The Amino-terminal Domain of Human STAT4
OVERPRODUCTION, PURIFICATION, AND BIOPHYSICAL CHARACTERIZATION*

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The multifunctional signal transducer and activator of transcription (STAT) proteins relay signals from the cell membrane to the nucleus in response to cytokines and growth factors. STAT4 becomes activated when cells are treated with interleukin-12, a key cytokine regulator of cell-mediated immunity. Upon activation, dimers of STAT4 bind cooperatively to tandem interferon-γ activation sequences (GAS elements) near the interferon-γ gene and stimulate its transcription. The amino-terminal domain of STAT4 (STAT4(1–124)) is required for cooperative binding interactions between STAT4 dimers and activation of interferon-γ transcription in response to interleukin-12. We have overproduced this domain of human STAT4 (hSTAT4(1–124)) in Escherichia coli and purified it to homogeneity for structural studies. The circular dichroism spectrum of hSTAT4(1–124) indicates that it has a well ordered conformation in solution. The translational diffusion constant of hSTAT4(1–124) was determined by nuclear magnetic resonance methods and found to be consistent with that of a dimer. The rotational correlation time (τc) of hSTAT4(1–124) was estimated from 15N relaxation to be 16 ns; this value is consistent with a 29-kDa dimeric protein. These results, together with the number of signals observed in the two-dimensional 1H-15N heteronuclear single quantum coherence spectrum of uniformly 15N-labeled protein, indicate that hSTAT4(1–124) forms a stable, symmetric homodimer in solution. Cooperativity in native STAT4 probably results from a similar or identical interaction between the amino-terminal domains of adjacent dimers bound to DNA.

Cytokines mediate communication between cells in the immune system by binding to specific receptors and stimulating the transcription of distinct sets of genes. The signals are relayed from the cell surface to the nucleus via the JAK-STAT pathway (1–4). JAK-STAT signaling commences when cytokine binding induces receptor dimerization, which brings cytoplasmic receptor-associated JAKs into apposition and enables them to self-activate by reciprocal transphosphorylation. The activated kinases phosphorylate a distal tyrosine on the cytoplasmic tail of the receptor, which can then be recognized by the SH2 domain of a specific STAT protein. Upon association with the receptor, a tyrosine residue near the carboxyl terminus of the STAT protein is phosphorylated by the JAK kinase. Now activated, the STAT protein can form homo- or heterodimers in which the phosphorytosine of one partner binds to the SH2 domain of the other. The STAT dimers then migrate to the nucleus, where they participate in transcriptional activation by binding to specific DNA sequences, termed interferon-γ activation sequence (GAS) elements.

Thus far, only seven different STATs and four JAK family members have been identified (2), which raises the question of how transcriptional specificity is achieved in cytokine signal transduction: that is, how can a relatively small number of JAKs and STATs elicit distinct responses to a much larger number of cytokines and growth factors, particularly when all but one of the STATs appear to bind preferentially to the same DNA sequence (2). Recently, Xu et al. (5) characterized authentic binding sites for STATs 1, 4, 5, and 6 within the first intron of the interferon-γ (IFN-γ) gene by DNase I footprinting. Remarkably, these experiments revealed that rather than binding to the same sites, as might have been expected, STATs 1, 4, and 5 each bound to a distinct pattern of adjacent sites, none of which bears a close resemblance to the high-affinity, consensus sequence identified by the random selection method. Instead, these binding sites consist of tandem arrays of imperfect GAS elements that are separated by 10 base pairs or about one turn of the helix in B-form DNA. The binding of STAT4 to these tandem sites is cooperative in nature; simultaneous occupancy of multiple sites is required to achieve a stable association with the DNA. The amino-terminal 124 residues of STAT4 are essential for cooperative binding to these adjacent low-affinity sites, but not for its ability to be phosphorylated, to dimerize, or to bind to, a single high-affinity site (5). Xu et al. (5) proposed that this cooperativity results from a direct interaction between the amino-terminal domains of STAT dimers bound to adjacent sites on DNA. A similar result has been obtained with STAT1 (6), and it seems likely that the other STATs also use their amino-terminal domains for cooperative binding (2).

