Biophysical and structural insight into the USP8/14-3-3 interaction

Citation for published version (APA):

DOI:
10.1002/1873-3468.13017

Document status and date:
Published: 01/04/2018

Document Version:
Accepted manuscript including changes made at the peer-review stage

Please check the document version of this publication:

• A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher’s website.
• The final author version and the galley proof are versions of the publication after peer review.
• The final published version features the final layout of the paper including the volume, issue and page numbers.

Link to publication

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the “Taverne” license above, please follow below link for the End User Agreement:
www.tue.nl/taverne

Take down policy
If you believe that this document breaches copyright please contact us at:
openaccess@tue.nl
providing details and we will investigate your claim.

Download date: 25. Apr. 2020
Biophysical and structural insight into the USP8/14-3-3 interaction

Federica Centorrinoa, Alice Ballonea, Madita Woltera, Christian Ottmannab,*

a Laboratory of Chemical Biology, Department of Biomedical Engineering and Institute for Complex molecular Systems, Eindhoven University of Technology, The Netherlands

b Department of Chemistry, University of Duisburg-Essen, Universitätsstr. 7, 45117 Essen, Germany

Eindhoven University of Technology, P.O. Box 513, 5600 MB, Eindhoven, The Netherlands.
Tel: +31 40 247 2835
Email: c.ottmann@tue.nl

Abstract

The USP8/14-3-3 protein-protein interaction has recently been shown to exert a significant role in the pathogenesis of Cushing’s disease (CD). USP8 is a deubiquitinase that prevents epidermal growth factor receptor (EGFR) degradation. Impairment of 14-3-3 binding leads to a higher deubiquitination of EGFR and results in a higher EGFR signaling and an increased production of adrenocorticotropic hormone (ACTH). Here we report the high-resolution crystal structure of the 14-3-3 binding motif of USP8 surrounding Ser718 in complex with 14-3-3ζ and characterize the interaction with fluorescence polarization and isothermal titration calorimetry. Furthermore, we analyze the effect of USP8 mutations identified in CD on binding to 14-3-3.

Keywords

X-ray crystallography; fluorescence polarization; isothermal titration calorimetry; 14-3-3 proteins; ubiquitin specific protease 8

Abbreviations

CD, Cushing’s disease; USP8, ubiquitin specific protease 8; ACTH, adrenocorticotropic hormone; EGFR, epidermal growth factor receptor; DUB, deubiquitinating enzyme; FP, fluorescence polarization; ITC, isothermal titration calorimetry

Introduction

Pituitary corticotroph adenomas, hypersecreting ACTH, are the most common cause of Cushing’s disease (CD) [1]. The chronic high level of ACTH stimulates the adrenal gland to produce an excess of glucocorticoids, inducing the typical manifestations of the syndrome such as weight gain, disturbed mood, decreased bone mineral density, impaired reproductive function, myopathy and weakening of the immune response. The first line treatment is transsphenoidal surgical resection (TSS) of the pituitary adenomas, with a success rate of 65-90% [2]. The molecular basis of the disease had been largely unknown until recently. Mutations in genes that cause syndromes associated with pituitary adenomas, such as multiple endocrine neoplasia type 4 (CDKN1B), multiple endocrine neoplasia type 1 (MEN1), familiar isolated pituitary adenomas (AIP) and Carney complex (PRKAR1A), do not account for the majority of corticotropinomas [3, 4]. The ubiquitin-specific protease 8 (USP8) is a deubiquitase implicated in the lysosomal trafficking of the epidermal growth factor receptor (EGFR). Specifically, by removing ubiquitin from activated EGF, it impedes the receptor downregulation [5]. USP8 activity is usually inhibited by binding to 14-3-3 proteins, after phosphorylation of serine 718 [6]. A recent study from Reincke et al. has highlighted the role of mutations in the USP8 gene in the pathogenesis of CD [7]. The mutations identified in patients with CD cluster in the 14-3-3 binding motif of the USP8 gene and lead to an impaired binding to 14-3-3. This results in an increased activation of EGFR signaling and subsequent induction of proopiomelanocortin (POMC) transcription and ACTH secretion [8]. Mutations in the USP8 gene have been identified in around one third of both adult and pediatric population affected by CD and have been connected to a higher rate of tumor recurrence after surgical resection of the pituitary adenomas [9, 10].

