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Structure of human Fyn kinase domain complexed with staurosporine

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Abstract

The tyrosine kinase Fyn is a member of the Src kinase family. Besides the role of Fyn in T cell signal transduction in concert with Lck, its excess activity in the brain is involved with conditions such as Alzheimer's and Parkinson's diseases. Therefore, inhibition of Fyn kinase may help counteract these nervous system disorders. Here, we solved the crystal structure of the human Fyn kinase domain complexed with staurosporine, a potent kinase inhibitor, at 2.8 Å resolution. Staurosporine binds to the ATP-binding site of Fyn in a similar manner as in the Lck- and Csk-complexes. The small structural differences in the staurosporine-binding and/or -unbinding region among the three kinase domains may help obtaining the selective inhibitors against the respective kinases.

Keywords: Fyn; Kinase domain; Non-receptor tyrosine kinase; Src family; Staurosporine; Inhibitor; X-ray crystal structure

Human Fyn is a member of the Src family of non-receptor type tyrosine kinases that collectively are involved in the cytoplasmic signal transduction cascade for a variety of membrane receptors [1]. The function of the Src family kinases Fyn and Lck plays a critical role in proximal T-cell antigen receptor signal transduction [2]. Inhibitors of these kinases may contribute to anti-inflammatory therapy [3]. Recently it was reported that Lck is the major contributor in T-cell receptor-based signals [2] and Lck-selective compounds with low Fyn inhibitory activity prevent heart allograft rejection [4]. Besides the role of Fyn in T-cell development and activation, it is involved in myelination of neurons [5] and in disorders of the central nervous system. Fyn induces synaptic and cognitive impairments in a transgenic mouse model of Alzheimer's disease [6] and is essential for haloperidol-induced catalepsy, which resembles Parkinson's disease, in mice [7]. Therefore, selective inhibition of Fyn kinase may help in counteract these disorders without immunosuppressive side effects.

The Src family kinases are of a modular nature, consisting of a unique N-terminal sequence, three protein modules including the SH3, SH2, and kinase domains, and a C-terminal tail (Fig. 1). The modules play an important role in enzyme reactions [8]. Crystal structures of the SH2 and SH3 domains of the Fyn kinase reveal its binding specificity for peptide inhibitors [9,10]. The structure of the kinase domain is also attractive for production of Fyn kinase inhibitors.

We believe the present study to be the first report of the structure of the Fyn kinase domain complexed with staurosporine, a well-known kinase inhibitor, and discuss the selectivity of Lck and Csk kinases based upon comparison with the reported structures of each of the staurosporine complexes [8,11].

Materials and methods

Expression and purification. A truncated version of the Fyn kinase domain (residues 260–537) was inserted into the baculovirus expression vector pFastBac1, incorporating a C-terminal hexahistidine purification tag. The enzyme was overexpressed in the baculovirus expression system using *Sf21* insect cells. Log-phase *Sf21* cells were infected at a multiplicity

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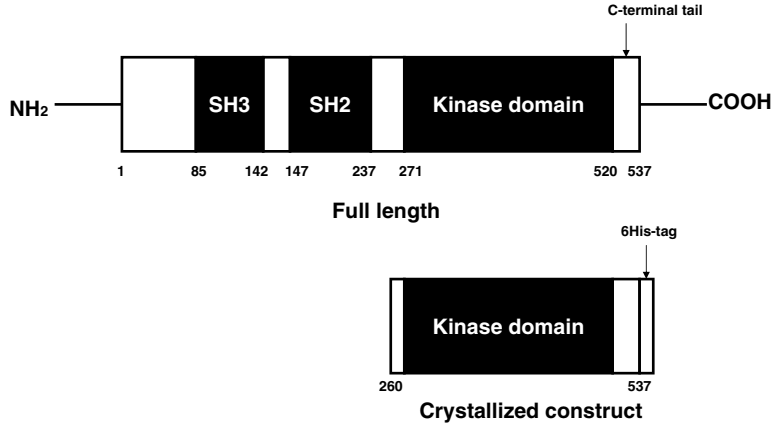

Fig. 1. Schematic representations of the intact Fyn tyrosine kinase and the crystallized kinase domain construct.

of infection of 1 and incubated for 48 h in a roller bottle, after which, the cells were collected by centrifugation, and the cell pellet washed with cold PBS and kept at −80 °C until used. Cell pellets were thawed on ice and resuspended in lysis buffer (50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole, 1% Nonidet P-40, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, and 2 μg/ml aprotinin). The suspension was cleared by centrifugation at 9000*g* for 20 min. All chromatographic steps were carried out at 4 °C. The supernatant was loaded on a His Trap HP column (GE Amersham Biosciences) using the AKTA prime system. After loading the supernatant, the column was washed in buffer (50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 20 mM imidazole, and 10% glycerol). Bound Fyn was eluted with elution buffer (50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 250 mM imidazole, 10% glycerol, and 5 mM DTT). Subsequent purification by size exclusion on a Superdex 200 column and Mono-Q column (GE Amersham Biosciences) yielded a homogeneous sample suitable for crystallization.