STAT4 is activated in response to interleukin-12 (IL-12), which plays a primary role in the development of T helper 1 (Th1) cells and in the induction of organ-specific autoimmune diseases (7, 8). Cooperative binding of STAT4 dimers to adjacent low-affinity GAS sites is required for transcriptional activation of IFN-γ (5), which is thought to mediate many of the effects of IL-12 (9). Therefore, a small molecule that can bind to the amino-terminal domain of STAT4 and prevent its self-
association might be an effective immunosuppressant. To facilitate the discovery of such a therapeutic agent, we have undertaken an effort to determine the three-dimensional structure of this domain in solution and elucidate the molecular details of its self-association. To this end, we have overproduced the amino-terminal domain of human STAT4 (hSTAT4(1–124)) in Escherichia coli and purified it to homogeneity. Furthermore, we have characterized this fragment of STAT4 by circular dichroism (CD) and nuclear magnetic resonance (NMR) techniques and report that it has a well ordered conformation in solution that is amenable to structure determination.

**MATERIALS AND METHODS**

**Plasmid Expression Vector**—The segment of a human STAT4 cDNA clone that corresponds to amino acid residues 1–124 was amplified by the polymerase chain reaction (PCR), using primers PE-11 (5′-TATATCGATTAGATGCAACTGAGTG-3′) and PE-12 (5′-ATTACATGTTGGATGCTCGGA-3′). The restriction sites within these primers that were used to cleave the PCR fragment prior to ligation (BspHI and BamHI) are underlined. Mismatches that were introduced within the PE-12 primer to change rarely used arginine and isoleucine codons to more common ones are indicated in lowercase type (see text for discussion). PCR amplification was performed with Pfu polymerase under the conditions recommended by the supplier (Stratagene). The PCR regimen entailed 25 cycles in a Perkin-Elmer Thermal Cycler 2400, with each cycle consisting of 30 s at 94, 55, and 72 °C, respectively. The PCR fragment was cleaved with BspHI and BamHI and then ligated with the Neo/ BamHI vector backbone of pET3d (10) to construct pDW474. The nucleotide sequence of the STAT4 DNA in pDW474 was confirmed experimentally.

**Protein Expression**—Cells from single, drug-resistant colonies of *E. coli* BL21/DE3 containing pDW474 and pDC952 were grown to saturation in LB broth (11) supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol at 37 °C. The saturated cultures were diluted 100-fold in the same medium and grown in shake-flasks to mid-log phase (A600 = 0.5–0.7), at which time isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. After 3 h, the cells were recovered by centrifugation. 15N-Labeled hSTAT4(1–124) was expressed in M. lysodeikticus (pLysS) at 30 °C.

**Protein Purification**—*E. coli* cells containing hSTAT4(1–124) were resuspended in 3 ml culture volume of lysis buffer (50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA) and disrupted by three successive passes through a French press at 4°C. The soluble extract was prepared by centrifuging the disrupted cell suspension at 14,000 × g for 15 min at 4°C. To remove nucleic acids and some acidic proteins from the soluble extract, polyethyleneimine (PEI) was added to a final concentration of 0.15%. After the mixture was kept on ice for 5 min, the precipitate was pelleted by centrifugation at 14,000 × g. Residual PEI was removed by a two-step ammonium sulfate precipitation at concentrations of 20 and 60%. The pellet from the 60% ammonium sulfate precipitation was resuspended in 50 mM Tris (pH 8.0), 1 mM EDTA, 5 mM dithiothreitol (DTT) (10 ml/liter of culture volume) and dialyzed extensively against the same at 4°C. The dialyzed material was clarified by centrifugation at 14,000 × g for 10 min and then passed over an anion exchange column (Q-Sepharose, 1.6 cm × 10 cm, Amersham Pharmacia Biotech) at 4°C. The bound protein was eluted from the column with a linear gradient of 0–1 M NaCl in 50 mM Tris (pH 8.0) and 5 mM DTT; hSTAT4(1–124) eluted at approximately 250 mM NaCl.

