Biochemical and Biophysical Research Communications 374 (2008) 1–5

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Crystal structure of unphosphorylated STAT3 core fragment

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ARTICLE INFO

Article history:
Received 18 March 2008
Available online 21 April 2008

Keywords:
STAT
Signal transduction
Dimerization

ABSTRACT

Signal transducers and activators of transcription (STATs) are latent cytoplasmic transcriptional factors that play an important role in cytokine and growth factor signaling. Here we report a 3.05 Å-resolution crystal structure of an unphosphorylated STAT3 core fragment. The overall monomeric structure is very similar to that of the phosphorylated STAT3 core fragment. However, the dimer interface observed in the unphosphorylated STAT1 core fragment structure is absent in the STAT3 structure. Solution studies further demonstrate that the core fragment of STAT3 is primarily monomeric. Mutations corresponding to those in STAT1, which lead to disruption of the core fragment interface and prolonged tyrosine phosphorylation, show little or no effect on the tyrosine phosphorylation kinetics of STAT3. These results highlight the structural and biochemical differences between STAT3 and STAT1, and suggest different regulation mechanisms of these two proteins.

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STAT proteins are latent cytoplasmic transcriptional factors that play an essential role in cytokine and growth factor signaling. Mammalian STATs share six structural regions: N-domain (ND), coiled-coil domain (CCD), DNA-binding domain (DBD), linker domain, SH2 domain, and transcriptional activation domain (Fig. 1A). Upon receptor activation, a single tyrosine residue (Y705 in STAT3) is phosphorylated. Once phosphorylated, STATs form homo- or hetero-dimers via reciprocal SH2-phosphotyrosine interactions, translocate into the nucleus, and activate gene transcription [1–3].

Crystal structures of unphosphorylated STAT1 and STAT5 showed that the core fragment (residues ~130 to ~680) forms a reciprocal dimer involving CCD and DBD [4,5]. This so-called "antiparallel" dimer has been shown to play an important role in STAT1 dephosphorylation [6,7]. To explore whether this interface is conserved in other STATs, we set out to determine the crystal structure of unphosphorylated STAT3.

Materials and methods

Molecular cloning, protein and peptidomimetic preparations. DNA sequence of corresponding mouse STAT3 residues 127–688 was cloned into pET20b(+) (Novagen). This protein was purified as published [8]. The peptidomimetic compound, PM50D (Fig. 1B), was synthesized as published [9]. QuikChange (Stratagene) was used to generate STAT3 mutations in the pRc/CMV vector. All of the STAT constructs and mutants were confirmed by sequencing.

Crystallization, data collection, structure determination, and refinement. The complex of STAT3 (residues 127–688) and PM50D was prepared by mixing the protein with the peptide in a 1:1.5 molar ratio. Crystals in hanging drops were obtained at 4 °C by mixing 0.8 μl of 20 mg/ml protein-PM50D mixture, 0.8 μl of reservoir solution (100 mM Bis–Tris, pH 5.5–5.9, 500 mM $(NH_4)_2SO_4$, 10–12% PEG3350), 0.4 μl 500 mM KCl, and 0.5 μl 250 mM NaI. The best crystals ($600 \times 80 \times 80\ \mu m^3$) were obtained in 3–4 days.

Crystals were transferred through a series of cryoprotection solutions with increasing concentrations of glycerol (100 mM Bis–Tris, pH 5.5, 500 mM $(NH_4)_2SO_4$, 12% PEG3350, 100 mM KCl, 50 mM NaI, and 5%–25% glycerol) and flash frozen in liquid N_2. Diffraction data were collected at Advanced Light Source (ALS) Beamline 8.3.1 and processed using HKL2000 [10]. Crystals grew in the trigonal space group $P3_121$ with unit cell dimensions $a = b = 254.8$ and $c = 123.8$ Å. There are two molecules of STAT3 per asymmetric unit. The structure was determined by molecular replacement using Phaser [11]. The published STAT3 structure [12] was used as the search model. Molecular model was then modified in O [13] and refined with CNS [14]. Partial twinning was detected using CNS and the refinement was carried out using the twinning operator $-h$, $-k$, l and twinning fraction of 0.373.