Crystallization and data collection. Purified Fyn was concentrated to 3 mg/ml against 20 mM Tris/HCl buffer, pH 8.0, 0.25 M NaCl, and 5 mM DTT. The Fyn–staurosporine complex solution was prepared by direct suspension of the inhibitor into the protein solution and incubated at 4 °C until the crystallization experiments. Hexagonal-plate crystals of the complex were obtained at 4 °C using a reservoir solution of 1.15 M ammonium phosphate dibasic, 0.2 M NaCl, and 0.1 M imidazole buffer, pH 8.0. After dipping into Paratone-N oil (Hampton Research), the crystals were frozen using a nitrogen gas stream at 100 K. Diffraction data were collected at a wavelength of 1.0 Å using the synchrotron radiation at Photon factory beamline NW12A and a CCD detector Quantum 210 (ADSC) with an exposure time of 10 s per image at a crystal to detector distance of 250 mm. X-ray diffraction data consisting of the 360 images were processed and scaled using the program HKL2000 (HKL).

Structure analysis. The structure of the complex was resolved and refined using the program AMoRe [12]. A starting model was obtained by homology modeling from the Lck structure [10] using the program DS MODELLER (Accelrys). After translation function computation, the correlation was 49% and the *R* value was 45%. At this stage, $F_{obs} - F_{calc}$ Fourier maps were calculated. These maps fitted the initial model without the C-terminal 20 amino acids, including the hexahistidine-tag. After removing the amino residues, the recalculated $F_{obs} - F_{calc}$ Fourier maps showed clear electron density for the inhibitor staurosporine at the active site. Model building and map fitting were performed using the program DS Modeling (Accelrys). Further refinement involved iterative manual rebuilding and maximum-likelihood refinement using the program CNS [13]. Coordinates of the final model have been deposited in the Protein Data Bank (PDB Accession No. 2DQ7). Details of the data collection and refinement are shown in Table 1. Structure comparisons with the Lck and Csk structures (PDB accession codes 1QPD and 1BYG, respectively) were performed using the program DS Modeling (Accelrys).

Table 1
Data collection and refinement statistics

Data collection	
Space group	$P3_221$
Unit cell (Å)	$a = 52.96$
	$c = 210.99$
Resolution (Å)	50–2.80
Observations	93294
Unique reflections	9281
Completeness (%)	99.9 (99.7)
R_{merge} (%)[a]	12.7 (33.1)
I/σ	35.8 (6.2)
Refinement statistics	
Resolution (Å)	50–2.80
Reflections	9281
Total atoms	2286
R-factor (%)	25.5 (32.6)
R_{free} (%)	28.1 (32.8)
R.m.s. deviations	
Bond length (Å)	0.008
Bond angle (°)	2.0

Values in parentheses are for the highest resolution shell.

[a] $R_{merge} = \sum_h \sum_j |I_{hj} - \langle I_h \rangle| / \sum_h \sum_j |I_{hj}|$, where *h* represents a unique reflection and *j* represents symmetry-equivalent indices. *I* is the observed intensity and $\langle I \rangle$ is the mean value of *I*.

Kinase assay. The kinase reactions were conducted in a 40 μl volume containing Fyn and 250 nM biotinylated gastrin peptide as substrate in 15 mM Tris/HCl, pH 7.5, 0.01% Tween 20, 20 mM $MgCl_2$, 20 μM ATP, and 2 mM DTT. Following incubation of 30 min at room temperature, the kinase reactions were terminated by adding 40 μl of 200 mM EDTA. Reaction mixture was then transferred to a streptavidin (SA)-coated 96-well plate (Perkin-Elmer). The wells were then washed four times, thoroughly, with 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, and 0.02% Tween 20 (wash buffer). To reduce background, SA-coated plates, containing captured substrate, were blocked for 30 min at room temperature with 0.1% BSA in wash buffer. Following blocking of non-specific binding, an HRP-conjugated mouse anti-phospho tyrosine (PY20) antibody (Santa Cruz) was added to each well and incubated for 30 min at room temperature. The antibody incubation was followed by a washing step as described before, after which, 3,3′,5,5′-tetramethylbenzidine (TMB) substrate was added and incubated for 5 min. The HRP–TMB reaction was terminated upon addition of an equal volume of 0.2 N H_2SO_4. Absorbance was read at 450 nm with a SpectraMAX microplate reader (Molecular Devices). The background signal was determined by incubating with a non-ATP control and subtracting. For inhibitor experiments,