Fractions containing hSTAT4(1–124) were pooled and dialyzed against 25 mM sodium phosphate, 12 mM sodium formate, 12 mM sodium acetate (pH 5.25), 5 mM DTT at 4°C. This material was then passed over a cation exchange column (SP-Sepharose, 1.6 cm × 10 cm, Amersham Pharmacia Biotech), and the bound protein was eluted from the column with a linear gradient of 0–1 M NaCl in 25 mM sodium phosphate, 12 mM sodium formate, 12 mM sodium acetate (pH 5.25), 5 mM DTT. Again, hSTAT4(1–124) eluted at approximately 250 mM NaCl. The fractions containing hSTAT4(1–124) were pooled, concentrated to at least 2 mg/ml, and dialyzed against 20 mM sodium acetate (pH 5.25), 25 mM NaCl, 5 mM DTT at 4°C. The final polishing step was performed on a preparative-grade gel filtration column (Superdex 75, 1.6 × 60 cm, Amersham Pharmacia Biotech) equilibrated with the same buffer.

**CD Spectroscopy**—The CD spectrum was recorded on a JASCO 720 spectropolarimeter. The protein sample was 25 μM in 20 mM sodium acetate-d3 (pH 5.3), 50 mM NaCl, 5 mM DTT. Spectra were recorded from 195 to 350 nm using a 0.1-mm path length demountable cell (Uvonics) at 22°C. Ellipticity was calculated per residue.

**NMR Spectroscopy**—All NMR spectra were acquired on a Varian Unity plus 600 spectrometer equipped with a 2-z-pulse triple resonance, pulsed-field gradient probe (Nalorac Corp., Martinez, CA) at 25°C. Self-diffusion measurements were made using the water-sLED experiment as described (12, 13), with a diffusion time of 134 ms, a recycle delay of 10 s, and 128 transients for each of the 25 values of the pulsed-field gradient ranging from 4 to 42 G·cm. The 1H-15N HSQC spectrum was acquired using coherence selection (14) and flipback pulses (15). 1H T1 and T2 relaxation times were measured using sensitivity enhanced experiments (16). The protein concentration for all NMR experiments was 0.8 mM in 20 mM sodium acetate-d3 (pH 5.3), 50 mM NaCl, 5 mM DTT, 90% H2O/10% D2O. RESULTS

**Overproduction of hSTAT4(1–124) in E. coli**—Xu et al. (5) produced amino acids 1–124 of human STAT4 as a hexahistidine-tagged polypeptide in *E. coli* and demonstrated that this fragment could competitively inhibit the cooperative binding of STAT4 homodimers to adjacent low-affinity GAS sites. This result indicates that the isolated domain is capable of folding into an “active” conformation in the *E. coli* cytoplasm. We elected to express the same fragment of human STAT4 in an untagged (native) form to alleviate any concern that the polyhistidine tag might reduce the solubility or otherwise alter the behavior of the protein in solution. Accordingly, residues 1–124 were amplified from a cDNA clone by PCR and inserted into the bacteriophage T7 promoter vector pET3d (10), as described under “Materials and Methods.”

We noted that this interval of the human STAT4 cDNA contains four arginine codons that are rarely used in *E. coli* (AGG and AGA), including two consecutive ones. Several studies have demonstrated that these arginine codons, which are frequently present in eukaryotic cDNAs, can significantly impair the yield of a recombinant protein in *E. coli* (17, 18) or even cause the incorporation of arginine at random (19), particularly when they occur in tandem. Fortuitously, the pair of consecutive arginine codons in STAT4 was close enough to the carboxyl terminus of this domain that they could be mutated to alternative codons simply by incorporating the appropriate mismatches into the PCR primer that was used to amplify the
coding sequence for ligation with the expression vector. Another way to circumvent the problems associated with rare arginine codons is to overproduce the cognate tRNA (the product of the argU gene) on a compatible plasmid vector (17, 19). Thus, as a precautionary measure, cells also contained the argU plasmid pDC952 (20).

E. coli BL21/DE3 cells containing the hSTAT4(1–124) expression vector (pDW474) and pDC952 were grown to mid-log phase in LB broth at 37 °C and induced for several hours with isopropyl-1-thio-D-galactopyranoside, after which samples of the total and soluble intracellular proteins were prepared and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); the results are shown in Fig. 1. Samples of the total intracellular protein from uninduced BL21/DE3 (pDW474) and from induced BL21/DE3 (pET3d + pDC952) cells were also prepared as controls. The results indicate that upon induction with isopropyl-1-thio-β-D-galactopyranoside, hSTAT4(1–124) accumulates to comprise approximately 30% of the total intracellular protein (lane 3), and virtually all of this material is soluble in the crude cell extract (lane 4). A significant amount of hSTAT4(1–124) accumulates in the uninduced cells as well (lane 2), suggesting that the mRNA is very efficiently translated in E. coli. As expected, no hSTAT4(1–124) is produced by otherwise isogenic cells harbor-

