14-3-3 protein masks the nuclear localization sequence of caspase-2

Aneta Smidova1, Miroslava Alblova1, Dana Kalabova1,2, Katarina Psenakova1,3, Michal Rosulek4,5, Petr Herman6, Tomas Obsil1,3 and Veronika Obsilova1

1 Department of Structural Biology of Signaling Proteins, Division BIOCEV, Institute of Physiology of the Czech Academy of Sciences, Vestec, Czech Republic
2 2nd Faculty of Medicine, Charles University, Prague, Czech Republic
3 Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Prague, Czech Republic
4 Division BIOCEV, Institute of Microbiology of the Czech Academy of Sciences, Vestec, Czech Republic
5 Department of Biochemistry, Faculty of Science, Charles University, Prague, Czech Republic
6 Institute of Physics, Faculty of Mathematics and Physics, Charles University, Prague, Czech Republic

Keywords
14-3-3 protein; caspase-2; fluorescence; nuclear localization sequence; protein–protein interactions; small angle X-ray scattering

Correspondence
T. Obsil, Department of Physical and Macromolecular Chemistry, Faculty of Science, Charles University, Prague, Czech Republic
Fax: +420 224919752
Tel: +420 221951303
E-mail: obsil@natur.cuni.cz

V. Obsilova, Department of Structural Biology of Signaling Proteins, Division BIOCEV, Institute of Physiology of the Czech Academy of Sciences, 252 50 Vestec, Czech Republic
Fax: +420 241 062 488
Tel: +420 325 873 513
E-mail: veronika.obsilova@fgu.cas.cz

Aneta Smidova, Miroslava Alblova and Dana Kalabova contributed equally to this work

(Received 17 August 2018, accepted 28 September 2018)
doi:10.1111/febs.14670

Caspase-2 is an apical protease responsible for the proteolysis of cellular substrates directly involved in mediating apoptotic signaling cascades. Caspase-2 activation is inhibited by phosphorylation followed by binding to the scaffolding protein 14-3-3, which recognizes two phosphoserines located in the linker between the caspase recruitment domain and the p19 domains of the caspase-2 zymogen. However, the structural details of this interaction and the exact role of 14-3-3 in the regulation of caspase-2 activation remain unclear. Moreover, the caspase-2 region with both 14-3-3-binding motifs also contains the nuclear localization sequence (NLS), thus suggesting that 14-3-3 binding may regulate the subcellular localization of caspase-2. Here, we report a structural analysis of the 14-3-3:caspase-2 complex using a combined approach based on small angle X-ray scattering, NMR, chemical cross-linking, and fluorescence spectroscopy. The structural model proposed in this study suggests that phosphorylated caspase-2 and 14-3-3ζ form a compact and rigid complex in which the p19 and the p12 domains of caspase-2 are positioned within the central channel of the 14-3-3 dimer and stabilized through interactions with the C-terminal helices of both 14-3-3ζ protomers. In this conformation, the surface of the p12 domain, which is involved in caspase-2 activation by dimerization, is sterically occluded by the 14-3-3 dimer, thereby likely preventing caspase-2 activation. In addition, 14-3-3 protein binding to caspase-2 masks its NLS. Therefore, our results suggest that 14-3-3 protein binding to caspase-2 may play a key role in regulating caspase-2 activation.

Database
The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.wwpdb.org (PDB ID codes 6GKF and 6GKG).

Abbreviations
CARD, caspase recruitment domain; CSP, chemical shift perturbation; NLS, nuclear localization sequence; NMR, nuclear magnetic resonance; PIDD, p53-induced protein with a death domain; proC2, caspase-2 residues 123-452 without the CARD domain; RAIDD, adaptor protein RIP-associated ICH-1/CAD-3 homologous protein with a death domain; SAXS, small angle X-ray scattering.
**Introduction**

Caspase activation coordinates the apoptotic pathway. Caspases cleave substrates at the C-terminal to aspartate residues. In particular, caspase-2 is a key switch between cellular metabolism and apoptosis and tumor suppressor [1]. This protease is crucial for human physiology and pathophysiology, as shown by its prominent downstream targets, such as protein kinase C delta and huntingtin protein. Accordingly, its activation must be strictly controlled in cells [2,3].

Caspase-2 is an initiator caspase (alongside caspase-8 and -9) activated by proximity-induced dimerization after recruitment to specific high molecular weight protein complexes and trans-cleavage of the caspase recruitment domain (CARD) and linker regions [4–7]. This results in the formation of the fully active enzyme, a heterodimer of two large (p19) and two small subunits (p12) which is responsible for triggering apoptosis. In turn, the PIDDosome, a complex formed by the scaffold protein p53-induced protein with a death domain (PIDD) and by the adaptor protein RIP-associated ICH-1/CAD-3 homologous protein with a death domain (RAIDD) [8,9] is a well-known caspase-2 activation platform.

In contrast to other caspases, endogenous caspase-2 is mainly localized in the nucleus and in the Golgi complex [10,11]. The nuclear localization of caspase-2 is driven by a classical nuclear localization sequence (NLS), located in the linker region between the CARD and the p19 domains (sequence P149LYKKLRL156), which is recognized by the importin α/β heterodimer [12]. Mutating a conserved Lys152 in this sequence has been shown to abolish caspase-2 nuclear localization and its binding to the importin α/β heterodimer. Moreover, a recent study has shown that DNA damage-induced caspase-2 activation primarily occurs in the nucleolus and depends on the PIDDosome and on the nucleolar phosphoprotein nucleophosmin. Conversely, cytoplasmic caspase-2 activation is driven by a PIDD-independent platform, albeit still requiring RAIDD [13].

In addition to the upstream regulation by its adaptor proteins, caspase-2 activation can also be fine-tuned by phosphorylation. In *Xenopus laevis*, abundant nutrient flux leads to strongSer164 phosphorylation (human numbering), which blocks caspase-2 binding to RAIDD and induces caspase-2 binding to the scaffolding protein 14-3-3, thereby keeping caspase-2 inactive [14]. In addition, Ser340 phosphorylation by Cdk1 kinase has also been shown to prevent caspase-2 activation during normal mitosis [15].

14-3-3 proteins, highly homologous dimeric molecules present in all eukaryotic organisms, are the main regulators of key cellular processes such as apoptosis, signal transduction, cell cycle regulation, or tumorigenesis [16–19]. More than 2000 14-3-3 protein-binding partners have been predicted and several hundred have been experimentally validated thus far [20,21]. In most cases, 14-3-3 protein binding is triggered by phosphorylation of the target protein. Thus, 14-3-3 proteins function as master regulators of their binding partners by switching their active and inactive states in complex cell signaling networks [22,23].

