-1 pRE oligodeoxynucleotides affected the genomic DNA binding activity of Stat2 (Fig. 1). Therefore, we performed a titration with the pRE to determine the DNA binding characteristics of the ISGF3-independent Stat2 complex(es). Cytoplasmic extracts of IFN-α-treated U266 cells were analyzed using GDAC in the presence of excess 2′-5′ OAS ISRE and increasing amounts of IRF-1 pRE oligodeoxynucleotides. The high salt eluates were examined for the presence of the IFN-α-induced STAT proteins Stat1, Stat2, and Stat3. Whereas genomic DNA binding of both Stat1 and Stat3 was largely eliminated, Stat2 was only mildly affected by the addition of 150 ng of pRE and, indeed, remained bound to genomic DNA in the presence of 600 ng of pRE oligodeoxynucleotides (Fig. 2B). These results indicate that the ISGF3-independent Stat2 complex(es), unlike homo- and heterodimers of Stat1 and Stat3, exhibits only a low affinity for the pRE.

DNA-binding STAT complexes contain dimers of STAT proteins formed through SH2-phosphotyrosine peptide interactions (11, 17, 33). These complexes are sensitive to competition by soluble phosphotyrosine (31). To confirm that STAT dimers are required for ISGF3-independent Stat2 complex formation, we analyzed extracts of IFN-α-treated U266 cells, using GDAC in the presence of ISRE oligodeoxynucleotides, for sensitivity to phosphotyrosine. The results shown in Fig. 2C demonstrate that the presence of phosphotyrosine abrogates the DNA binding activity of the ISGF3-independent Stat2 complex(es), whereas phosphothreonine or phosphoserine had no effect.

ISGF3-independent Stat2 complex(es), as assayed in GDAC, exhibit a relatively low affinity for the IRF-1 pRE (see Fig. 2B). These results suggest that IFN-α-induced Stat2 complexes may bind to the pRE under specific conditions that would allow detection in the EMSA. In order to optimize detection of Stat2 in complex with the pRE, we incubated IFN-α-treated cytoplasmic extracts with a large amount (up to 50 ng) of IRF-1 pRE. Following EMSA, complexes were detected by immunoblotting with antibodies to Stat2. A pRE-dependent mobility shift complex, that migrated with a higher mobility than the ISRE Stat2 complex (Fig. 3A, upper panel). Apparently, the Stat2-pRE complex forms with relatively low affinity, since we were able to detect the ISGF3-ISRE complex using as little as 0.5 ng of ISRE probe. Immunoblotting with antibodies to p48 revealed the association of p48 with the ISRE-Stat2 complex and not the pRE-Stat2 complex (Fig. 3B, lower panel). This ISGF3-independent Stat2 complex was not found in extracts of untreated cells, nor did it form...
when IFN-α-treated cell extracts were incubated with a mutant 2'-5' OAS ISRE as probe (Fig. 3B). Addition of antibodies against Stat1 and Stat2 to the protein-DNA reaction mixture affected the mobility of the pIRE-Stat2 complex in the native gel. Antibodies to Stat3, however, had no effect (Fig. 3B). These results indicate that a Stat2:1 complex can bind, albeit with low affinity, to the IRF-1 pIRE and confirm our results with GDAC.

IFN-α Induces a Stat2:3 Complex—The data in Fig. 2C suggest that the ISGF3-independent Stat2 complex(es) contains a dimer(s) of STAT proteins. Immunoprecipitation experiments were performed to identify STAT proteins that dimerize with Stat2 in an IFN-α-dependent manner. Using antisera to Stat2, we observed a co-immunoprecipitation of both Stat1 and Stat3 in IFN-α treated cells (Fig. 4A). As the formation of these complexes is dependent on IFN-α, this eliminates the possibility that immunoprecipitation of Stat3 is a result of the anti-Stat2 antisera cross-reacting with Stat3. In control experiments using preimmune antisera for immunoprecipitation, no STAT proteins were detected. The results shown in Fig. 4B demonstrate that the IFN-α-induced Stat2:3 complex is sensitive to competition by soluble phosphorylase and not phosphothreonine or phosphoserine. Taken together, our data suggest that heterodimers of Stat2:1 and Stat2:3 are formed following treatment of cells with IFN-α.

DISCUSSION

Application of GDAC to Identify IFN-α-Induced STATs—To date, the procedure to identify STAT complexes was dependent on the prior identification of the target DNA elements involved (23, 24, 32, 34–36). Our approach employing GDAC permits the identification of STAT complexes irrespective of the DNA recognition elements and, hence, has general application in studies of cytokine-induced STATs. Using this approach we were able to identify the individual IFN-α-induced STATs, as well as the complexes they form. Having analyzed a number of IFN-α-sensitive cell lines, we conclude that within the first 15 min of IFN-α treatment only activation of STATs that are recognized by antibodies to Stat1, Stat2, and Stat3 occurs (Fig. 1 and data not shown). The different functional isoforms of IFN-α-induced STATs were revealed (Fig. 1). Our results are in agreement with earlier studies suggesting that both forms of Stat1, Stat1α and Stat1β, are involved in STAT complex formation (13). Based on their electrophoretic mobility, we demonstrate that antibodies to Stat3 recognizes three different proteins. The two slower migrating factors likely represent Stat3 isoforms that differ at the level of serine phosphorylation (37). The third, faster migrating factor may be analogous to the Stat1 isoform, Stat1β, i.e. a differentially spliced product of the same gene. Such a factor has been described recently (38) and designated Stat3β. Only a single isoform of Stat2 was identified.