Multiangle light scattering (MALS) analysis. Purified proteins [STAT3α (residues 1–770), STAT3 (residues 127–688), and STAT3β

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doi:10.1016/j.bbrc.2008.04.049

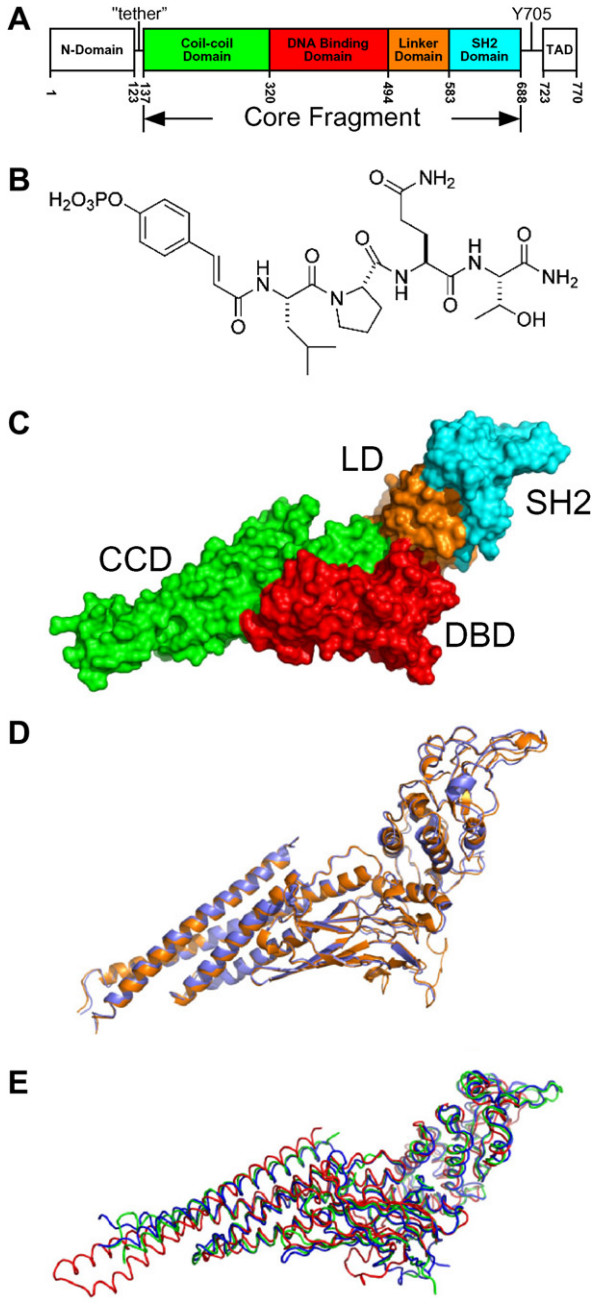

Fig. 1. (A) Domain structure of STAT3. The six domains of STAT3α are N-domain, coiled-coil domain (green), DNA binding domain (red), linker domain (orange), SH2 (cyan), and transcriptional activation domain. Between SH2 and TAD there is a tail segment that contains the phosphorylation site Y705. (B) Chemical structure of compound PM50D. (C) Crystal structure of monomeric STAT3 (127–688). (D) Superimposition of phosphorylated (orange, PDB code 1BG1) and unphosphorylated (slate) STAT3 core fragments. (E) Superimposition of unphosphorylated STAT1 (blue; 1YVL), STAT3 (green), and STAT5 (red; 1Y1U) core fragments.

(residues 127–722)] were characterized by MALS coupled with size exclusion chromatography. A sample was injected onto a KW-803 column (Shodex) equilibrated in higher salt buffer (100 mM Bis–Tris, pH 5.5, 500 mM $(NH_4)_2SO_4$, 100 mM KCl, 50 mM NaI), low salt buffer (100 mM Bis–Tris, pH 5.5, 0.1 M KCl, 50 mM NaI), or PBS (137 mM NaCl, 10 mM Na/K phosphate, 2.7 mM KCl, pH 7.4). The experiments were performed essentially as previously published [4].

Tissue culture and immuno blots. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum at 37 °C with 5% CO_2. One day prior to transfection, cells were plated at a density of 1.5×10^5 cells per well in a 6-well plate. Transfection was carried out using FuGENE 6 transfection reagent (Roche) with 0.25 μg plasmid per plate and a 1:4 DNA/FuGENE 6 ratio. After 40 h, cells were washed twice and fed with DMEM without serum overnight. The next day cells were first stimulated with human recombinant EGF (100 ng/ml) (Upstate Biotechnology) for 15 min. The media were then aspirated and new media containing 10 μM AG1478 were fed to cells. Cells were then harvested at given time points and whole-cell lysates were prepared and resolved in 8% SDS–PAGE gels. Phosphorylated and unphosphorylated STAT3 levels were detected by Western blot with anti-phosphotyrosine 705 of STAT3 (Cell Signaling) and anti-STAT3 (BD Transduction Labs) antibodies, respectively.

