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Structural characterization of 14-3-3ζ in complex with the human Son of sevenless homolog 1 (SOS1)

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A B S T R A C T
The deviant Ras activation machinery is found in approximately 30% of all human cancers. SOS1 is an important protagonist of this pathway that plays a key-role in aberrant cell proliferation and differentiation. Interaction of SOS1 with 14-3-3 proteins modulates SOS1 activity in Ras-MAPK signaling. In the present study, we analyze the 14-3-3/SOS1 protein-protein interaction (PPI) by different biochemical assays and report the high resolution crystal structure of a 13-mer motif of SOS1 bound to 14-3-3ζ. These structural and functional insights are important for the evaluation of this PPI interface for small-molecule stabilization as a new starting point for modulating the Ras-Raf-MAPK pathway.

1. Introduction
Ras is a small GTP-ase that regulates the mitogen-activated protein (MAP) kinase pathways by coupling cell surface receptors to intracellular signaling involved in the control of cell proliferation and differentiation (Boguski and McCormick, 1993). The activation of Ras is mediated by a variety of regulatory proteins such as guanine nucleotide exchange factors (RasGEFs) that catalyze the exchange of GDP (inactive state) for GTP (active state). The full activation of Ras is synchronized by the binding with the Ras guanine-nucleotide exchange factor Son of sevenless (SOS) (Bar-Sagi, 1994). The membrane recruitment of SOS starts via the association of the adapter protein Grb2 which binds phospho-tyrosine motifs on the activated receptor (Aronheim et al., 1994). Subsequently, SOS binds Ras via an allosteric conserved pocket of SOS corresponding to the domain from residue 605–1068 (Margarit et al., 2003) leading to sequential activation of RAF, MEK and ERK kinases. One third of human cancers involve Ras mutations (Simanshu et al., 2017); in particular, the deviant activation of Ras via SOS binding can be found in different human cancers (Stephen et al., 2014) such as pancreatic cancer (Karnoub and Weinberg, 2008) (Schubbert et al., 2007).

14-3-3 proteins are a family of conserved regulatory proteins expressed in all eukaryotic organisms; in human cells there are seven 14-3-3 isoforms β, γ, s, η, ζ, τ, σ (Aitken, 2006) (Mhawech, 2005). They have the capability of binding several hundred signaling proteins (Babula and Liu, 2015) and regulate their physiological effects by modulating their subcellular localization, enzymatic activity or their ability to interact with other proteins (Aghazadeh and Papadopoulos, 2016; Bier et al., 2016).

Many of the several hundred 14-3-3 interaction partners characterized are disease-relevant proteins involved in key cellular processes (Aghazadeh and Papadopoulos, 2016). Recent studies have shown that the ribosomal S6 kinase (RSK), component of the Ras-MAPK signaling, phosphorylates SOS homolog 1 (SOS1) in vivo at Ser1134 and Ser1216 (Saha et al., 2012) and this mechanism creates 14-3-3 binding sites on SOS1. The binary complex 14-3-3/SOS1 might reduce SOS1 catalytic activity or prevent its interaction with Ras. Alternatively, 14-3-3 might decrease the ability of SOS1 to bind to the plasma membrane and, in this way, attenuate Ras-MAPK signaling. Along these lines, 14-3-3 plays a key-role on the relocation of SOS1 to the cytosol, thus the interaction between 14-3-3 and SOS1 might be fundamental to downstream signaling proteins that are responsible for uncontrolled cell growth. In this context, the structural characterization of 14-3-3 binding sites in human SOS1 could be the starting point for the development of new therapeutic strategies to interfere with irregular cell proliferation and differentiation.

In the present study, biochemical assays and X-ray crystallography were carried out to provide structural and functional information about the 14-3-3-binding site surrounding SOS1pSer1216.

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2. Materials and methods

2.1. Peptide synthesis

The following sequence was used: Son of sevenless homolog 1 ([Homo sapiens] (UniProt identifier: Q07889-1) 1155-PRRRPE{pSer1161}APAESS-1167 (SOS1pSer1161). Both labeled and unlabeled synthetic peptides used in this study were synthetized using solid-phase peptide synthesis (SPPS) technique with Fmoc chemistry, on an automated Intavis MultiPep RSi peptide synthesizer. Protected amino acids and chemicals were purchased from Novabiochem and Sigma-Aldrich. The resin Rink amide AM resin (Novabiochem; 0.59 mmol/g loading) was used for the synthesis of the phosphorylated peptide. Preceding the deprotection, the resin for each peptide was divided into two portions. Fluorescein isothiocyanate (FITC) (Sigma-Aldrich) was used to label one portion to perform Fluorescence Polarization measurements (FP). The remainder of the resin was used to obtain the (unlabeled) peptide for Isothermal Titration Calorimetry (ITC), Differential Scanning Fluorimetry (DSF) assays and crystallization studies; the peptide was acetylated at the N terminus (1:1:3 Acetic anhydride/pyridine/NMP) before resin cleavage. Crude peptides were analyzed and purified by high pressure liquid chromatography (HPLC) using a preparative reverse phase column with MS detection. After purification, peptides were freeze dried and stored at 243 K.