This work focuses on the elucidation of the structural basis of the 14-3-3-USP8 protein-protein interaction (PPI). The 14-3-3 family of proteins is a class of small (25-30 kDa) eukaryotic adapter proteins with seven isoforms in humans, identified
with the Greek letters β, γ, ε, η, σ, τ, ζ [11]. They influence different physiological processes, including cell-cycle regulation, signal transduction, protein trafficking and apoptosis [12]. 14-3-3 proteins interact with several hundreds of partner proteins via short phosphorylated sequences, modulating their partners’ subcellular localization, enzymatic activity or their ability to interact with further proteins [13]. In the present study, we report a biophysical characterization and a structural elucidation of the complex formed by the motif surrounded USP8 Ser718 and 14-3-3ζ protein. Additionally, we provide a biophysical evaluation of the effect of the mutations identified in CD in the binding motif to 14-3-3. These findings might provide valuable insights for further investigations on this important PPI.

Materials and methods

Peptide synthesis

USP8pS718 peptide was synthesized via Fmoc solid-phase peptide synthesis (SPPS), making use of an automated Intavis MultiPep Rsi peptide synthesizer and a Rink amide AM resin (Novabiochem; 0.59 mmol/g loading). The peptide used for ITC measurements and crystallography was acetylated at the N-terminus (1:1:3 Ac2O/pyridine/DMF) before resin cleavage.

The peptide used for FP assay was labeled with FITC (Sigma-Aldrich) attached to a beta-Alanine linker. The peptides were purified by preparative reversed-phase HPLC with MS detector. Detailed synthetic procedures and final LC-MS run (Figure S2) are described in the Supporting Information.

Materials and methods

Peptide synthesis

USP8pS718 peptide was synthesized via Fmoc solid-phase peptide synthesis (SPPS), making use of an automated Intavis MultiPep Rsi peptide synthesizer and a Rink amide AM resin (Novabiochem; 0.59 mmol/g loading). The peptide used for ITC measurements and crystallography was acetylated at the N-terminus (1:1:3 Ac2O/pyridine/DMF) before resin cleavage.

The peptide used for FP assay was labeled with FITC (Sigma-Aldrich) attached to a beta-Alanine linker. The peptides were purified by preparative reversed-phase HPLC with MS detector. Detailed synthetic procedures and final LC-MS run (Figure S2) are described in the Supporting Information. USP8 mutant peptides Ser718del, Ser718Cys, Ser718Pro, Pro720Arg and [Leu713Arg;Tyr717Cys] were purchased from GenScript (Hong Kong) in purity higher than 95%.

14-3-3 expression and purification

His-tagged 14-3-3 isoforms full-length and 14-3-3ΔC (C-terminally truncated after T234) were expressed in NiCo21 (DE3) competent cells via a pPROEX HTb plasmid and purified using nickel-affinity chromatography. The proteins were dialyzed against ITC buffer before usage. The ΔC variant for crystallography was treated with TEV-protease to cleave off the His6-tag, followed by a second nickel-affinity chromatography and a size exclusion chromatography.

Fluorescence polarization assay

The FITC labeled peptides were dissolved in FP buffer (10 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Tween20, 1.0 mg x mL⁻¹ BSA) to a final concentration of 100 nM. Dilution series of 14-3-3 isoforms were made on Corning black, round-bottom, low-binding 384-well plates. Their polarization was measured in presence of 100 nM peptide, with a Tecan Infinite F500 plate reader (excitation: 485 nm; emission: 535 nm). FP data were analyzed using GraphPad Prism 5 software (GraphPad Software, San Diego, USA). Detailed procedures for 14-3-3 titration are described in the Supporting Information. See Figure S8 for a comparison between the affinities of the full length construct (used for the binding assays) and the C-terminal truncated construct (used for crystallography).

Isothermal titration calorimetry

Isothermal titration calorimetry experiments were performed using a Malvern MicroCal ITC200 instrument (Malvern Instruments Ltd., Malvern, UK) in ITC buffer (25 mM HEPES pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 0.5 mM TCEP). The USP8pS718 peptide (0.45 mM) was titrated (2 x 18 injections of 2 μL) to 14-3-3β (0.060 mM). Two repetition series were performed at 25 °C (reference power: 5 μCal/sec., initial delay: 60 sec., stirring speed: 750 rpm, spacing: 180 sec) and the data were merged using ConCat32 software (Malvern Instrument Ltd.). The association constant (K_a= 1/K_d), the molar binding stoichiometry (N), the molar binding enthalpy (ΔH) and entropy (ΔS) were determined by fitting the binding isotherm by a single binding site model, using Origin software (OriginLab Corporation, Northampton, MA, USA). The data presented
are the mean of three replicates (Figure S1). More detailed experimental procedures are described in the Supporting Information.

