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REVIEW

Look for the Scaffold: Multifaceted Regulation of Enzyme Activity by 14-3-3 Proteins

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Summary

Enzyme activity is regulated by several mechanisms, including phosphorylation. Phosphorylation is a key signal transduction process in all eukaryotic cells and is thus crucial for virtually all cellular processes. In addition to its direct effect on protein structure, phosphorylation also affects protein-protein interactions, such as binding to scaffolding 14-3-3 proteins, which selectively recognize phosphorylated motifs. These interactions then modulate the catalytic activity, cellular localisation and interactions of phosphorylated enzymes through different mechanisms. The aim of this mini-review is to highlight several examples of 14-3-3 protein-dependent mechanisms of enzyme regulation previously studied in our laboratory over the past decade. More specifically, we address here the regulation of the human enzymes ubiquitin ligase Nedd4-2, procaspase-2, calcium-calmodulin dependent kinases CaMKK1/2, and death-associated protein kinase 2 (DAPK2) and yeast neutral trehalase Nth1.

Key words

14-3-3 protein • Scaffold • Enzyme • Kinase • Procaspase-2 • Nedd4-2 • CaMKK2 • DAPK2

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Introduction

Scaffold proteins are major regulators of many signalling pathways, playing a key role in cell biology. These proteins form platforms that facilitate the assembly of protein complexes, re-localisation of signalling molecules, and coordination of positive or negative feedback signals and ensure the correct course of signalling pathways. The first scaffold protein discovered was the yeast MAPK scaffold Ste5, which helps to link multiple protein kinases in the MAP kinase cascade in *S. cerevisiae* [1]. Binding of the scaffold molecule to the target protein is often facilitated by various stimuli, one of which is phosphorylation, the most prevalent post-translational modification in eukaryotes [2]. Protein phosphorylation occurs mostly on the side chains of serine, threonine and tyrosine residues, and the introduction of a negatively charged phosphate group then directly affects the structure of the protein and/or mediates new interactions, often through scaffold domains or proteins that specifically recognize phosphorylated motifs. Such proteins/domains include, among others, 14-3-3 proteins, WW, Polo-box, WD-40, leucine-rich repeats, BRCT and FHA domains [3].

14-3-3 proteins were discovered through studies on the phosphorylation-dependent regulation of tryptophan hydroxylase and Raf kinase [4-9]. 14-3-3 proteins are a family of highly evolutionarily conserved 30 kDa acidic proteins abundantly expressed in all eukaryotes, often in multiple isoforms, which form homo- and heterodimers [10-13]. In humans,

seven 14-3-3 protein isoforms denoted by the Greek letters (β, γ, ε, ζ, η, σ and τ) were identified. Numerous structural studies have shown that the 14-3-3 dimer adopts a rigid cup-shaped structure capable of accommodating two phosphorylated segments of the target protein(s) [8,14-17]. 14-3-3 binding motifs are often found in disordered regions of target proteins and bordering structured domains. Moreover, the affinity of the interaction between 14-3-3s and a particular phosphorylated motif appears to be strictly influenced by the sequence surrounding the phosphoresidue [18-20].

Three canonical 14-3-3 binding motifs have been described thus far: R[S/Φ][+](pS/pT)XP (mode I), RX[S/Φ][+](pS/pT)XP (mode II) and (pS/pT)X_{1-2}-COOH (mode III), where Φ means any hydrophobic amino acid and X means any amino acid [8,17,21]. However, the motifs of many well-characterised 14-3-3 binding partners deviate significantly from these canonical motifs. In fact, 14-3-3 proteins have also been shown to recognize unphosphorylated or glycosylated motifs (reviewed in [13]). Recently, 14-3-3 protein have also been shown to target viral proteins, such as N protein from SARS-CoV-2, as demonstrated by crystal structure and SAXS-based analysis [22-24]. This, together with the involvement of 14-3-3 proteins in the regulation of a number of other pathophysiological processes, suggests that 14-3-3 proteins are a promising drug target.