Many 14-3-3-binding partners contain two or more 14-3-3-binding motifs, which could be simultaneously used to engage both protomers within the 14-3-3 dimer [21,24,25]. It has previously been suggested that the 14-3-3 protein binding to caspase-2 requires phosphorylation not only at Ser164 but also Ser139 (Fig. 1A) [14,26,27]. Indeed, we have recently shown that the complex formation between caspase-2 and 14-3-3 requires simultaneous phosphorylation of both these sites [28]. However, the structural details of this interaction and the exact role of 14-3-3 in regulating caspase-2 activation remain unclear. Moreover, the presence of both 14-3-3-binding motifs and of the NLS in the same caspase-2 region suggests that 14-3-3 binding may regulate the subcellular localization of caspase-2. Therefore, such mode of regulation likely requires a direct interaction between 14-3-3 and the NLS of caspase-2 that masks the interaction between the NLS and the nuclear import machinery, yet no such interaction has been shown thus far.

In this context, we aimed to analyze 14-3-3 protein binding to caspase-2 toward understanding how this interaction affects caspase-2 structure and regulation. For this purpose, we performed a structural analysis of the complex between 14-3-3 and phosphorylated caspase-2 using a combined approach based on small angle X-ray scattering (SAXS), NMR, chemical cross-linking, and fluorescence spectroscopy.

**Results**

**The complex between caspase-2 and 14-3-3 has a globular and compact shape**

The main goal of this work is to provide the structural basis for the role of 14-3-3 protein binding in regulating caspase-2 activation. Because all our previous attempts to crystallize the complex between Ser139- and Ser164-phosphorylated caspase-2 (residues 123–452 without the CARD domain) [28] (hereafter referred to as
**Fig. 1.** SAXS data analysis. (A) Domain structure of human caspase-2. The N-terminal part with the CARD domain (not shown in the proC2 construct) is highlighted in gray, the linker sequence between the CARD and p19 domains in brown, the p19 domain in salmon and the p12 domain in yellow. The positions of phosphorylation sites and of the NLS are indicated in red and blue, respectively. The positions of Tyr and Phe residues mutated to Trp and used in fluorescence experiments are indicated by green arrows. (B) Experimental scattering intensity $I(s)$ is plotted as a function of the scattering vector $s$ ($s = 4\pi\sin(\theta)/\lambda$, where $\theta$ is the scattering angle, and $\lambda$ is the wavelength). Data were collected at the following protein concentrations: proC2 (1.9 mg/mL, blue), 14-3-3f (2.2 mg/mL, green), and the proC2:14-3-3f complex mixed at 1 : 2 molar stoichiometry (3.6 mg/mL, red). Guinier plots (ln$I(s)$ versus $s^2$) for scattering curves are shown as inset. (C) Distance distribution functions $P(r)$ calculated from scattering data using the program GNOM [56]. (D) Dimensionless Kratky plots. Gray lines mark the maximum at a value of 1.104 for $sR_g = 1.73$, a typical attribute of scattering data of compact globular proteins [35]. (E) Ab initio shape reconstruction of proC2:14-3-3f complex (wheat envelope) calculated from SAXS data with a superimposed AllosMod-FoXS model of the proC2:14-3-3f complex shown in two perpendicular views. The linker sequence in the groove, p19, p12 domains and phosphorylation sites are indicated in deep teal, salmon, yellow, and red spheres. The calculated molecular envelope was aligned to the structural model using the program SUPCOMB [66]. The ab initio shape reconstruction was performed using the program DAMMIF [67]. (F) Comparison of the calculated scattering curve of proC2 (red line) with the experimental scattering data (black line).
A. Smidova et al.

Structure of the 14-3-3:caspace-2 complex

proC2) and 14-3-3ζ had been unsuccessful, we decided to use small angle X-ray scattering (SAXS) combined with NMR, with chemical cross-linking coupled to MS and with fluorescence spectroscopy to characterize the solution structure and conformational behavior of this complex. We and others have successfully used a similar approach to characterize conformationally flexible 14-3-3 protein complexes [29–34].

X-ray scattering data were collected for proC2, 14-3-3ζ, and the proC2:14-3-3ζ complex (mixed in 1 : 2 molar stoichiometry) at different concentrations (Table 1 and Fig. 1B). The complex was prepared at concentrations at least two orders of magnitude higher than the previously reported $K_D$ value of ~230 nm [28]. The values of the average diffusion coefficient ($D_{max}$), maximum particle distance ($D_{max}$), and Porod volume ($V_p$) were calculated from the measured SAXS profiles. The linearity of Guinier plots indicated the absence of aggregation in all samples (Fig. 1B). The apparent $D_{max}$ values of the proC2, 14-3-3ζ and the proC2:14-3-3ζ complex estimated from the forward scattering intensity $I(0)$ and $V_p$ matched their expected $M_w$ values (Table 1). The apparent $M_w$ values of the proC2:14-3-3ζ complex and its $V_p$ values are consistent with a 1 : 2 m stoichiometry, thus corroborating our previously published data [28].

The distance distribution functions $P(r)$ showed that the $R_g$ values of the complex are only slightly larger than those of proC2 and 14-3-3ζ alone (Fig. 1C), thus suggesting that the proC2:14-3-3ζ complex adopts a rather compact conformation. This was further confirmed by dimensionless Kratky plots ($sR_g^2I(s)/I(0)$ versus $sR_g$), which indicate the conformational flexibility of a protein, regardless of its size [35]. In this plot, both the 14-3-3ζ dimer and the proC2:14-3-3ζ complex exhibited a bell-shaped profile, typical of a globular and rigid molecule with a maximum value of ($sR_g^2I(s)/I(0)$ of 1.04 at $sR_g = 1.73$ (Fig. 1D). Conversely, the dimensionless Kratky plot of proC2 alone exhibits a higher maximum of 1.2 at $sR_g = 1.9$, thus indicating that the proC2 molecule has a greater flexibility than the complex.

Subsequently, we performed chemical cross-linking experiments using disuccinimidyl suberate (DSS) and disuccinimidyl glutarate (DSG) bifunctional agents to determine the distance restraints of the proC2:14-3-3ζ complex for structural modeling purposes. The inter-molecular cross-links identified can be divided into three groups (Tables S1 and S2). The first group consists of cross-links connecting the N-terminal amino group of proC2 (Ser123) to the loops between the helices H3-H4 (containing Lys68 and Lys75) and H4-H5 (Lys138) of 14-3-3ζ. The second group includes cross-links connecting Lys153 of proC2, located in the linker between the phosphorylation sites Ser139 and Ser164, to the H3-H4 loop and to the helix H1 of 14-3-3ζ containing Lys68 and Lys11, respectively. Cross-links from the last group connect Lys68 from the H3-H4 loop of 14-3-3ζ to either Lys72 or Lys81 located in the loop preceding the Q6 helix of the proC2 p12 domain, thus indicating the proximity of this domain to the 14-3-3ζ surface.