**Protein crystallization and structure elucidation**

Crystals of the 14-3-3ζ / USP8pS718 binary complex were grown by mixing 12.5 mg/ml 14-3-3ζ in a molar ratio of 1:2 with USP8pS718 peptide, in 20 mM Hepes pH 7.5, 2mM MgCl$_2$, and 2mM BME, and incubating overnight at 277 K. The complex was then set up for crystallization using the hanging drop method by mixing 1:1 with 0.1M phosphate citrate pH 4.2, 40% PEG 300. For reproduction and optimization 0.1M phosphate citrate pH 4.4 and 40% PEG 300 were used. Crystals were harvested after 1 week incubation at room temperature and directly flash cooled in liquid nitrogen. Data collection was performed at the Swiss Light Source, beamline PXII, detector Pilatus 6M. A total set of 720° was obtained with an oscillation of 0.25° per image. Data were indexed and integrated using iMosflm [14], and scaled and merged using AIMLESS [15]. Molecular replacement was carried out using MOLREP [16] and 4HKC (wwpdb.org) as a template. The electron density of the peptide without the amino acids modelled in is shown in Figure S5. The obtained model was subjected to reiterative rounds of model building and refinement using COOT [17], REFMAC [18] and PHENIX [19]. PHENIX software was used for the final round of model refinement. See Table S1 for further details of the crystallography data and refinement statistics. The protein-peptide interactions were analyzed using LigPlot software (University College London, United Kingdom), see Figure S6 [20]. Figures were prepared using VMD software [21].
Results

Binding of the USP8pS718 peptide to 14-3-3

The peptide (T12KLKRSypSSPDITQ22), corresponding to the USP8 binding domain to 14-3-3, was synthesized with the phosphorylated serine 718 surrounded by six amino acids of the natural sequence on each side. After N-terminal labeling with fluorescein isothiocyanate (FITC), fluorescence polarization (FP) measurements were performed to analyze the binding of the labeled peptide to 14-3-3 isoforms. The results showed that the peptide binds 14-3-3 with strong affinity, with Kd in the order of one micromolar. In particular, 14-3-3η and γ bind with the strongest affinities (Kd = 0.42 ± 0.02 μM and Kd = 0.48 ± 0.02 μM respectively), followed by β, ζ, τ, ε, and σ (Kd = 3.27 ± 0.11μM) (Figure 1B).

Isothermal titration calorimetry (ITC) experiments of the unlabeled USP8pS718 peptide were performed against 14-3-3β. A Kd of 1.16 ± 0.15 μM was measured, confirming the value observed in FP (Figure 1C).

Crystal structure of the 14-3-3/USP8pS718 complex

In order to elucidate the structural basis of the USP8/14-3-3 interaction, the USP8pS718 peptide was co-crystallized with 14-3-3ζ and the structure solved to a resolution of 1.6 Å [Protein data bank (PDB) code 6F09]. The crystals are in the triclinic space group P1. Each singly phosphorylated peptide is bound to a 14-3-3 monomer in its conserved amphipathic groove with an extended conformation, covering the whole length of this groove (Figure 2A and S7). The structure shows...
four crystallographically independent views of the interaction between a 14-3-3ζ monomer and the USP8pS718 peptide. The analysis of the symmetry-related molecules of the crystal lattice allows to identify the physiological dimers typical of 14-3-3 proteins (Figure S3). Figure 2B displays one of the monomers in which it was possible to observe interpretable electron density for 10 out of 13 amino acids of the peptide (see Figure S4 for further details). The analysis of the interface reveals how both polar contacts and some hydrophobic interactions are relevant for the binding mode of this peptide to 14-3-3. The phosphate group of pSer718 is located in the basic pocket of 14-3-3, making polar contacts with Arg56, Arg127 and Tyr128, consistent with previously reported structures of phosphorylated peptides with 14-3-3 [22-24]. Additional polar contacts are established between Lys49, Glu180, Asn173, Asn224 and Asp213 of the protein with the peptide’s backbone and between the sidechains of Trp220 and Ser716, and the sidechains of Lys120 and Asp721. Hydrophobic interactions are formed between Leu172, Leu216, Leu220 of 14-3-3 and Tyr719, Pro720 and Ile722 of USP8pS718 peptide (Figure 2C). Comparison of the 14-3-3 binding motif of USP8pS718 with other structurally elucidated 14-3-3 binding sequences [25-28] (Figure 3) reveals how, despite the differences in the nature of the side chains, the relative positions at -1 and +2 from the phosphorylation site are similar. The position of the N-terminal Arg715 overlaps with the one of the N-terminal Arg of c-Raf and the Lys of β2 integrin, while a different conformation of the Arg can be observed in MLF1 peptide. The C-terminus of USP8pS718 shows a similar orientation compared to YAP, despite the C-terminus of YAP seems to be oriented more deeply into the groove. The situation is more different for the C-terminus of MLF1, where the phenyl ring points into a shallow depression and shows an interaction that does not resemble the other binding motifs.