Thanks to advances in structural studies of 14-3-3 protein complexes, we now have a much better understanding of how these scaffolds regulate their binding partners. The mechanisms of these regulations can be divided into three basic modes: (1) direct conformational change of the target protein; (2) partial masking of the surface of the target protein; and (3) scaffolding facilitating the interaction between two proteins, as previously reviewed in [11-13,25-28]. In some cases, the regulatory mechanism is based on a combination of different modes. This mini-review focuses on mechanisms of 14-3-3-medited regulation of several enzymes recently studied in our research groups: (i) the yeast neutral trehalase Nth1; (ii) the ubiquitin ligase Nedd4-2; (iii) procaspase-2; (iv) death-associated protein kinase 2 (DAPK2); and (v) calcium-calmodulin dependent kinases (CaMKK).

14-3-3 protein complexes are often highly conformationally flexible and heterogeneous. For this reason, we use hybrid approaches to study these complexes, combining several structural biology methods such as protein crystallography, NMR, small-angle X-ray scattering (SAXS), chemical crosslinking and H/D exchange. While Nth1 activation and Nedd4-2 modulation exemplify mode 1 (direct structural change), caspase-2 activation inhibition is an example of mode 2 (masking of surfaces important for the localisation and activation of the enzyme). The protein kinases CaMKK and DAPK2 are inhibited similarly, with 14-3-3 proteins masking their key regulatory phosphorylation sites, the calmodulin binding site and the access to the catalytic center. In addition, 14-3-3 proteins further contribute to DAPK2 inhibition by stabilizing its dimerization, i.e., *via* regulatory mode 3.

Allosteric activation of yeast neutral trehalase Nth1

The main function of the neutral trehalase 1 (Nth1) from *Saccharomyces cerevisiae* is the hydrolysis of trehalose (α-D-glucopyranosyl-(1-1)-α-D-glucopyranoside) into two glucose molecules, which helps the yeast survive adverse conditions such as heat shock, starvation or oxidative stress [29-32]. Nth1 has two structured domains: a calcium-binding domain containing an EF-hand-like motif and a catalytic trehalase domain, which is responsible for the enzymatic activity. In addition, the unique N-terminal extension of Nth1, present only in the yeast trehalases, contains five serine residues whose phosphorylation by cAMP-dependent protein kinase (PKA) or by cyclin-dependent kinase 1 (Cdk1) generates 14-3-3 binding motifs [33-36]. The catalytic activity of Nth1 is entirely controlled by phosphorylation-dependent 14-3-3 (in yeast Bmh1/2) protein binding, with Ca^{2+} binding playing a modulatory role [34-39]. This regulatory mechanism has been shown to be common to yeast neutral trehalases from both budding yeast, such as *Saccharomyces cerevisiae* [35,40] or *Kluyveromyces lactis* [41] and fission yeast, such as *Schizosaccharomyces pombe* [37] or *Candida albicans* [42].

Nth1 is phosphorylated during the transition from the G1 to the S phase of the cell cycle through interplay between PKA and Cdk, which determines the ratio of different Nth1 phospho-isoforms (Fig. 1A) [43-46]. PKA phosphorylates Ser60 and Ser83, both of which are docking sites for 14-3-3 proteins [36]. However, PKA also phosphorylates residues Ser20 and Ser21, which also participate in Nth1 activation as they may represent a binding site for phosphatase PP2A, whose binding is reduced in the phospho-state (pSer20/pSer21). Under nutrient-rich conditions, when