**Fig. 2. Purification of hSTAT4(1–124).** A, Coomassie-stained SDS-polyacrylamide gel (Nu-PAGE, 10% BisTris) showing samples of hSTAT4(1–124) after each step in the purification scheme. Lanes: 1, molecular size standards (kilodaltons); 2, total intracellular protein; 3, soluble extract; 4, 20% NH₄SO₄ supernatant; 5, 60% NH₄SO₄ pellet (resuspended); 6, pooled fractions from anion exchange chromatography; 7, pooled fractions from cation exchange chromatography; 8, pooled fractions from size exclusion chromatography. B, silver-stained SDS-polyacrylamide gel. Lanes: 1, 60% NH₄SO₄ pellet (resuspended); 2, pooled fractions from size exclusion chromatography. C, Isoelectric focusing gel. Lanes: 1, IEF standards (Novex); 2, pooled hSTAT4(1–124) fractions from size exclusion chromatography.

**Fig. 3. Secondary structure of hSTAT4(1–124).** A, amino acid sequence and predicted secondary structure of human STAT4(1–124). The hSTAT4(1–124) secondary structure is anticipated to be the same as that of murine STAT4(1–124), which was solved experimentally (22). Predicted α-helices are indicated by cylinders. B, CD spectrum of 23 µM hSTAT4(1–124) in 20 mM sodium acetate-ᴅ₃ (pH 5.3), 50 mM NaCl, 5 mM DTT.
ing the unmodified pET3d expression vector (lane 1). No difference in the yield of hSTAT4(1–124) was observed in the absence of pDC952 (data not shown).

**Purification of hSTAT4(1–124)**—We used a combination of bulk fractionation methods and column chromatography to purify the recombinant hSTAT4(1–124) to homogeneity from an *E. coli* cell extract. The purity was monitored by SDS-PAGE after each step (Fig. 2A). The cells were lysed with a French press and then the nucleic acids and some endogenous proteins were precipitated by the addition of PEI. Next, additional *E. coli* proteins were precipitated by adding ammonium sulfate to a final concentration of 20% (w/v), which is the highest concentration that did not precipitate any of the hSTAT4(1–124).

After the insoluble debris was removed by centrifugation, the ammonium sulfate concentration was increased to 60% (w/v), at which point nearly all of the hSTAT4(1–124) was recovered in the pellet. Some contaminating proteins remained in the supernatant at this point, which was discarded. Following bulk fractionation with PEI and ammonium sulfate, the hSTAT4(1–124) was already approximately 50% pure, as gauged by densitometric scanning of SDS-polyacrylamide gels (Fig. 2A, lane 5).

After the residual ammonium sulfate was removed by dialysis, the dialysate was applied to an anion exchange column. hSTAT4(1–124) bound to the column and was eluted with a NaCl gradient. Next, because the isoelectric point of hSTAT4(1–124) is close to neutral, we were also able to find conditions under which the protein would bind reversibly to a cation exchange column. After both forms of ion exchange chromatography were used in succession, hSTAT4(1–124) was free of detectable contaminants except for a multimeric form of the protein that results from an intermolecular disulfide bond. These cross-linked multimers were readily removed by gel filtration, however.

The final preparation of hSTAT4(1–124) is extremely pure, yielding just a single band on a silver-stained SDS gel (Fig. 2B, lane 2). Liquid chromatography electrospray mass spectrometry analysis revealed that this material consists of a single component with a molecular weight of 14,473 (data not shown). The predicted molecular weight of hSTAT4(1–124) without the amino-terminal methionine residue is 14,472. Because the initiator methionine of hSTAT4(1–124) is followed by a serine, we expected that it would be removed posttranslationally by *E. coli* methionine amino peptidase (21). This conjecture was confirmed by amino-terminal sequencing (data not shown). Finally, we note that our preparation of hSTAT4(1–124) appears as a single, sharp band on an isoelectric focusing gel (Fig. 2C).

The measured isoelectric point is approximately 6.7, which is only slightly higher than the predicted value of 6.5. Hence, by a variety of rigorous criteria, hSTAT4(1–124) produced in *E. coli* and purified as described appears to be a chemically homogeneous protein.