Results and discussion

Structure determination and general architecture

The crystal structure of the unphosphorylated STAT3 core fragment was determined to 3.05 Å resolution. The final twinned R_{work} is 25.3% and twinned R_{free} 27.5%. There are two molecules per asymmetric unit. A monomer is shown in Fig. 1C. Several loops (residues 185–193, 371–378, 399–400, 419–430 in both molecules, and residues 659–662 in the second) are disordered and not included in the final model. PM50D was not convincingly seen in the electron density map and thus was omitted from the final structure. Table 1 summarizes the data collection and refinement statistics.

As was the case for STAT1, there is little conformational change within the core fragment of STAT3 before and after tyrosine phosphorylation (Fig. 1D). The r.m.s.d. for more than 501 C_α atoms between our structure and that of the phosphorylated monomer is 0.9 Å. Also, similar to STAT1 and STAT5, the core fragment has a contiguous hydrophobic core that is an integral structural unit [4,5,15] (Fig. 1E). Interestingly, in the crystal structures of unphosphorylated STAT1 and STAT5, there is a core fragment dimer interface formed by residues from the CCD and DBD [4,5]. However, this interface is not seen in this STAT3 structure.

Oligomerization state of unphosphorylated STAT3

To investigate the oligomerization state of STAT3 in solution, we used MALS to measure the masses of the core fragment and full-

Table 1
Summary of crystallographic analysis

Data collection statistics	
Space group	$P3_121$
Unit cell	$a = b = 254.78$, $c = 1.12$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$
Energy (wavelength)	11110.8 eV (1.115891 Å)
Resolution range (Å)	30.0–3.05 (3.16–3.05)
Completeness (%)	93.4 (70.6)
$I/\sigma(I)$	11.1 (2.1)
R (%)	11.3 (44.7)
Refinement statistics (30–3.05 Å)	
Reflections	
Working set	67,834
Test set	7254
Number of atoms	8544
Rmsd bonds (Å)	0.011
Rmsd angles (°)	1.48
Twinned $R_{conventional}$ (%)	25.0
Twinned R_{free} (%)	26.9
Ramachandran plot	
Most favored	75.6%
Additionally allowed	21.4%
Disallowed	0.0%

Table 2
Molecular masses of STAT3 proteins as determined by MALS

	Protein	BSA (monomeric)	STAT3 (127–688)	STAT3 (127–688) + PM50D	STAT3 (1–770)
	$MW_{calc.}$ (kDa)	66	64.34	65.02	88.07
Low salt buffer (pH 5.5)	$MW_{meas.}$ (kDa)	66.85	66.54	67.36	170.5
	Polydispersity	1.000	1.001	1.001	1.000
High salt buffer (pH 5.5)	$MW_{meas.}$ (kDa)	64.05	63.98	64.18	116.5
	Polydispersity	1.000	1.001	1.002	1.010
PBS (pH 7.4)	$MW_{meas.}$ (kDa)	63.99	75.82	ND[a]	170.5
	Polydispersity	1.000	1.000	ND	1.000

[a] Not determined.