2.2. 14-3-3 expression and purification

All seven 14-3-3 isoforms, both full length and C-terminally truncated (ΔC, devoid of the last 18C-terminal residues), were expressed using BL21(DE3) cells employing pPROEX HTb expression plasmids and purified via a nickel column. After purification, for the truncated isoforms, the His6-tag was cleaved with TEV protease and a second purification was performed by size-exclusion chromatography as described previously (Schumacher et al., 2010). Each isoform was concentrated to 60 mg/ml, aliquoted, flash-frozen in liquid nitrogen and stored at 193 K.

2.3. Fluorescence polarization (FP) assay

FP measurements were performed at room temperature and with a 1 h incubation. The FP buffer consisted of 0.1 M Hepes, 1.5 M NaCl, 0.1% Tween-20, 1 mg/mL BSA, Milli-Q, pH 7.4. The FITC labeled peptide was dissolved in Milli-Q to a final concentration of 100 nM. Dilution series of all the 14-3-3 isoforms were performed on Corning black round -bottom 384-well plates. The affinity of this peptide against the different isoforms was determined on a Tecan Infinite F500 plate reader using a 485 nm excitation and 535 nm emission filter. FP values are expressed in millipolarization (mP) units. All statistical
analyses were performed with GraphPad Prism 5 (GraphPad Software, San Diego, USA) using a non-linear regression analysis method (single site binding model) in Prism 5.0.

2.4. Isothermal titration calorimetry (ITC) assay

ITC experiments were carried out on a Malvern MicroCal iTC200. In the cell, a solution of 0.035 mM 14-3-3β was placed and titrated by two repetitions of a series of 19 2-μL aliquots of 0.35 mM solution of SOS1pSer1161 at 25 °C (reference power 5 μCal/s; stirring speed 750 rpm). Both protein and peptide were dissolved in ITC buffer containing 25 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl2, 0.5 mM TCEP. Raw Data were analyzed with Origin 7.0 software for ITC data analysis; baseline corrections were done manually. By a single binding site model, the binding isotherm was determined together with the thermodynamics parameters: the association constant $K_a$ ($K_d = 1/K_a$), molar binding stoichiometry (N) and molar binding enthalpy ($\Delta H$) isotherm.

Fig. 2. Crystal structure of SOS1pSer1161 peptide bound to 14-3-3ζ dimer. (A) Crystallographic organization consisting of 4 monomers of 14-3-3ζ bound to SOS1pSer1161 peptide. The 14-3-3ζ dimer shows the typical W-like shape with the phospho peptides bound to the amphipathic groove. (B) View of monomer B (pink surface) turned by 90° around the y-axis. SOS1pSer1161 peptide (cyan rods) is depicted and fills about three fourth of the amphipathic groove. The final electron density map of the peptide is shown (2Fo-Fc, white mesh, contoured at 1σ). (C) Polar contacts (black dashed lines) and hydrophobic interactions (wireframe white spheres) between the residues of 14-3-3ζ monomer B (red rods and pink cartoon) and the pSer1161 binding site of SOS1 (cyan rods). See Table 1 for data collection and refinements.
The observed and calculated structure factors. $R_{\text{free}}$ is calculated as for $R_{\text{work}}$, but from a Microsoft Excel. Data analysis was performed as described by Nielsen A. Ballone et al.

measurement of re
molecular replacement using PDB ID: 1QJB as the search model. The with an oscillation of 0.25° per image. The structure was solved by
of Hamburg (Germany). For the high-resolution setting the crystal-to-
dataset of 1.9 Å was collected at the Deutsches Elektronen-Synchrotron
2.6. Crystallization of the binary complex

To obtain the 3D structure of the 14-3-3/SOS1pSer$^{1161}$ peptide complex, protein and peptide were mixed in a 1:2 M ratio to a final concentration of 10 mg/mL in 140 mM 1.5x SYPRO orange in 100 mM Heps, pH 7.5, 150 mM NaCl used as control and 3 other replicas together with 200 μM SOS1pSer$^{1161}$ to determine the affinity protein-peptide. The samples were heated from 25 °C to 95 °C with increments of 1 °C/minute, and fluorescence was measured at each step. To obtain the melting temperature, the data analysis was performed in Microsoft Excel. Data analysis was performed as described by Nielsen et al. (Niesen et al., 2007).