![Figure 2](image-url)
USP8 mutants lose 14-3-3 protein binding

FITC labeled peptides bearing the single amino acid substitutions Pro720Arg, Ser718Cys, Ser718Pro, Ser718del and the double amino acids substitution [Leu713Arg;Tyr717Cys] were tested in FP assay against all human 14-3-3 isoforms (Figure 4). These mutation sites have been identified by Reincke et al. [7] in the tumor tissues of patients with CD. This work showed how the USP8 mutants lose 14-3-3 binding by co-immunoprecipitation experiments. Specifically, Ser718del and Pro720Arg mutations resulted in the most dramatic inhibition of 14-3-3ε binding, Ser718Cys and Ser718Pro had the second highest impact, while [Leu713Arg; Tyr717Cys] retained a weak binding. Additionally, the double mutations [Leu713Arg and Tyr717Cys] were analyzed separately and Tyr717Cys revealed to have the highest impact on EGFR endocytosis. These findings prompted us to investigate the effect of peptides bearing the previously described amino acids substitutions on the binding to 14-3-3 using FP assay. All these data, combined with our crystal structure of the USP8pS718 binding motif to 14-3-3, allowed us to infer fundamental structural information on the relative importance of the amino acids involved in this interaction.

Our FP data confirm the substantial loss of binding to 14-3-3 isoforms for Ser718Cys, Ser718Pro and Ser718del. This finding can be explained by the loss of the relevant polar contacts made by the phosphate group with Arg56, Arg127 and Tyr128; besides it points out how these contacts are the driving force for the USP8/14-3-3 interaction. In agreement with the previously reported data, we have observed a weaker binding for [Leu713Arg;Tyr717Cys] compared to the wild type. This observation can be partially explained from our structure with the loss of the hydrophobic contacts made by Tyr717, which was reported as the site having the highest impact on EGFR endocytosis, compared to Leu713Arg. Interestingly, in our FP assay Pro720Arg still showed binding to all 14-3-3 isoforms, in contrast with the data reported by Reincke et al.[7] in which this mutation was described as one originating the most dramatic reduction in 14-3-3 binding to USP8. As we have observed that the influence of the phosphate group is of crucial importance for the interaction, we speculate that mutations in the Pro720Arg site would negatively influence the phosphorylation of Ser718 and lead to the impairment of 14-3-3 binding previously described in the cellular experiments.
Figure 4. Binding between USP8 mutant peptides and 14-3-3. (A) Sequences of the peptides representing the wild type USP8 and the mutation identified between amino acids 713-720. FP assay of the FITC labeled peptides bearing the singly amino acid substitutions Ser718del (B), Ser718Pro (C), Ser718Cys (D), Pro720Arg (E), and the double amino acids substitution [Leu713Arg; Tyr717Cys] (F), with 14-3-3 isoforms. Background polarization was subtracted from all values. The data presented are the mean of three experiments; SD error bars are smaller than the data points symbols.

Discussion

USP8 is a deubiquitinating enzyme (DUB) that belongs to the ubiquitin-specific protease (USP) family. Ubiquitination is a reversible post-translational modification that regulates the fate and functions of various proteins in eukaryotic cells [29]. Conjugated ubiquitin molecules can be removed from target proteins by DUBs [30]. Among the different DUBs encoded by the human genome, USP8 is involved in the lysosomal trafficking of ligand-activated EGFR and by removing ubiquitin molecules from the receptor, it impedes its downregulation and sustains epidermal growth factor (EGF) signaling. The high incidence of USP8 mutations in corticotroph adenomas of patients with CD, might play a key-role in driving the constitutively activated EGFR signaling and ACTH production observed in the pituitary tumors. Moreover, the disease-associated mutations cluster around 14-3-3 binding motif, suggesting the importance of the USP8/14-3-3-protein−protein interaction (PPI) in CD, and prompted us to investigate the structural basis of the PPI interaction. Proteins manage long-range effectiveness through wider interactomes, highly organized and responsive networks, the so-called protein−protein interactions (PPIs) [31]. In this regard, proteins function not merely as single, isolated entities but display their roles by interacting with other cellular components. Hence, in many biological processes and particularly in the case of diseases where PPI interactions are implied, it is of utmost importance not only to elucidate the PPI network [32] but also gain
mechanistic insights in these PPIs. This is especially the case with hub proteins like 14-3-3 where the structural knowledge of the regulatory complexes lag significantly behind the biological knowledge of the (biomedical) importance of these interactions [33].