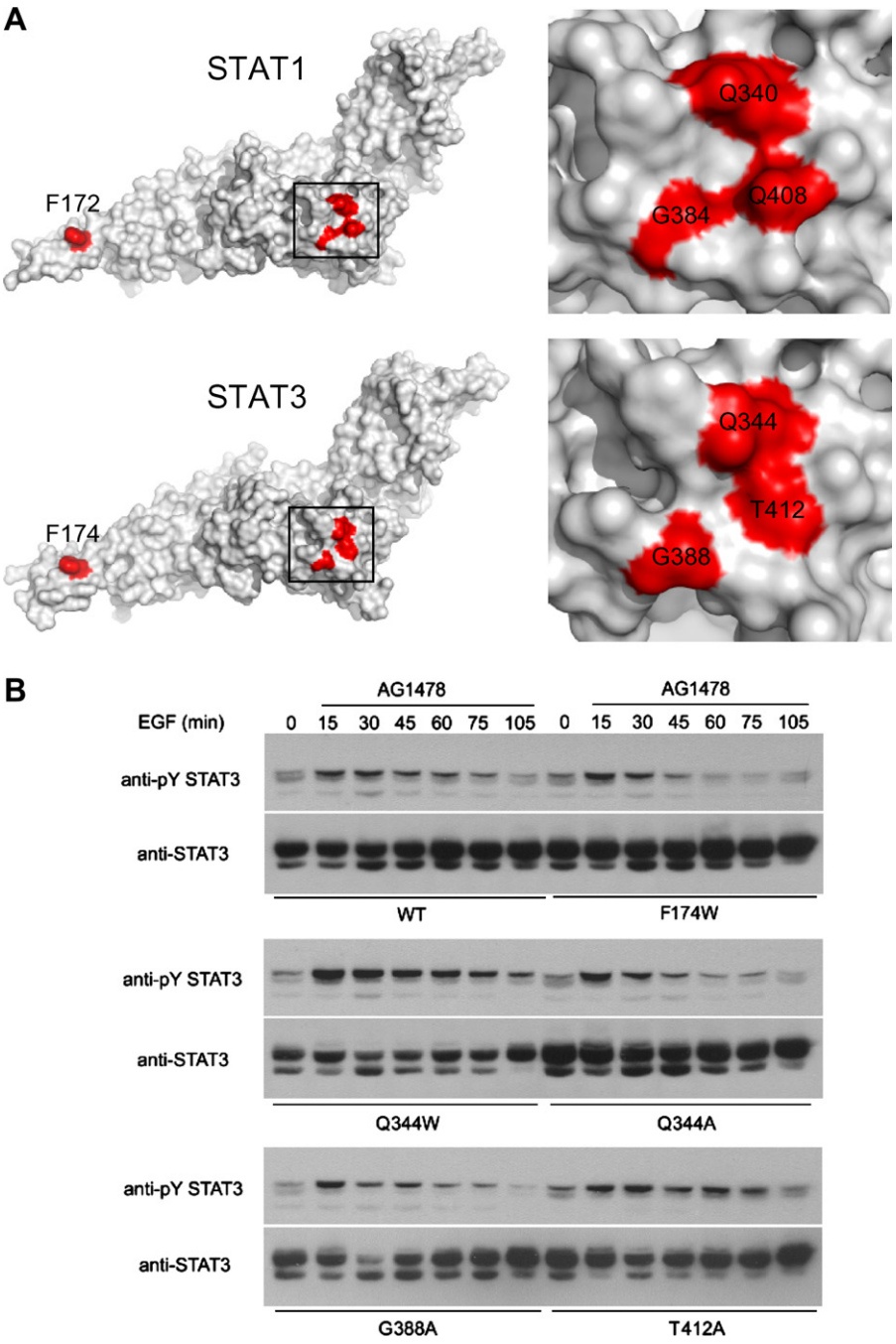

Fig. 2. (A) Structural diagrams showing the locations of the equivalent mutations in STAT1 and STAT3 core fragments, respectively. (B) Time course of tyrosine phosphorylation of wild-type and mutant STAT3α. COS-7 cells were transfected with STAT3 wild-type and mutants. At 48 h after transfection, cells were treated with 100 ng/ml EGF for 15 min, and then AG1478 was added to 10 μM. Cells were harvested at the times indicated (time marked are minutes after EGF stimulation). Top panel of each set shows Western blot with anti-STAT3 (pY705) antibody. The membranes were stripped and re-blotted with anti-STAT3 antibody (bottom).

length STAT3 in different buffers, including high-salt buffer (crystallization reservoir solution without PEG3350), low-salt buffer [high-salt buffer without $(NH_4)_2SO_4$], and phosphate buffered saline (PBS). As shown in Table 2, in all of these conditions, STAT3 (residues 127–688) was predominantly monomeric. However, its molecular mass in PBS was slightly higher than calculated. Considering that the p*I* of STAT3 (residues 127–688) is ~8, upon the pH change from 5.5 (high- and low-salt buffers) to 7.4 (PBS), the protein may tend to aggregate, leading to a higher apparent molecular mass. In comparison, full-length STAT3α is dimeric in low-salt buffer and PBS. In high-salt buffer, the apparent molecular mass is in between, suggesting a weakened dimer. All of these experiments were performed with a protein concentration of 1–2 mg/ml. We also used a much higher protein concentration (~50 mg/ml) for STAT3 (residues 127–688). In high and low salt buffers, the MW of the major peak is, respectively, 66.3 and 74.3 kDa. Also, as shown in Table 2, the oligomerization state of this protein is not affected by PM50D. Taken together, STAT3 (residues 127–688) exists primarily as a monomer in solution.

It was reported that a slightly longer construct, STAT3β (residues 127–722), existed as a monomer by gel filtration, whereas the phosphorylated form of the construct was dimeric [16]. Using MALS we confirmed those observations (K. Zhang and X.C., unpublished observations). In an analytical ultracentrifugation study, Braunstein et al. reported that STAT3β (residues 127–722) had "a significant increase of the presence of monomeric species", as compared to full-length STAT3 [17]. Since the N-domains of STATs can dimerize in a homotypic manner [18] and full-length STAT3 is a stable dimer [17], it is possible that the full-length dimer is mostly mediated by the N-domain interactions. This conclusion has been substantiated for STAT1 [4].