The initial structural model of the complex was created using the crystal structures of 14-3-3ζ (PDB ID: IQJB) and caspase-2 (PDB ID: 3R7S) [36,37]. The two 14-3-3-binding motifs of caspase-2 are separated by 25 amino acid residues, similarly to the 14-3-3-binding motifs of yeast neutral trehalase Nth1, whose crystal

| Table 1. Structural parameters determined from SAXS data. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| proC2           | monomer         |
| 1.0             | 1.9             |
| 27              | 48              |
| 25.5 ± 1.6      | 26.8 ± 1.7      |
| 26.7 ± 0.3      | 27.6 ± 0.2      |
| 98              | 95              |
| 75              | 72              |
| 30              | 47              |
| 14-3-3ζ         | dimer           |
| 2.2             | 4.1             |
| 39              | 73              |
| 29.8 ± 1.7      | 29.1 ± 0.9      |
| 30.1 ± 0.1      | 29.6 ± 0.1      |
| 104             | 93              |
| 91              | 88              |
| 48              | 49              |
| 50              | 55              |
| Complex         | Complex         |
| 1.7             | 3.6             |
| 18              | 38              |
| 32.9 ± 1.0      | 33.0 ± 0.3      |
| 33.1 ± 0.1      | 33.4 ± 0.1      |
| 119             | 115             |
| 152             | 155             |
| 83              | 81              |
| 98              | 84              |
| 95              | 101             |

- Molar concentration of the 14-3-3ζ dimer and the proC2:14-3-3ζ complex with 1 : 2 molar stoichiometry. b Calculated using Guinier approximation. c Calculated using the program GNOM [56]. d The excluded volume of the hydrated particle (Porod volume). e Molecular weight was estimated by comparison of the forward scattering intensity $I(0)$ with that of the reference solution of bovine serum albumin. f The theoretical molecular weights of the proC2, 14-3-3ζ dimer and of the proC2:14-3-3ζ complex (with 1 : 2 stoichiometry) are 38.2, 56 and 94.2 kDa, respectively. g Molecular weight was estimated from the Porod volume ($M_w = 0.625 \times V_p$).
Structure of the 14-3-3:caspase-2 complex

A. Smidova et al.

structure in complex with 14-3-3 was recently reported by our group [38]. Therefore, the conformation of the proC2 N-terminal segment containing both 14-3-3-binding motifs and its interaction with the 14-3-3 dimer were modeled using the structure of the Nth1:14-3-3 complex (PDB ID: 5N6N). The Allos-Mod-FoXS method based on conformational sampling, combined with the rigid-body modeling of SAXS profiles and distance restraints assessed by intermolecular cross-links between 14-3-3ζ and the proC2 p12 domain, was used to calculate a structural model consistent with the experimental SAXS data [39]. The best-scoring model fitted the experimental SAXS data with $\chi^2 = 1.3$ (Fig. 1F), and its theoretical $R_g$ and $D_{max}$ values of 33.1 Å and 123 Å, respectively, are consistent with the experimental values (Table 1). In this model, the proC2 p19 and p12 domains are positioned within the central channel of the 14-3-3 dimer and the α7 helix of the p12 domain interacts with the loop between helices H3 and H4 of 14-3-3ζ (Fig. 2A). The N-terminal segment of proC2 containing both 14-3-3-binding motifs and the NLS is located within the central channel of 14-3-3ζ, with the NLS region squeezed between the bottom of the 14-3-3 central channel and proC2 p12 domain helices α6 and α7. The positions of the proC2 p12 and p19 domains are apparently stabilized by interactions with the C-terminal H9 helices of both 14-3-3ζ protomers. The structural model of the proC2:14-3-3ζ complex is consistent with the ab initio molecular envelope calculated from the scattering data (Fig. 1E). The filtered averaged envelope of the complex shows a globular shape, and one side bulkier than the other. The superimposition shown in Fig. 1E suggests that the narrower part corresponds to the linker between the proC2 p19 and p12 domains. Projecting the cross-links identified (Tables S1 and S2) onto the structural model revealed that all observed cross-links are within the cross-linking distance limit for DSS and DSG (< 28 Å). The length of the spacer arm is 7.7 and 11.4 Å for DSS and DSS, respectively, thus adding 6.5 Å for each cross-linked lysine side-chain (20.7 and 24.4 Å) and allowing conformational dynamics (~ 30 Å) (Fig. 2B,C) [40].

14-3-3ζ proC2-binding surface mapping by NMR

We then performed a detailed analysis of the 14-3-3ζ proC2-binding surface by NMR spectroscopy. The previously published sequence-specific backbone assignment of 14-3-3ζΔC (protein construct missing the last C-terminal 12 amino acid residues) was used to analyze changes in 14-3-3ζ backbone amide signals [41]. NMR titration experiments revealed that numerous 14-3-3ζ residues showed either a change in signal intensity or a significant change in the NMR signal position upon proC2 addition (Figs 3 and 4), thus suggesting their involvement in the interaction or their conformational change induced by proC2 binding. The summary of chemical shift perturbations and intensity change analyses of the 1H-15N HSQC spectrum of 14-3-3ζΔC upon proC2 binding is shown in Fig. 4B. The most affected regions include not only segments from helices forming the ligand-binding groove (H3, H5, H7, and H9) but also helices H4, H8, and H6. These changes are consistent with the SAXS-based structural model which suggested that the p19 and p12 domains of proC2 are held within the 14-3-3ζ central channel through contacts with the C-terminal helix H9 (Fig. 1C). In addition, Fig. 4C analysis shows that the affected 14-3-3ζ residues are protruding from the inside of the dimer to the outer surface. We assume that the binding effect propagates to 14-3-3ζ protein residues with no direct interaction with proC2 due to allosteric structural changes, as previously shown for other proteins [30,42,43].

14-3-3 binding reduces the conformational flexibility of proC2 including the NLS region

To examine the 14-3-3-binding effect on the conformational behavior of proC2, we performed time-resolved tryptophan fluorescence intensity and anisotropy decay measurements. We prepared five single tryptophan containing proC2 mutants (Trp151, Trp188, Trp218, Trp385, Trp426). However, the sequence of proC2 contains only one tryptophan residue at position Trp385. Therefore, we prepared other mutants by mutating Trp385 to Phe and subsequently placing the tryptophan residue at the position of interest (mutations Y151W, F188W, F218W, F218W, and F426W). We assessed the stability of all mutants by measuring the midpoint temperatures of the protein-unfolding transition by differential scanning fluorimetry. No significant changes were observed, thus suggesting that the mutations introduced caused no change in the stability of the proC2 structure. This approach allowed us to monitor conformational changes in five different regions of proC2: Trp151 is located in the NLS region between the 14-3-3-binding motifs; Trp188 and Trp218 are located at the C terminus of helix α1 and the loop between the helix α2 and strand β1 of p19 domain, respectively; and Trp385 and Trp426 are located in the N terminus of helix α6 and the C-terminal segment of p12 domain, respectively (Figs 1A and 5A).