2.6. Crystallization of the binary complex

To obtain the 3D structure of the 14-3-3/SOS1pSer$^{1161}$ peptide complex, protein and peptide were mixed in a 1:2 M ratio to a final concentration of 10 mg/mL in 100 mM Heps, 2 mM MgCl$_2$, 2 mM DTT, pH 7.5 and incubated at 4 °C overnight. Protein/peptide complexes were initially set-up for crystallization in 0.1 M phosphate citrate pH 4.2, 40%

(v/v) PEG 300. For reproduction and optimization, 36% (v/v) PEG 300 was used. Crystals grew within a week at room temperature and could be directly flash-cooled in mother liquor using liquid nitrogen. A dataset of 1.9 Å was collected at the Deutsches Elektronen-Synchrotron of Hamburg (Germany). For the high-resolution setting the crystal-to-detector distance was set to 0.322 m and 1440 images were obtained with an oscillation of 0.25° per image. The structure was solved by molecular replacement using PDB ID: 6F08 as the search model. The obtained second model was subjected to iterative rounds of model building and refinements using Coot (Emsley et al., 2010), REFMAC (Murshudov et al., 1997) and Phenix (Adams et al., 2010). Figures were created using VMD (Humphrey et al., 1996). Hydrogen bonds and hydrophobic contacts were established by the schematic diagram protein-
ligand interactions generated by LIGPLOT program (Wallace et al., 1995). The structure was deposited in the Protein Data Bank under PDB ID: 6F08.

3. Results

3.1. Binding of SOS1pSer$^{1161}$ to 14-3-3

Since the previous data suggested a classical phosphoserine 14-3-3 binding motif around residue 1161 (Saha et al., 2012), we synthesized the motif with the phosphorylated serine 1161 in the center surrounded by six amino acids of the natural sequence on each side to investigate the binding of this motif to all human 14-3-3 isoforms. Our biophysical cascade consisted of the use of FP, ITC and DSF. FP was chosen as the assay format to consider 14-3-3 isoform dependence. The 13-mer peptide was labeled on the N-terminus with FITC and used as a tracer to measure pSer$^{1161}$ binding. The result from the assays showed the motif to be a strong binder to all seven isoforms (Fig. 1a) with Kd values typically in the low micromolar range. 14-3-3ζ binds the strongest, followed by β, γ, ε, τ, ζ, and ξ respectively (Table 1). ITC measurements on the peptide showed the same trend in binding to 14-3-3ζ as observed in FP assay characterized by a Kd of 5.29 ± 0.56 μM (Fig. 1b). Additionally, by DSF we measured the effect of the 13-mer peptide on the melting temperature of 14-3-3ζ and we observed a significant positive shift of about ΔT_m = 3.82 ± 0.12 °C (Fig. 1c).

To investigate the molecular details of the protein/peptide interaction we employed X-ray crystallography and solved the crystal structure of SOS1pSer$^{1161}$ in complex with 14-3-3ζ. Crystallization experiments were initiated with commercial crystal screens including JCSG core suite – from I to IV – (Qiagen) and JCSG plus (Qiagen), all containing 96 different buffer conditions. The Mosquito robot was utilized to create the hanging drops containing the protein solution and crystallizing buffer in 1:2 ratio. For the initial screening we extensively used 14-3-3 α, γ at different concentrations. After protein and buffer administration, the plates were sealed and stored in parallel at room temperature as well as at 2–8° storage. Crystal formation was observed daily and later monthly; for 14-3-3ζ we found suitable crystals yielded within one week at room temperature.

The protein data structure was solved to a resolution of 1.9 Å [PDB ID code 6F08] (Fig. 2) using the PDB 1QJB as a template for the molecular replacement run. Interpretable density for 9 out of 12 amino acids of the SOS1pSer$^{1161}$ peptide was found (Fig. 2b and c). For 14-3-3ζ we found interpretable density for 226 out of 231 residues. Data and refinement statistics are summarized in Table 2. As expected from previously solved 14-3-3 structures, the protein crystallizes as a dimer with the typical W-like shape (Fig. 2a) showing that two phosphorylated binding motifs are simultaneously bound to one 14-3-3 dimer composed by eighteen helices. The pS$^{1161}$ is located in the typical binding groove establishing hydrogen bonds to Lys49 (2.90 Å), Arg56 (2.91 Å), Arg127 (2.87 Å), Tyr128 (2.94 Å) (Fig. 2c). Outside of this pocket at the position of Glu1165 (Fig. 2b). In this context, the mode II binds the strongest, namely between Pro165, Ala1162 and 14-3-3ζ’s Leu172 and 14-3-3ζ’s Pro165, Ala1162 and 14-3-3ζ’s Leu172 plus Glu$^{1169}$ and 14-3-3ζ’s Val$^{176}$.