In this work we provided a structural elucidation of the USP8pS718/14-3-3 interaction, which has been shown to exert a significant role in the pathology of CD. This might represent the fundamental knowledge for auxiliary studies on the modulation of the PPI. Furthermore, we performed a series of binding assays to analyze how peptides bearing CD-associated mutations affect binding to 14-3-3.

Since the USP8 binding domain to 14-3-3 is located in a disordered region of the protein, we could make use of synthetic phospho-peptides in order to mimic the binding motifs. Our binding assays correlate with the data previously observed in cellular experiments. The USP8pS718 wild-type peptide was shown to bind all 14-3-3 isoforms, with affinities in the order of one micromolar, while overall the mutations in the binding motifs impair 14-3-3 binding, with the ones on the phosphorylation site showing the strongest impact.

The binding mode presents the characteristic features of the mode I binding to 14-3-3 (RXX[pS/pT]XP) accommodated by the conserved amphipathic groove of 14-3-3.

Our studies on the mutations of the USP8 binding motif and the structural elucidation of the PPI interface provide an overview of the relative importance of the clinically observed amino acid substitution in the binding sequence. The dramatic loss of binding to 14-3-3 isoforms for Ser718Cys, Ser718Pro and Ser718del peptides can be explained by the loss of the relevant polar contacts made by the phosphate group with Arg56, Arg127 and Tyr128. This finding confirms the prominent role of the phospho-site in driving the interaction, consistently with other previously studied 14-3-3 PPIs. Another important interaction appears to be the hydrophobic contact made by Tyr717; this can explain the weaker binding of the double mutant [Leu713Arg; Tyr717Cys] peptide, compared to the wild type. Our FP data showed that Pro720Arg can still bind 14-3-3, in contrast with the data reported by Reincke et al. [7]. We speculate that the mutation in this site might negatively influence the phosphorylation of Ser718 and lead to the impairment of 14-3-3 binding previously observed.

All together these findings offer some insights into this important PPI and might provide a platform for further studies and structure-based drug discovery programs on its modulation. Targeting the complex network of protein-protein interactions has now been widely recognized as an attractive mean to therapeutically intervene in disease states and the last two decades have seen an increasing number of successful examples of PPI modulators [34-37]. In recent years, it has been shown that the hub-protein 14-3-3, which is involved in a wide range of cellular processes and diseases [38], can be targeted by natural products and their derivatives [39-41], small molecules [42], modified peptides [43,44], and supramolecular ligands [45,46]. These compounds may act as inhibitors or stabilizers of 14-3-3 PPIs and induce the desired effect on the activity, dimerization, or cellular localization of the binding partner. Developing tool compounds able to target and modulate the USP8/14-3-3 PPI, could serve as a starting point for the development of alternative therapeutic agents for the treatment of CD. Most cases of Cushing’s syndrome are caused by hypersecretion of ACTH from corticotroph adenomas of the anterior pituitary. Surgery is the treatment of choice, although a significant percentage of patients experience a relapse. Alternatively, drug therapy (glucocorticoid receptor blockers, inhibitors of either pituitary ACTH release or adrenal cortisol synthesis) may ameliorate the clinical manifestations of the disease; however, each of these drugs induce adverse effects [3]. Thus, in order to improve the control of the disease, new pharmacological strategies are needed. The elucidation of USP8pS718 peptide/14-3-3 interface and the structural findings in light of the disease-linked mutations might provide a starting point for structure-based investigations with the aim of targeting and stabilizing the PPI interface as a new potential therapeutic strategy for CD.

Acknowledgements

This work is funded by the H2020 Marie Curie Actions of the European Commission through the TASPPI project, grant Agreement 675179. We thank Dr. David Bier and the staff of beamline PXII of the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland for performing the data collection.
References