Then, we evaluated changes in the tryptophan environment induced by interaction with 14-3-3ζ proC2.
fluorescently silent 14-3-3f containing W59F and W228F mutations) by time-resolved emission and fluorescence anisotropy decays. We have previously shown that these mutations have no effect on 14-3-3f-binding properties [44,45]. Because our single-tryptophan proC2 proteins exhibited complex multiexponential emission decays, we decided to use mean fluorescence lifetime (τmean) as a sensitive indicator reflecting changes in Trp microenvironment upon complex formation (Table 2 and Fig. 5B). A direct interaction between proC2 and 14-3-3fNoW or a binding-induced conformational change of proC2 significantly increased the τmean of most mutants by approximately 0.4 ns.

The only exception is the Trp188 mutant, which exhibits an unusually short lifetime, unresponsive to 14-3-3fNoW binding. The increase in τmean likely resulted from the decrease in intramolecular quenching or from the increase in fluorophore shielding from solvent molecules in the presence of 14-3-3fNoW.

The results from the time-resolved emission anisotropy experiments are summarized in Table 2. The hydrodynamic properties of all five proC2 mutants differ in the presence and absence of 14-3-3fNoW. Three classes of correlation times were found. The first two include the fast Trp wobbling and the segmental motion reflected by the short correlation times φ1 and...
...φ₂ located near 0.4 and 1.3–3.0 ns, respectively. The third class refers to the long correlation time φ₃, which can be assigned to the rotational motion of the whole molecule or molecular complex. Binding-induced changes in the rotational freedom of Trp within each mutant were assumed from the sum of β₁ + β₂, denoted as βFAST, reflecting the overall amplitude of the fast motions. As shown in Fig. 5C and in Table 2, the Trp¹⁵¹ and Trp⁴²⁶ mutants exhibit a significantly lower βFAST upon complex formation, which suggests rigidization of the Trp neighborhood upon 14-3-3noW binding. The Trp mobility of the Trp¹₈₈ mutant was also reduced in the complex. However, it should be noted that a rather short fluorescence lifetime prevents an accurate assessment of the long correlation times of this mutant. In contrast, Trp²¹₈ and Trp³⁸⁵ exhibited a rather rigid Trp microenvironment, which was only weakly modulated by the formation of the complex. Thus, these data suggested a decrease in the conformational flexibility of several regions of proC2, namely around Trp¹⁵¹ and Trp⁴²⁶.

To further explore the 14-3-3-induced structural changes of proC2, we performed quenching experiments probing the fluorophore accessibility to acrylamide. The values of the bimolecular quenching constant kᵣ calculated from the Stern-Volmer plots are presented in Table S3 and compared in Fig. 5D. Trp¹₈₈ accessibility dramatically decreased by approximately 46% in the proC2:14-3-3noW complex. Trp³⁸⁵ showed a smaller decrease (~ 32%), whereas Trp¹₈₈ accessibility remained unchanged upon complex formation.

Together, these results suggested that the accessibility and mobility of Trp¹⁵¹ located in the NLS region and of Trp⁴²⁶ located in the C-terminal part of proC2 significantly decrease upon complex formation, thus corroborating the SAXS-based structural model in which these residues are opposite to each other on the interface between the two proteins (Fig. 5A). The behavior of Trp²¹₈, located in the loop preceding the proC2 helix α₂, exhibited an increase in both r_mean and βFAST, and a dramatic decrease in kᵣ upon the complex formation, likely resulting from improved shielding from the quencher upon complex formation. The Trp¹₈₈ mutant behaves distinctly because neither its lifetime nor its accessibility to the quencher changes upon complex formation. However, after 14-3-3noW binding, its motion virtually freezes. This is also consistent with the structural model proposed suggesting that this residue is located at the C terminus of the proC2 helix α₁ located near the helix H9 of a 14-3-3C₇ protomer.

14-3-3 protein binding protects proC2 from proteolytic degradation in vitro

Structural modeling together with chemical cross-linking and fluorescence spectroscopy data suggested that the 14-3-3C₇ dimer extensively interacts with the whole proC2N-terminal linker region containing both 14-3-3-binding motifs and the NLS (Fig. 1A and Table 2, Tables S1 and S2). Therefore, complex formation presumably protects this region against proteolysis. To test this hypothesis, we performed limited proteolysis experiments. The results of protease digestion of proC2 alone and in complex with 14-3-3C₇ by low levels of trypsin and chymotrypsin are presented in Fig. 6. Under the conditions used in this experiment, the proC2 alone was highly sensitive to proteolysis, and the cleavage resulted in the formation of a smaller fragment with an apparent Mₗ of 30 000 in both cases.
Fig. 4. Specific proC2 binding-induced changes in the NMR spectra of 14-3-3ζΔC. proC2 binding to 14-3-3ζ was evaluated by NMR titration of 150 μM of 15N-labeled 14-3-3ζΔC with unlabeled proC2 to a final molar ratio 2 : 1. (A) Comparison of representative signals of free (red) and proC2-bound (green) 14-3-3ζΔC from the 1H-15N HSQC spectra. (B) The summary of chemical shift perturbations (CSPs) and intensity change analyses of the 1H-15N HSQC spectra of 14-3-3ζΔC upon proC2 binding. The relative CSPs were calculated as an averaged Euclidean chemical shift change: $d = \frac{1}{2} \sqrt{d_H^2 + d_N^2}$, where $d_H$ and $d_N$ are the weighted average of shifts in the free and bound states for 1H and 15N, respectively [68]. The interaction induced both the change in positions (red bars) or a significant change in the intensities of the signals (yellow bars) in the 1H-15N HSQC spectrum of 14-3-3ζΔC. The regions of the protein backbone that could not be unambiguously assigned are highlighted in gray. The secondary structure elements of 14-3-3ζ are indicated at the top. (C) Range of the most significant CSPs and intensity changes mapped onto the 14-3-3ζ structure [36]. The 14-3-3ζ residues most affected by the proC2 binding are highlighted in dark red.
The 14-3-3 protein was resistant to both proteases within the time course of this experiment, whereas the presence of 14-3-3 substantially slowed proC2 cleavage by both proteases. Mass spectrometry analysis of proC2 bands formed after 30 min of protease digestion by both trypsin and chymotrypsin revealed that the smaller fragment corresponds to the caspase-2 sequence 156-452 (the first identified peptide from the N-terminal part was peptide 156-161 phosphorylated at Ser164 with the m/z signal 692.29). The peptide containing the first phosphorylation site Ser139 (peptide 131–152 with the m/z signal 1965.95) was observed only in nonprotease-treated samples of proC2. These data indicate that the 14-3-3 masks the cleavage site located within the NLS region of proC2, thus corroborating the SAXS-based structural model of the complex.