Although the SOS1pSer$^{1161}$ peptide used contains a proline at the +2 position, which would suggest that the peptide chain exits the binding groove (Schumacher et al., 2010) the peptide chain returns into the pocket at the position of Glu$^{1165}$ (Fig. 2b). In this context, the mode II binding motif would seem to be more of interest because on comparison with other structures such as 5NAS (Fig. 3; (Boura and Eisenreichova, n.d.) the SOS1pSer$^{1161}$ peptide shows a comparable mode II binding motif (Fu et al., 2000).
4. Discussion

Structurally, all seven 14-3-3 isoforms share a high degree of similarity, however their functionality and their contribution to different types of cancers is very diverse and their role in pathogenesis has not been completely understood yet (Stevers et al., 2017) (Cau et al., 2017). For this reason, a better functional and mechanistic understanding of 14-3-3 PPIs might help for the development of bioactive tool compounds and ultimately new drugs in targeted cancer therapy. Achieving this goal relies, as first starting point, on elucidating structures of distinct isoforms that binds new protein binding partners; within this context, we might be able to design possible therapeutic approaches related to 14-3-3 (Zhao et al., 2011) by expanding the knowledge of the 14-3-3 PPI network (Ottmann, 2013).

Although in vitro studies established the phosphorylation of SOS1 via RSK (Saha et al., 2012) as well as the possible binding of 14-3-3 to SOS1 in response to the negative feedback control in RAS-MAPK signaling, the interaction between SOS1 and 14-3-3 remains poorly understood. In this sense, it was useful to determine the exact 14-3-3 binding site(s) of SOS1, a notion of importance for further studies on the modulation of PPI.

Biochemical assays of the 13-mer with all isoforms of 14-3-3 showed that there is no specific preference among 14-3-3 isoforms and the short, SOS1-derived peptide (Fig. 1); moreover the electrostatic interactions found by X-ray crystallography indicates that the high-affinity binding of this complex is dependent on phosphorylation of pSer\textsuperscript{1161}.

To qualify as a potential 14-3-3 binding site, the criteria described previously have been applied (Rose et al., 2012): a serine or threonine has to be present, and the site has to be accessible for both phosphorylation by a kinase as well as interaction with 14-3-3.

For practical reason, 14-3-3\textsubscript{ζ} has been used in crystallography experiments besides the fact this isoform is the most reported in the 14-3-3 literature. As the binding groove is highly conserved among the different isoforms (Aitken, 2006), 14-3-3\textsubscript{ζ} should therefore provide useful information of the binding between SOS1 and 14-3-3. In this study, the crystal structure shows that the SOS1 peptide bind in the conserved amphipathic groove of 14-3-3\textsubscript{ζ} as most of the structure solved as far (Fig. 3) (Yaffe et al., 1997).

The same X-ray protein crystallography screening procedure has been performed for the SOS1pSer\textsuperscript{1134} peptide, shown in the literature to be phosphorylated by RSK and interacting with 14-3-3 (Saha et al., 2012). However, no crystals could be observed in any of these conditions. For this reason we focused here on investigating the SOS1pSer\textsuperscript{1161} peptide/14-3-3\textsubscript{ζ} interaction.

In this framework, the complex structure 14-3-3\textsubscript{ζ}/SOS1pSer\textsuperscript{1161} would provide an initial first step for investigating and manipulating the binding mechanisms of binding of SOS1 to 14-3-3, in particular with small molecules identified, for example, by fragment-based approaches (Sijbesma et al., 2017) to stabilize the 14-3-3\textsubscript{ζ}/SOS1 interface. Because of the structural rigidity 14-3-3 proteins, X-ray crystallography is an useful method for the design and the optimization of new small molecules modulators (Mori et al., 2013).

In a more biological context, it has been demonstrated that the modulation of 14-3-3 PPIs (Bartel et al., 2014; Doveston et al., 2017; Hartman and Hirsch, 2017) in particular by natural products and their derivatives (Camoni et al., 2011; de Boer and de Vries-van Leeuwen, 2011).
2012; Molzan et al., 2013; Anders et al., 2013, Stevers et al. 2016), can induce the desired effect on the activity, dimerization, or cellular localization of the binding partner. In addition, the chemical toolbox to target 14-3-3-ss has been extended by modified peptides (Glás et al., 2014; Milroy et al., 2015), supramolecular ligands (Bier et al., 2013, 2017), and fragments (Sijbesma et al., 2017). Since the stabilization of the negative regulation of 14-3-3 to oncogenic SOS1 could be a valuable new therapeutic approach in certain cancer, this study could represent the starting point towards a pharmacological intervention in tumor cells with aberrant Ras-MAPK signaling.

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