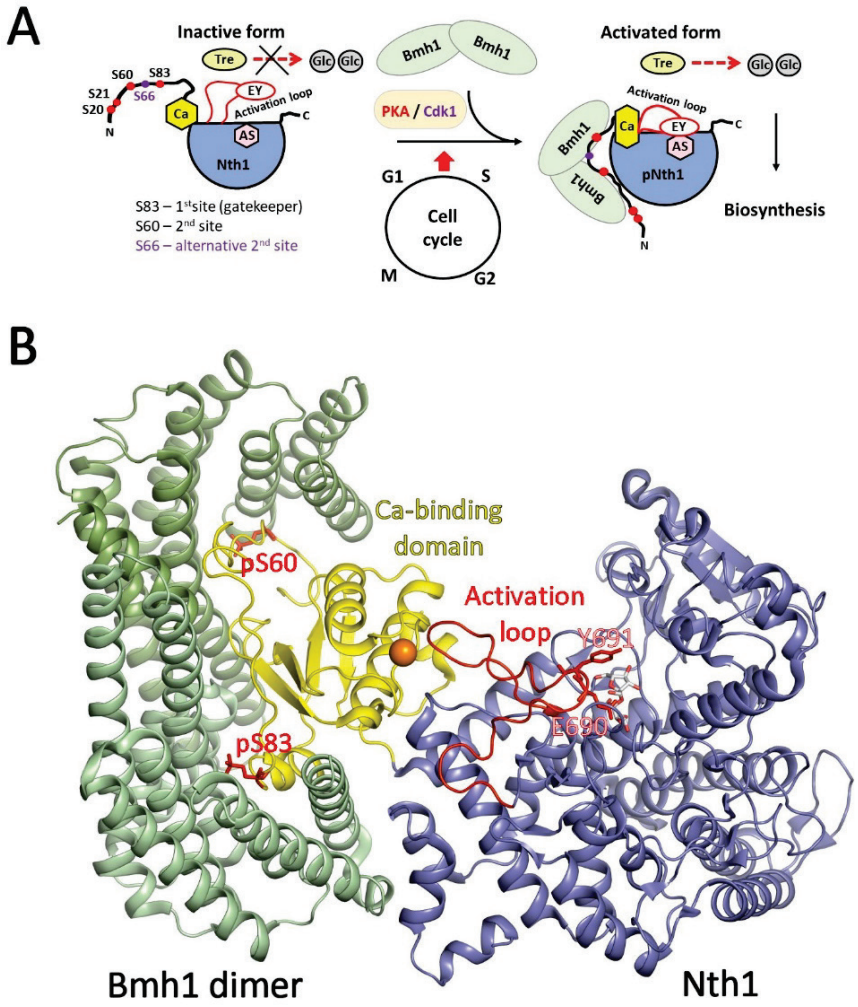

Fig. 1. Allosteric regulation of Nth1 by 14-3-3 protein. (**A**) Schematic model of 14-3-3-dependent regulation of Nth1. The inactive form of Nth1 is unable to hydrolyze trehalose to glucose. The N-terminal segment of Nth1 contains five phosphorylation sites, of which four are recognized by PKA and the other by Cdk1. The Ser83-containing motif serves as a gatekeeper, the dominant site for 14-3-3 protein binding, while the Ser60-containing motif functions as a secondary site [36,38,40]. An alternative secondary site is the Ser66-containing motif phosphorylated by Cdk1. The Ser20 and Ser21 sites are part of the binding site for the phosphatase, but this interaction is suppressed by phosphorylation [44]. Phosphorylated Nth1 is recognized by the yeast 14-3-3 protein during the G1 to S cell cycle transition [43]. 14-3-3 protein binding induces a conformational change in both the calcium and catalytic domains, creating a loop with all the residues necessary for catalysis. The active form of Nth1 then begins to metabolize trehalose to glucose. (**B**) Crystal structure of the Bmh1:Nth1 complex (PDB: 5N6N) [40]. The protomers of the Bmh1 homodimer are shown in two shades of green. The N-terminal extension and the calcium-binding domain are shown in yellow, and the catalytic domain is highlighted in blue. The phosphorylated Ser60 and Ser83, the regulatory loop and its two residues crucial for catalysis (Glu690 and Tyr691) are shown in red. The calcium ion is shown in orange. The figure was prepared with PyMOL (https://pymol.org/2/).

PKA has high activity, all four PKA sites Ser20, Ser21, Ser60 and Ser83 are phosphorylated. However, during slow growth, Ser60 is not efficiently phosphorylated by PKA, but Cdk phosphorylates a nearby Ser66 under these conditions, allowing its use as an alternative binding site for the 14-3-3 protein [44]. Thus, 14-3-3 protein binding to Nth1 is likely mediated by pSer60+pSer83 or pSer66+pSer83 phospho-motif pairs through the joint action of PKA and Cdk.