**Structural characterization of the 14-3-3-binding motifs of caspase-2**

Previous studies have suggested that caspase-2 has two 14-3-3-binding motifs around pSer139 and pSer164 (Fig. 1A) [14,27,28]. Neither of these motifs is similar to canonical 14-3-3-binding motifs because they lack...
basic residues at positions -3 and -4 relative to the phosphorylated residue [36,46]. To elucidate the structural basis of interactions between 14-3-3 protein and the 14-3-3-binding motifs of caspase-2, we solved the crystal structures of phosphopeptides pepS139 (sequence YDLpSer139LPFP) and pepS164 (sequence VEHpSer164LDNK) with 14-3-3$c$ (D$\alpha$ denotes the missing ~15-residue-long flexible C-terminal tail). The 14-3-3$c$ isoform was selected based on crystal quality. The pepS139 and pepS164 phosphopeptide complex structures were determined at 2.6 and 2.8 Å resolution, respectively, with four 14-3-3 dimers with bound phosphopeptides in the asymmetric unit (Table 3). Both structures were solved by a molecular replacement with 14-3-3$c$ (PDB ID: 2B05) as search model. The final electron density allowed us to trace all eight residues of the pepS139 peptide in all eight 14-3-3$c$ protomers present in the asymmetric unit (Fig. 7A,B). In the case of pepS164, the electron density allowed us to build only six of eight peptides, and the longest traced peptide contained six residues. We were unable to trace the last two residues (Asn167 and Lys168).

### Table 2. Summary of time-resolved tryptophan fluorescence measurements.

<table>
<thead>
<tr>
<th>proC2 variant</th>
<th>$\tau_{\text{mean}}^{a,b}$ (ns)</th>
<th>$\phi_1^c$ (ns)</th>
<th>$\phi_2^c$ (ns)</th>
<th>$\phi_3^d$ (ns)</th>
<th>$\beta_1$</th>
<th>$\beta_2$</th>
<th>$\beta_3$</th>
<th>$\beta_{\text{int}}^e$</th>
<th>$\beta_{\text{F3}} = \beta_1 + \beta_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp151</td>
<td>3.8</td>
<td>0.4</td>
<td>0.040</td>
<td>1.9</td>
<td>0.074</td>
<td>65</td>
<td>0.123</td>
<td>0.114</td>
<td></td>
</tr>
<tr>
<td>Trp151$+14$-3-3$c$</td>
<td>4.2</td>
<td>1.5</td>
<td>0.032</td>
<td>20</td>
<td>0.069</td>
<td>0.136</td>
<td>0.032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp188</td>
<td>1.8</td>
<td>3.0</td>
<td>0.050</td>
<td>28</td>
<td>0.187</td>
<td>0.050</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp188$+14$-3-3$c$</td>
<td>1.8</td>
<td>2.5</td>
<td>0.002</td>
<td>20</td>
<td>0.217</td>
<td>0.018</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp218</td>
<td>3.6</td>
<td>1.8</td>
<td>0.012</td>
<td>21</td>
<td>0.188</td>
<td>0.037</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp218$+14$-3-3$c$</td>
<td>4.0</td>
<td>1.2</td>
<td>0.016</td>
<td>65</td>
<td>0.221</td>
<td>0.016</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp385</td>
<td>6.5</td>
<td>1.3</td>
<td>0.029</td>
<td>18</td>
<td>0.153</td>
<td>0.055</td>
<td>0.029</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp385$+14$-3-3$c$</td>
<td>6.8</td>
<td>2.0</td>
<td>0.025</td>
<td>26</td>
<td>0.087</td>
<td>0.125</td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp426$+14$-3-3$c$</td>
<td>4.2</td>
<td>0.5</td>
<td>0.039</td>
<td>2.8</td>
<td>0.068</td>
<td>0.130</td>
<td>0.107</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trp426</td>
<td>4.7</td>
<td>0.5</td>
<td>0.013</td>
<td>2.1</td>
<td>0.035</td>
<td>0.189</td>
<td>0.048</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Mean lifetimes were calculated as $\tau_{\text{mean}} = \Sigma f_i \tau_i$, where $f_i$ is an intensity fraction of the $i$-th lifetime component $\tau_i$. $^b$ SD value is $\pm 0.05$ ns. $^c$ Fluorescence anisotropies $\phi(t)$ were analyzed for a series of exponentials by a model-independent maximum entropy method without setting assumptions about the shape of the correlation time distributions, $\phi(t) = \Sigma \beta_i \exp(-t/\tau_i)$. The rotational correlation times, $\tau_i$, and their amplitudes, $\beta_i$, represent the characteristics of the peaks in the corresponding distributions. $^d$ SD value is $\pm 5$ ns. $^e$ Limiting anisotropy value of the longest correlation time related to the rotational diffusion of the whole protein or protein complex. Due to the short fluorescence lifetimes, the exact value of the correlation time was unresolved. $^f$ $\beta_{\text{F3}}$ represents the overall amplitude of fast molecular motions reflecting the rotational freedom of Trp within the molecule. Higher $\beta_{\text{F3}}$ indicates higher Trp mobility. $^g$ The human 14-3-3-3$\zeta$noW protein mutant missing all Trp residues (mutations W59F and W228F) was used in all proC2 tryptophan measurements [44,45].

### Fig. 6. Limited proteolysis of the proC2:14-3-3$c$ complex. Limited proC2 proteolysis in the absence and in the presence of 14-3-3 digested with trypsin (A) and chymotrypsin (B) for 10, 20, and 30 min. The protease/proC2 ratio was 1 : 1000 (w/w). The reactions were stopped by boiling the samples with SDS/PAGE loading buffer at the times indicated before they were analyzed by SDS/PAGE. The resulting proC2 peptides were analyzed by MALDI-TOF-MS.
suggesting that these two residues are already disordered (Fig. 7C,D).

The recognition of the phosphate group, the main-chain conformation and other contacts in the 14-3-3-binding groove of both motifs are similar to those previously observed in other 14-3-3 protein complexes [25,36,38,47–49]. The pSer 139 moiety of pepS139 is coordinated by the side-chains of the 14-3-3 residues Lys 50, Arg 57, Arg 132, and Tyr 133; other contacts include hydrogen bonds between the side-chains of the 14-3-3 residues Glu 185, Asn 229, and Asn 178 and the main-chain atoms of the residues Tyr 136, Leu 138, and Leu 140, respectively (Fig. 7B). Very similar contacts were also observed in the case of the pepS164 peptide (Fig. 7D). The only differences are the presence of two intramolecular hydrogen bonds within the N-terminal half of the peptide and the contact between the side-chain of the 14-3-3 residue Lys 50 and the main-chain carbonyl group of Leu 165 of pepS164.