Mutations in STAT3 corresponding to those in STAT1 do not affect the course of phosphorylation in vivo

In STAT1, the interaction between CCD and DBD plays a role in the dephosphorylation of pY701 [6,7]. Mutations that disrupt the core fragment dimer interface (F172W, Q340W, G384A, and Q408W) lead to prolonged STAT1 tyrosine phosphorylation (up to 2 h) and insensitivity to nuclear phosphatase TC45. Fig. 2A shows the location of these residues in STAT1 (top panel). Upon dimerization between CCD and DBD the side chain of F172 is inserted into the "pocket" involving residues Q340, G384, and Q408. To examine whether an analogous interface exists in STAT3 in cells, we substituted the corresponding residues in full-length STAT3α: F174W, Q344A, Q344W, G388A, and T412A. The bottom panel of Fig. 2A shows the locations of these residues.

COS-7 cells were transiently transfected with each of these single mutants, stimulated with EGF, and then treated with an EGF receptor kinase inhibitor, AG1478. Cells were then harvested at defined time points and the levels of phosphorylated STAT3 were analyzed by Western blot. First we determined the half-life of phosphorylated STAT3 in this system to be approximately 20 min (data not shown). This result was consistent with the report of a previous study with a different inhibitor of EGFR kinase, PD157655 [19]. We then studied the time course of phosphorylation of each STAT3 mutant. As shown in Fig. 2B, STAT3 was phosphorylated upon stimulation with EGF for 15 min. Upon addition of AG1478, the phosphorylation level of STAT3 decreased and all returned back to pre-treatment level after 90 min. In summary, there was no significant difference in the phosphorylation time course between these mutants and wild-type STAT3 in COS-7 cells, suggesting that either the CCD:DBD does not exist in STAT3 or, if there is such an interface, its disruption does not give rise to the prolonged phosphorylation phenotype.

Recently, Zhang et al. reported that in NIH-3T3 cells ND-deleted STAT3 with a C-terminal FLAG tag responded similarly to the full-length protein (with FLAG) to IL-6 treatment. Also, in STAT3$^{-/-}$ MEF's, both constructs displayed similar dephosphorylation and nuclear accumulation kinetics [20]. Similar results were obtained earlier in COS-1 cells stimulated with EGF and HepG2 cells with IL-6 [21]. These results on STAT3 are drastically different from those of STAT1, suggesting a difference in the surface features of these two homologous proteins.

With regard to the STAT1 phenotype of prolonged tyrosine phosphorylation, it is interesting to note that the phenotype can be caused by four different mechanisms: (i) complete or partial removal of the ND [6,22]; (ii) mutations that either disrupt the ND dimer interface directly (e.g., F77A/L78A double mutant) or indirectly by presumably destabilizing the ND structure, e.g., R31A and E39A [22]; (iii) mutations that disrupt the CCD–DBD dimer interface (e.g., F172W, Q340W, G384A, and Q408W), and (iv) shortening of the flexible tether connection between the ND and the core fragment [6], which likely weakens one or both interfaces. One common feature in all these scenarios is that one or both of the dimer interfaces in the unphosphorylated STAT1 is compromised, either directly or indirectly. In this sense, STAT3 is different from STAT1. As demonstrated in this study and those of others [20,21], both STAT3 mutants corresponding to F172W and the "pocket" mutants in the STAT1 core fragment and the complete removal of the ND apparently have no significant effect on STAT3 tyrosine phosphorylation. STAT4 presents yet another intriguing situation: mutations disrupting the ND dimer interface lead to impaired tyrosine phosphorylation upon IFN-α stimulation [18]. It seems that although different STAT proteins share several characteristics, each has its unique structured and biochemical features. How these differences directly correlate with their specific biologic functions (e.g., STAT1 as a tumor suppressor vs. STAT3 as an oncogene) remains to be elucidated.

Acknowledgments

We thank Drs. James Holton and George Meigs (Beamline 8.3.1) of ALS for help with data collection. This work is supported by the grants from the NIH to X.C. (R01 GM068566) and J.S.M. (R01 CA096652). Z.R. is supported by a fellowship from the Odyssey Program and The Cockrell Foundation Award for Scientific Achievement at UTMDACC. The coordinates and structure factors have been deposited into the PDB (accession code 3CWG).

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