The molecular mechanism of 14-3-3 protein-mediated Nth1 activation was elucidated by solving the crystal structure of the complex of Nth1 phosphorylated at Ser60 and Ser83 with the yeast 14-3-3 protein Bmh1 [40]. This structure revealed that the Bmh1 dimer binds both phosphorylated motifs of the N-terminal segment of Nth1 and acts as an allosteric modulator by allowing the calcium-binding domain to interact with the catalytic domain. The interaction of these domains then stabilizes the conformation of the flexible loop (Fig. 1B, shown in red), which contains residues crucial for catalysis. In the absence of Bmh, the N-terminal segment of Nth1, including the calcium-binding domain, is flexible and does not interact with the catalytic domain. As a result, the loop containing the catalytic residues is disordered, so these residues are absent from the active center of the enzyme. Furthermore, Ca^{2+} binding to the site at the interface between the calcium-binding and catalytic domains further stabilizes interactions between these domains, thus explaining the potentiating effect of Ca^{2+} binding on Nth1 activity. This mechanism is consistent with our previous results from hydrogen-deuterium exchange coupled to mass spectrometry (HDX-MS), which have suggested that both Nth1 domains, especially the Ca-binding domain, undergo a significant conformational change upon 14-3-3 and/or Ca^{2+} protein binding [38,47].

Structural modulation of human ubiquitin ligase Nedd4-2

The main function of Neural precursor cell expressed developmentally downregulated 4 (Nedd4) E3 ligases is to tag proteins for ubiquitination as the final step in the ubiquitination cascade [48,49]. The Nedd4-2 ubiquitin ligase belongs to the Nedd4 family, whose members have a similar domain structure consisting of three distinct domains, namely the N-terminal C2 domain, which is responsible for membrane binding, two to four WW domains, responsible for binding to proline-rich sequence (PY motif) of protein substrates, and the catalytical HECT domain [50]. Nedd4-2 activity must be tightly regulated due to its pivotal role in animal physiology as this E3 ligase targets numerous membrane proteins [51,52]. We and others have shown that Nedd4-2 is regulated through phosphorylation and 14-3-3 protein binding, with three 14-3-3 binding motifs that contain Ser342, Ser367 and Ser448 and border the WW2 domain (Fig. 2A) [53-56].

Detailed biophysical analysis of the interaction between Nedd4-2 and 14-3-3η using analytical ultracentrifugation and protein crystallography confirmed the need for bidentate Nedd4-2 binding to the 14-3-3 protein dimer [56]. The highest binding affinity was observed in Nedd4-2 phosphorylated at Ser342 and Ser448. These sites are phosphorylated by SGK or PKA, and the motif containing pSer448 is the dominant site, also known as the gatekeeper. As shown by structural characterization of the Nedd4-$2_{335-455}$ and its complex with 14-3-3η using small angle X-ray scattering (SAXS) and chemical cross-linking, in the absence of 14-3-3η, the WW2 and WW3 domains interact with the HECT domain. Conversely, the WW3 domain is sequestered into the central channel of the 14-3-3η dimer in the complex [56]. In the complex, the WW3 and WW4 domains are sterically blocked and less mobile, whereas the catalytic site in the C-lobe of the HECT domain is more exposed and mobile, key findings that were revealed in a subsequent study based on time-resolved fluorescence measurements with 1,5-IAEDANS-labeled variants of Nedd4-2 [57]. Complex formation had little effect on the mobility of the WW2 domain and no effect on the WW1 domain, whose mobility remains unchanged.

In conclusion, 14-3-3 protein binding induces a structural rearrangement of Nedd4-2 by affecting interactions between its structured domains. As such, 14-3-3 proteins regulate the dynamic processes of membrane protein ubiquitination depending on the accessibility of individual WW domains of Nedd4-2. In addition, our data provided a solid platform for subsequent studies aimed at targeting the Nedd4-2:14-3-3 complex for potential therapeutic purposes, e.g., for the treatment of Nedd4-2-related diseases such as Parkinson's and renal disease, hypertension, and other conditions [58,59].