**Discussion**

Previous studies have shown that caspase-2 activation is inhibited by phosphorylation in a process involving interaction with the scaffolding 14-3-3 protein, which recognizes two 14-3-3-binding motifs within the linker between the CARD and the p19 domains of caspase-2 (Fig. 1) [14,26–28]. However, the inhibitory mechanism and the role of 14-3-3 in this process remain unresolved.

Caspase-2 is activated by proximity-induced dimerization after recruitment to specific high molecular weight protein complexes and trans-cleavage [4–7]. Caspase-2 dimerization has been previously shown to require both the CARD domain and residues from the C-terminal p12 domain [4]. The structural analysis of the complex between phosphorylated proC2 and 14-3-3 reported in this study suggested that most of the p12 domain surface is masked by 14-3-3. In the model, the p12 domain faces the central channel of the 14-3-3 dimer, and its helix α7 interacts with the loop between 14-3-3 helices H3 and H4 and the proC2N-terminal linker, located at the bottom of the 14-3-3 dimer central channel (Fig. 2A). The proximity of the p12 domain to the 14-3-3 surface was confirmed by cross-linking experiments (Fig. 2B,C and Tables S1 and S2) and by fluorescence quenching measurements, which revealed that tryptophan residues Trp 385 and

<table>
<thead>
<tr>
<th>Table 3. Crystallographic data collection and refinement statistics.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex</td>
</tr>
<tr>
<td>14-3-3γΔC:pepS139</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
</tr>
<tr>
<td>Space group</td>
</tr>
<tr>
<td>Unit-cell parameters</td>
</tr>
<tr>
<td>a, b, c (Å)</td>
</tr>
<tr>
<td>α, β, γ (°)</td>
</tr>
<tr>
<td>Asymmetric unit contents</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
</tr>
<tr>
<td>Unique reflections</td>
</tr>
<tr>
<td>Data multiplicity</td>
</tr>
<tr>
<td>Completeness (%)</td>
</tr>
<tr>
<td>〈I/σ(I)〉</td>
</tr>
<tr>
<td>R&lt;sub&gt;work&lt;/sub&gt;</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;</td>
</tr>
<tr>
<td>R&lt;sub&gt;free&lt;/sub&gt;</td>
</tr>
<tr>
<td>No. of protein atoms</td>
</tr>
<tr>
<td>No. of waters</td>
</tr>
<tr>
<td>Average B factors (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>Protein</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>R.m.s. deviations from ideal values</td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
</tr>
<tr>
<td>Bond angles (°)</td>
</tr>
<tr>
<td>Ramachandran favored (%)</td>
</tr>
<tr>
<td>Ramachandran allowed (%)</td>
</tr>
<tr>
<td>Ramachandran outliers (%)</td>
</tr>
</tbody>
</table>

* Values in parentheses are for the highest resolution shell. * R<sub>work</sub> = Σ(|I(hkl)| − |⟨I(hkl)⟩|) / Σ|I(hkl)|, where |I(hkl)| is the intensity of reflection hkl, |⟨I(hkl)⟩| = Σ|I(hkl)| / ΣN(hkl), and N(hkl) the multiplicity. * R.m.s., root mean square.
Trp426, both located within the p12 domain, are less exposed to the solvent upon complex formation (Fig. 5D). The proC2 construct used in this study (residues 123–452) did not include the N-terminal CARD domain (residues 32–121). However, because the linker between the CARD and the p19 domains containing both 14-3-3-binding motifs (residues 123–169) is buried within the central channel of the 14-3-3ζ dimer (Fig. 2A, shown in brown), the preceding CARD will likely be close to the 14-3-3ζ dimer surface also. Therefore, SANS-based structural modeling together with chemical cross-linking and fluorescence data suggested that both regions required for caspase-2 dimerization are located close to the 14-3-3ζ surface, thus indicating that the 14-3-3 protein binding may prevent caspase-2 oligomerization and/or its binding to RAIDD.

Caspase-2 is the only known caspase that shuttles to the nucleus. The nuclear localization of caspase-2 zymogen is driven by the NLS, which is located between the 14-3-3-binding motifs within the linker connecting CARD and p19 domains [10–12]. 14-3-3 proteins are well-known regulators of the subcellular localization of their binding partners. Furthermore, because both 14-3-3-binding motifs and the NLS are located in the same region of caspase-2, 14-3-3 binding may play a role in the subcellular localization of caspase-2. However, such mode of regulation would likely require a direct interaction between 14-3-3 and NLS.

Fig. 7. Interactions between 14-3-3 and the 14-3-3-binding motifs of caspase-2. (A) Crystal structure of the 14-3-3ζ:pepS139 complex. The 2Fo–Fc electron density map is contoured at 1σ. (B) Detailed view of contacts between 14-3-3ζ and the pepS139 peptide. The caspase-2 residues are labeled in red, and the 14-3-3ζ residues are labeled in black. (C) Crystal structure of the 14-3-3ζ:pepS164 complex. The 2Fo–Fc electron density map is contoured at 1σ. (D) Detailed view of contacts between 14-3-3ζ and the pepS164 peptide. The caspase-2 residues are labeled in red, and the 14-3-3ζ residues are labeled in black.
Our structural model of the proC2:14-3-3ζ complex suggested that the NLS region of caspase-2 is buried within the central channel of the 14-3-3 dimer constricted between the bottom of the channel and the p12 domain of proC2 (Fig. 2A). This position of NLS region on the interface between 14-3-3 and p12 was confirmed by chemical cross-linking experiments (Fig. 2B,C and Tables S1 and S2). In addition, fluorescence quenching and time-resolved fluorescence measurements of tryptophan residue inserted at position 151 within the NLS revealed a significant suppression of both its flexibility and solvent accessibility (Fig. 5C, D and Tables 2 and S3). Thus, these results indicated that the 14-3-3 protein binding masks the NLS region of caspase-2. However, whether this steric occlusion translates into the inhibition of caspase-2 nuclear localization in vivo, remains to be shown.