**CD Study**—The secondary structure of hSTAT4(1–124) is predicted (22) to be composed entirely of α-helices and connecting loops, with no β-structure (Fig. 3A). As shown in Fig. 3B, the far-UV CD spectrum of hSTAT4(1–124) corroborates this prediction. The CD spectrum exhibits a strong peak of negative ellipticity at 222 nm, which is characteristic of a high degree of α-helical character and further serves to demonstrate that hSTAT4(1–124) has a well ordered structure in solution. Quantitative analysis of the spectrum yields an estimate of ~75% α-helix (23), which is in good agreement with the predicted secondary structure (Fig. 3A).

**NMR Analysis**—Because it was proposed that the amino-terminal domain of STAT4 mediates cooperative DNA binding through self-association, we sought to determine whether the isolated domain exists as a dimer in solution. Initially, we used NMR pulsed-field gradient self-diffusion measurements (12) as an experimental tool for this purpose. The data obtained from these experiments are summarized in Fig. 4. As shown in Fig. 4, the self-diffusion coefficient, $D_{S0}$, of $8.6 \times 10^{-6}$ cm$^2$ s$^{-1}$ corresponds to a dimer for hSTAT4(1–124), based on comparisons with two monomeric proteins of similar size (lysozyme, 14.1 kDa, $D_S = 1.04 \times 10^{-6}$ cm$^2$ s$^{-1}$; NusB, 15.7 kDa, $D_S = 1.01 \times 10^{-6}$ cm$^2$ s$^{-1}$) and a dimeric protein, which is slightly larger than hSTAT4(1–124) (IL-10, 37.4 kDa dimer, $D_S = 0.82 \times 10^{-6}$ cm$^2$ s$^{-1}$) (12, 13).

A second assessment of the quaternary structure of hSTAT4(1–124) was made based on $^{15}$N relaxation measurements, utilizing a sample of uniformly labeled $^{15}$N-hSTAT4(1–124) prepared and purified as described under "Materials and Methods." The rotational correlation time was estimated from the ratio of $^{15}$N $T_1/T_2$ (24), which yielded a value of 16 ns at 25 °C (data not shown). The estimated correlation time clearly indicates that the protein is not monomeric when compared with values of 9.3 ns for ribonuclease H at 27 °C (17.6 kDa) (25) or 10.7 ns for human immunodeficiency virus protease at 27 °C (21 kDa) (26) and is in the proper range for a dimeric system with an effective size of 29 kDa. A recent detailed analysis of a two-domain protein, which is similar in size to the hSTAT4(1–124) dimer, determined a value of 13.1 ns for the correlation time at 40 °C (27). This value would be expected to be longer at 25 °C, which is what we observed for hSTAT4(1–124).

To characterize the structure of this domain in greater detail, we have examined its two-dimensional $^1$H-$^{15}$N HSQC
NMR spectrum (Fig. 5). This spectrum reports a resonance for each H-1N pair that does not undergo rapid exchange of the H-N with solvent H2O, including side chain NH2 groups. There are approximately 110 readily discernible backbone amide signals present in this spectrum; the remaining expected resonances cannot be unambiguously counted because of spectral overlap. Hence, the dimer must be symmetric, giving rise to a single set of equivalent resonances for each monomer. Any disruption of the symmetry would lead to a significant increase in the number of observed resonances.

DISCUSSION

Although it is not required for binding to single, high-affinity GAS sites, the amino-terminal domain of STAT4 is essential for cooperative binding to tandem arrays of low affinity sites within the IFN-γ gene and for the stimulation of its transcription in response to IL-12 (5). Like IFN-γ, many genes probably will utilize tandem arrays of low-affinity sites for activation. If so, the amino-terminal domains of the STATs may be attractive targets for therapeutic agents that seek to attenuate cytokine signal transduction.

The experiments reported here indicate that hSTAT4(1–124) has a well-ordered conformation in solution that is amenable to structure determination by heteronuclear NMR spectroscopy. Furthermore, they reveal that hSTAT4(1–124) forms a stable, symmetric homodimer at high micromolar concentrations. The existence of a dimeric form of hSTAT4(1–124) is consistent with current models of transcriptional activation, which postulate that cooperative binding of STATs to adjacent low affinity GAS sites involves a physical interaction between their amino-terminal domains (22). This suggests that it should be possible to develop compounds of this type with high specificity for a particular STAT.

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