14-3-3 inhibits procaspase-2 activation by masking its dimerization surface

Procaspase-2 (proC2) functions as an initiator caspase, linking cellular metabolism to apoptosis [60,61]. But while most studies associated proC2 with tumor suppression, proC2 can also promote cancer by triggering neuroblastoma development [62]. Structurally, proC2 consists of three domains, namely the N-terminal caspase activation and recruitment domain (CARD) and large (p19) and small (p12) subunits [63]. In turn, proC2 activation requires dimerization and autocatalytic processing to form the p19:p12 mature active caspase-2 dimer [64].

The increase in NADPH levels generated by the pentose phosphate pathway leads to proC2 phosphorylation at Ser139 (human caspase-2 numbering) by calcium/calmodulin-dependent kinase II. This phosphorylation prevents the interaction between proC2 and the death domain-containing protein CRADD and enables protein 14-3-3 binding [65,66], thereby blocking both caspase-2 activation and apoptosis. Moreover, caspase-2 is also the most conserved and the only caspase known to be regulated by 14-3-3 protein [67].

As shown by various biochemical and biophysical studies, the interaction of human proC2 with 14-3-3 proteins is controlled by pSer139- and pSer164-containing motifs located in the linker between the CARD and p19 domains [68]. Both motifs are required for proC2 to establish stable interactions with 14-3-3 proteins, and pSer139 is the dominant site. An important aspect of caspase-2 activation is its localization. As the only caspase that is mainly located in the nucleus and Golgi complex, caspase-2 is activated both in the nucleus and in the cytoplasm [69-72]. Interestingly, the nuclear localisation signal (NLS) of caspase-2 is located between the two 14-3-3 binding motifs, suggesting that 14-3-3 protein binding could mask this signal sequence. Indeed, structural analysis of the proC2:14-3-3 complex using a combination of fluorescence spectroscopy, SAXS, NMR, chemical crosslinking, H/D exchange

coupled to MS and X-ray crystallography has revealed that 14-3-3 protein binding masks two important regions of proC2, NLS and the surface of the p12 domain around Cys436, which is involved in caspase-2 dimerization during its activation (Fig. 2B) [73,74]. Thus, our results indicate that 14-3-3 proteins play a dual role in regulating caspase-2 activation as they (i) block its activation by masking its dimerization surface and (ii) influence its cellular localisation by blocking its NLS.