The SAXS-based structural analysis together with chemical cross-linking, NMR and fluorescence spectroscopy data revealed that phosphorylated proC2 and 14-3-3ζ form a compact and rigid complex in which the proC2 p19 and p12 domains are positioned within the central channel of the 14-3-3 dimer, where their position appears to be stabilized through interaction with the C-terminal helices H9 of both 14-3-3ζ protomers (Figs 1, 4 and 5). Compared with previously reported structures of 14-3-3 protein complexes, this architecture resembles the structures of complexes between 14-3-3ζ and serotonin N-acetyltransferase [50] and between 14-3-3ζ and the DNA-binding domain of the forkhead transcription factor FOXO4 [31] (Fig. 8). In addition, crystallographic analysis revealed that the phosphate group recognition, the main-chain conformation, and other contacts of both previously suggested 14-3-3-binding motifs of caspase-2 are similar to those observed in other 14-3-3 protein complexes [25,36,38,48,49]. Therefore, both phosphorylated motifs containing pSer139 and pSer164 are indeed the 14-3-3-binding motifs.

In conclusion, the structural analysis of the 14-3-3:caspase-2 complex reported in this study suggested that 14-3-3 protein binding may inhibit caspase-2 activation through interference with caspase-2 oligomerization and/or its nuclear localization by sterically occluding caspase-2 p12 domain as well as NLS, which is bordered by the two phosphorylated 14-3-3-binding motifs of caspase-2. Thus, these results corroborate the hypothesis that 14-3-3 binding is an important

---

Fig. 8. Comparison of the structural model of the 14-3-3ζ:proC2 complex (A) with the crystal structure of the 14-3-3ζ:AANAT complex (B) [50] and with the model of the 14-3-3ζ:FOXO4-DBD complex (C) based on FRET experiments [31].
regulatory element of caspase-2 activation. Further research should be directed to study the effect of 14-3-3 on the caspase-2 dimerization and cellular localization in vivo.

Materials and methods

Heterologous expression, purification and phosphorylation of proC2

Human proC2 was expressed, purified and phosphorylated as previously described [28]. The mutants of proC2 containing a single-tryptophan residue (mutations Y151F, F188W, F218W, and F426W) were created using the QuikChange™ approach (Stratagene, San Diego, CA, USA). All mutations were confirmed by sequencing.

Heterologous expression and purification of 14-3-3 protein isoforms

The mutant version of human 14-3-3αnoW with no tryptophan residues (mutations W59F and W228F) and 14-3-3 ϵAC (residues 1–230) were prepared as previously described [44,50].

Crystallization, data collection, and structure determination

The 14-3-3γΔC and the pepS139 (sequence YDLpSer139LPFP) or pepS164 (sequence VEHpSer164LDNK) were mixed in a 1:10 M stoichiometry, respectively, in a buffer containing 20 mM HEPES (pH 7.0), 2 mM MgCl2 and 2 mM TCEP. Crystallization was performed using the hanging-drop vapor-diffusion method at 293.15 K. Crystals of the 14-3-3γΔC:pepS139 peptide complex were grown from drops consisting of 3 μL of 8 mg·mL⁻¹ protein and 3 μL of 100 mM Tris-HCl (pH 8.5), 32.5% (w/v) PEG 4000 and 200 mM sodium acetate. Crystals of the 14-3-3γΔC:pepS164 peptide complex were grown from drops consisting of 2 μL of 8 mg·mL⁻¹ protein and 4 μL of 100 mM Tris-HCl (pH 8.5), 32.5% (w/v) PEG 4000, 200 mM lithium sulfate. Crystals were cryoprotected using 20% (v/v) PEG 400 and flash frozen in liquid nitrogen before data collection in oscillation mode at beamline 14.2 of the BESSY synchrotron. Diffraction data processing was performed using the packages XDS and XDSAPP [51,52]. Crystal structures of both complexes were solved by molecular replacement in MOLEPREP [53], using the structure 14-3-3γ (PDB ID: 2B05) as search models, and refined at resolutions of 2.6 and 2.85 Å, respectively, with PHENIX [54]. The atomic coordinates and structure factors of both complexes have been deposited in the RCSB PDB with accession codes 6GKF and 6GKG. All structural figures were prepared with PyMOL (https://pymol.org/2/).

Small angle X-ray scattering (SAXS)

SAXS data were collected on the European Molecular Biology Laboratory (EMBL) P12 beamline on the storage ring PETRA III (Deutsches Elektronen Synchrotron (DESY), Hamburg, Germany). The proC2 and 14-3-3δ were measured in the concentration ranges of 0.5–3.7 and 1.2–8.1 mg·mL⁻¹, respectively. ProC2:14-3-3δ protein complex was measured in the concentration range of 0.9–6.6 mg·mL⁻¹ in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM TCEP, 3% (w/v) glycerol. Data analysis was performed using the ATSAS software suite (ATSAS software, EMBL, Hamburg, Germany). The data were averaged after normalization to the intensity of the transmitted beam, and the scattering of the buffer was subtracted using PRIMUS [55]. The forward scattering I(θ) and the radius of gyration Rg were evaluated using the Guinier approximation. The distance distribution function P(r) and the maximum particle dimension Dmax were determined by the indirect Fourier transformation of the scattering data I(s) using GNOM [56]. The solute apparent molecular mass (MMeq) was estimated by comparing the forward scattering with that from reference solutions of bovine serum albumin (molecular mass 72 kDa). The Porod volume, Vp (excluded volume of the hydrated particle), ab initio molecular envelopes were computed using DAMMIF. For each protein, 20 surfaces were generated and averaged using DAMAVER [57]. The averaged surfaces were then used as the final SAXS three-dimensional structure. Calculated molecular envelopes were superimposed to the structural models using the SUPCOMB program EMBL, Hamburg, Germany.

Structural modeling

The three-dimensional model of proC2 (residues 123–452) was generated using the AllosMod-FoXS method, SAXS data, and distance restraints calculated by intermolecular cross-links between 14-3-3γ and the proC2 p12 domain [58]. For proC2 (123–452) modeling, the crystal structure of caspase-2 was used (PDB ID: 3R7S). The starting model of the complex was prepared using the crystal structure of caspase-2 and the 14-3-3γ with phosphopeptides (PDB ID: 1QJB). The conformation of the proC2N-terminal segment containing both 14-3-3-binding motifs and its interaction with the 14-3-3 dimer were modeled using the structure of the Nth1:14-3-3 complex (PDB ID: 5N6N). The best-scoring AllosMod model was chosen according to the lowest χ² and the distances derived from intermolecular cross-links.