IFN-α-Induced STAT Complexes—Functionally diverse cytokines and growth factors have been shown to activate both Stat1 and Stat3. These include, in addition to IFN-α, IFN-γ, IL-6, and other gp130-mediated signaling proteins, epidermal growth factor, and platelet-derived growth factor (7, 12). To date, IFN-α is the only cytokine known to activate Stat2. Moreover, Stat2 is a critical regulator of IFN-α signal transduction, since the activation of Stat1 by IFN-α is dependent on the prior activation of Stat2 (15, 39). Once activated, STAT proteins apparently exhibit overlapping functions. Studies of gene activation by IFN-α and IFN-γ have revealed functional similarities between the Stat2:1 dimer of the IFN-α-induced ISGF3 and the Stat1:1 dimer induced by IFN-γ (40). Analysis of the DNA binding specificity of chimeric STAT proteins has localized the DNA binding domain between amino acid residues 300–500 (41, 42). Stat2 exhibits a high degree of amino acid identity to the other STATs in this region (43). Moreover, point mutational analysis of the DNA binding domain of Stat3 has identified specific residues important for DNA binding activity (41). These residues are also highly conserved in Stat2. To date, four distinct IFN-α-inducible STAT complexes have been described in the literature: homo- and heterodimers of Stat1 and Stat3 and a Stat2:1 heterodimer, which binds DNA via an adapter protein p48. However, in the light of recent data suggesting that Stat2 may possess an intrinsic DNA-binding domain, Stat2 may function in STAT complexes in addition to ISGF3.

Using GDAC we performed competition experiments with oligodeoxynucleotides corresponding to the pIRE of the IRF-1 gene and the ISRE of the 2'-5' OAS gene to identify IFN-α-
induced STAT complexes (Fig. 1). Our studies revealed a Stat2-containing STAT complex(es) distinct from those identified previously. This complex(es) is capable of binding genomic DNA in the presence of excess ISRE (Fig. 2A). Competition experiments with the ISRE indicated that the ratio of ISGF3 to total DNA-binding Stat2 is lower in the nucleus than the cytoplasm (Fig. 2A). As p48 is a component of ISGF3 and is required for DNA binding activity, this result likely reflects a lower ratio of p48 to total DNA-binding Stat2 in the nucleus compared with the cytoplasm. An excess of IRF-1 pIRE (600 ng) partially competed the DNA binding activity of the ISGF3-independent Stat2 complex(es) (Fig. 2B), suggesting that this complex(es) can bind to this element. However, as the DNA binding activity of both Stat1 and Stat3 was largely eliminated by excess pIRE, these Stat2-containing complex(es) likely bind with relatively low affinity. Nevertheless, following prolonged autoradiographic exposure, residual low levels of DNA binding Stat1 and Stat3 were apparent in the IFN-α-inducible cell extracts from which ISRE- and pIRE-binding complexes had been deleted (data not shown). The implications are that the ISGF3-independent Stat2 complex(es) may require Stat1 and/or Stat3 for DNA binding. The results in Fig. 2C reveal that these complex(es) bind DNA as a dimer. Consistent with the observed low affinity binding of Stat2 complex(es) to the pIRE in GDAC (Fig. 2B), low affinity, pIRE-binding Stat2:1 heterodimers were detected in EMSA (Fig. 3A and B). Immunoprecipitation experiments identified an IFN-α-inducible Stat2:1 dimer and a novel Stat2:2 dimer (Fig. 4). Moreover, in vitro studies involving tyrosine-phosphorylated Stat2 purified from baculovirus infected insect cells, identified Stat2 homodimerization. 2 Taken together, these data suggest that the ISGF3-independent Stat2 complexes identified using GDAC likely contain the STAT dimers Stat2:1, Stat2:2, and Stat2:3. However, in the mobility shift assay we detected only a Stat2:1 heterodimer in complex with the pIRE. The absence of both Stat2:2 and Stat2:3 complexes may be a consequence of a lower affinity and/or abundance of these complexes compared with the Stat2:1 complex. Alternatively, Stat2:2 and Stat2:3 complexes may bind to DNA elements distinct from the consensus ISRE and pIREs. We propose that one or more high affinity DNA recognition elements for the ISGF3-independent Stat2 complexes exist that are related to the IRF-1 pIRE.

Recent evidence suggests that Stat2, within the complex of ISGF3, does not contact DNA directly. Instead it participates in the DNA binding activity of ISGF3 by interacting with the DNA-binding protein p48 (17). Originally we hypothesized that the ISGF3-independent Stat2 complex(es) may also require a p48-like adapter protein for DNA binding. The DNA binding activity of ISGF3 is sensitive to alkylation by N-ethylmaleimide (NEM) (30). The addition of extracts from untreated cells to NEM treated extracts of IFN-α-induced cells, restores ISGF3, suggesting that only p48 is sensitive to NEM. We found that, although the ISGF3-independent Stat2 complex(es) was sensitive to NEM treatment, the DNA binding activity could not be restored by the addition of extracts from untreated cells (data not shown). Given the relatively high frequency of cysteine residues within STAT proteins, these results are not surprising; the conditions we employed for NEM treatment promote alkylation of cysteine residues, thus inactivation of STAT dimer binding to either an adapter protein, or directly to DNA, could be expected. This NEM sensitivity of STAT dimer(s) precludes the use of this assay to predict the presence of a p48-like protein(s) within STAT complex(es).

In this report, we demonstrate for the first time Stat2 DNA binding activity independent of ISGF3. We propose that these
Stat2-containing STAT dimers regulate transcription by interacting with an element(s) related to the IRF-1 pRE that is present in the promoter region of specific ISGs. Identification of this promoter element(s) will shed light on the role of these complexes in IFN-α signal transduction.

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