NMR spectroscopy

NMR spectra were acquired at 298 K on a Bruker Avance III™ HD 850 MHz spectrometer equipped with a 1H/13C/15N cryoprobe. For NMR experiments, 15N-labeled
14-3-3\textsubscript{C} was expressed in enriched minimal medium containing 15\textsuperscript{N}-ammonium sulfate. The sample volume was 160 \textmu L of 150 \textmu M 15\textsuperscript{N}-labeled 14-3-3\textsubscript{C} in the NMR buffer containing 50 mM sodium phosphate at pH 6.8, 100 mM NaCl and 10\% D\textsubscript{2}O/90\% H\textsubscript{2}O. One hundred and sixty out of 245 (65\%) backbone amide signals in the 2D \textsuperscript{1}H-15\textsuperscript{N} HSQC spectrum of 14-3-3\textsubscript{C} were assigned based on published sequence-specific backbone NMR assignment [41]. The binding of proC2 was evaluated by NMR titrations of 150 \textmu M 15\textsuperscript{N}-labeled 14-3-3\textsubscript{C} with unlabeled proC2. A reference and four titration points were collected in 2 : 0.25, 2 : 0.5, 2 : 0.75, and 2 : 1 (14-3-3\textsubscript{C}:proC2) molar ratios. \textsuperscript{1}H-15\textsuperscript{N} HSQC spectra were acquired at experimental times ranging from 90 min (16 scans, initial concentration) to 24 h (256 scans, final concentration).

**Differential scanning fluorimetry**

ThermoFluor assay was performed using real-time PCR LightCycler 480 II (Roche, Basel, Switzerland) at a final protein concentration of 1 mg mL\textsuperscript{-1}, as previously described [59].

**Chemical cross-linking combined with mass spectrometry**

ProC2 in the complex with 14-3-3\textsubscript{C} was cross-linked using DSS and DSG cross-linkers, and they were analyzed as described previously [40,60]. For chemical cross-linking, all proteins were transferred to 20 mM HEPES (pH 7.5) buffer with 150 mM NaCl and 1 mM TCEP, assessing the following protein concentrations: 0.37 mg mL\textsuperscript{-1} proC2(dp) and 0.53 mg mL\textsuperscript{-1}14-3-3\textsubscript{C}. Eluting peptides were separated at 60 °C on a reversed-phase analytical Acclaim PepMap\textsuperscript{TM} 100, C18 column (0.075 × 150 mm, 3 \textmu m; Thermo Fisher Scientific, Waltham, MA, USA) UltiMate3000 RSLCnano System ( Dionex, Sunnyvale, California, USA). The nano-UHPLC system was coupled to the CaptiveSpray ion source of a Solarix XR FT-ICR mass spectrometer (Bruker Daltonics, Billerica, Massachusetts, USA) equipped with a 12-T superconducting magnet. Data were acquired in positive broad-mode over the m/z range 245–2000, with 1M data points transient and 0.4 s ion accumulation and four scans were accumulated per spectrum. Data acquisition was performed using Solarix Control program, Bruker Daltonics, Billerica, MA, USA.

**Limited proteolysis and mass spectrometric analysis of proC2**

Samples containing 11.8 \textmu g of proC2 or 25.4 \textmu g 14-3-3\textsubscript{C} and the proC2:14-3-3\textsubscript{C} complex were digested by trypsin or chymotrypsin for 10, 20, and 30 min at 25 °C in a buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM TCEP, 10\% (w/v) glycerol (protease/protein ratio was 1 : 1000, w/w). Undigested protein was used as a zero time point. The reactions were terminated by boiling in the presence of SDS/PAGE loading buffer. Protein bands were cut off the gel and destained. Cysteines were in gel reduced with 100 mM DTT for 45 min at 60 °C, and free cysteines were alkylated with 100 mM iodoacetamide for 30 min at room temperature in the dark. Trypsin digestion proceeded overnight at 37 °C. Extracted peptides were analyzed by MALDI-FT-ICR mass spectrometer (Bruker Daltonics). Data were processed by mMass software [61].

**Time-resolved fluorescence and tryptophan quenching**

Time-resolved fluorescence intensity, fluorescence anisotropy, and tryptophan fluorescence quenching experiments were performed and analyzed as previously described [62–64]. For time-resolved measurements, tryptophan fluorescence was excited, at 298 nm, using a frequency-tripled Ti: sapphire laser, and the emission was detected at 355 nm using monochromator with a stack of UG1 and BG40 glass filters placed in the front of input slit. Decays were accumulated in 1024 channels with a time resolution of 78 ps/channel, until typically 10\(^7\) counts per decay were reached. Steady-state fluorescence quenching experiments were performed in a ISS PC1 photon counting fluorimeter, using a 1-nm bandpass on both excitation and emission monochromators. Stern-Volmer plots were constructed from changes in fluorescence intensity after adding acrylamide aliquots dissolved in the protein buffer. Trp emission was excited and measured at 297 and 340 nm, respectively; data were fitted and fitted with Eqn (1):

\[
\frac{I_0}{I} = 1 + k_q \tau_{\text{mean}} |Q| e^{VQ},
\]

where \(I_0\) and \(I\) is the fluorescence intensity in the absence and presence of quencher, respectively. \(|Q|/|V|\) is acrylamide concentration; \(k_q\) is the bimolecular quenching constant, \(\tau_{\text{mean}}\) denotes the mean fluorescence lifetime in the absence of quencher, and \(V\) is the static quenching constant. \(\tau_{\text{mean}}\) was calculated as \(\tau_{\text{mean}} = \Sigma f_i \tau_i\), where \(f_i\) is an intensity fraction of the \(i\)-th lifetime component \(\tau_i\). Corrections for the inner filter effect were performed as previously described [65]:

\[
I_c = I \text{antilog} \left( \frac{A_{ex} + A_{em}}{2} \right).
\]
Acknowledgments

We thank EMBL SAXS beamline P12 (Petra III DESY, Hamburg) for the allocated experimental beam time. We thank Prof Jaroslav Vecer for the help with tryptophan fluorescence data analysis, Dr Vaclav Veverka with NMR analysis, Dr Petr Novak with cross-linking analyses, as well as Dr Carlos V. Melo for proofreading the article.

Funding

This study was funded by the Czech Science Foundation (VO, grant number 17-00726S), the Grant Agency of Charles University (DK, grant number 296216), the Czech Academy of Sciences (RVO:67985823 of the Institute of Physiology), project BIOCEV (CZ.1.05/1.1.00/28.0109) and MEYS CR (LM2015043 CIISB, Biocev, Biophysical methods, Structural Mass Spectrometry). PH acknowledges EU Operational Program OP VaVpI No. CZ.1.05/4.1.00/16.0340 and Center of Nano- and Bio-Photonics UNCE/SCI/010.

Author contributions

VO and TO designed research; AS, MA, DK, KP, MR, PH, and VO performed research; AS, KP, MR, PH, TO, and VO analyzed data; and TO and VO wrote the paper.

Conflict of interests

The authors declare that they have no competing interests.

References


Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Intermolecular cross-links between proC2 and 14-3-3ζ using DSG.

**Table S2.** Intermolecular cross-links between proC2 and 14-3-3ζ using DSS.

**Table S3.** Results of acrylamide quenching of tryptophan fluorescence.