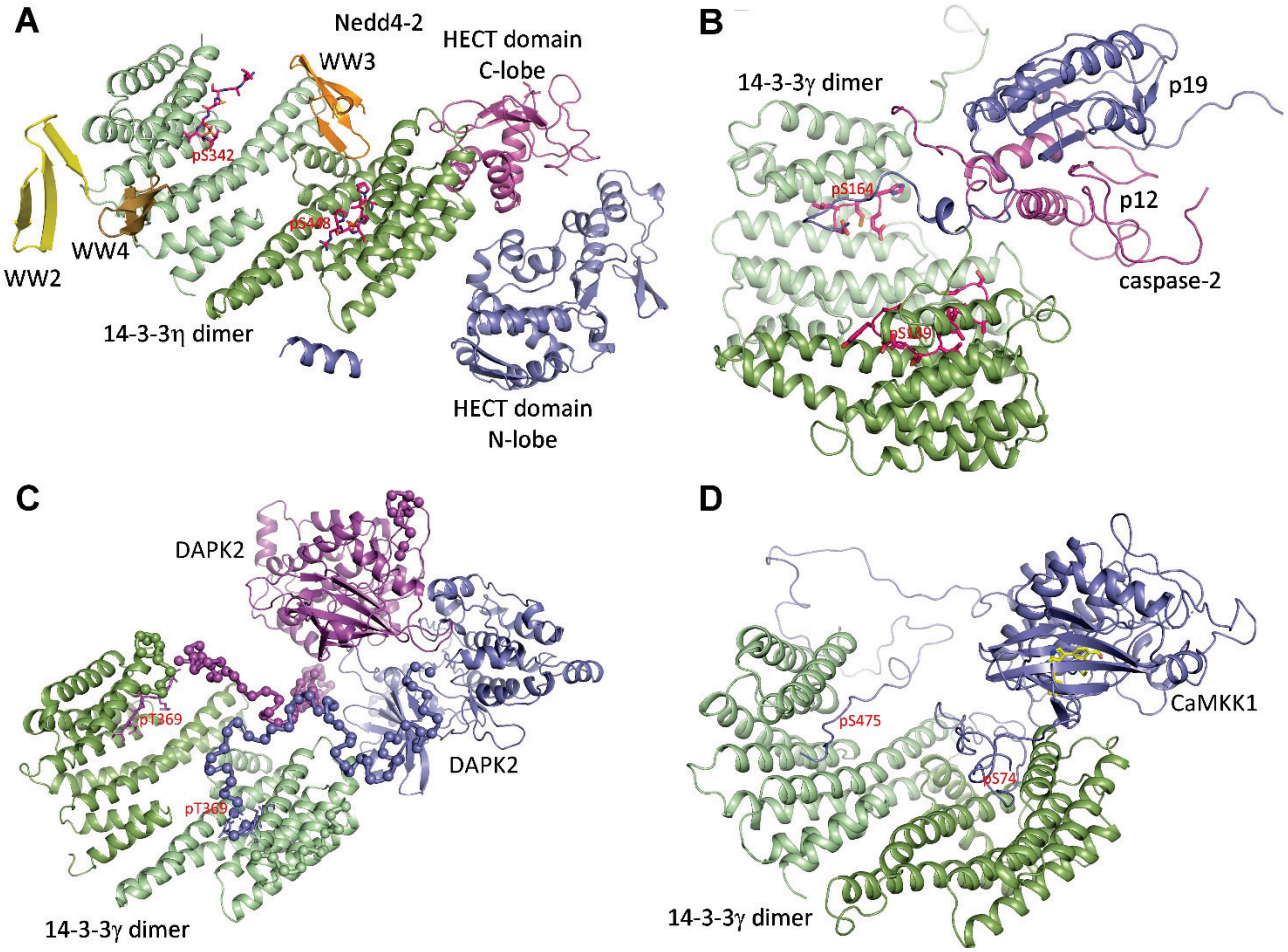

Fig. 2. Ribbon representation of SAXS-based structural models of select 14-3-3 protein complexes. The protomers of the 14-3-3γ and 14-3-3η homodimer are shown in two shades of green in all panels. (**A**) The best-scoring CORAL model of the Nedd4-2:14-3-3η complex constructed using crystal structures of HECT domain (PDB ID: 5HPK) [96], solution structures of WW1-3 domains (PDB ID: 1WR3, 1WR4, 1WR7) and 14-3-3γ with bound Nedd4-2 phosphopeptides pSer342 and pSer448 (PDB ID 6ZBT, 6ZC9)[56]. WW2-4 are shown in yellow, orange and sand, the N- and C-lobes of the HECT domain are shown in blue and magenta, respectively. (**B**) The best-scoring AllosMod-FoXS model of the procaspase-2:14-3-3γ complex [73] constructed using the crystal structure of caspase-2 (PDB ID: 3R7S) [97] and the 14-3-3γ with bound caspase-2 peptide phosphorylated on Ser139 and Ser164 (PDB ID 6SAD) [74]. p19 and p12 domains of procaspase-2 are shown in blue and magenta, respectively. (**C**) The best-scoring CORAL model of the DAPK2:14-3-3γ complex constructed using crystal structures of autoinhibited DAPK2 (PDB ID: 2A2A) [80,81] and 14-3-3γ with bound C-terminal DAPK2 phophopeptidepeptide pThr369 (PDB ID: 7A6R) [83]. The unstructured segments missing in the crystal structures were modelled as dummy residue chains, shown as spheres. Two protomers of DAPK2 dimer are shown in blue and magenta. (**D**) The best-scoring AllosMod-FoXS model of the pCaMKK1:14-3-3γ complex constructed using crystal structures of the kinase domain of CaMKK1 (PDB ID: 6CD6) and 14-3-3γ with bound CaMKK2 phosphopeptides (PDB ID: 6FEL and 6EWW) [94,95]. The position of the active site is indicated by the position of inhibitor in the crystal structure of CaMKK1 (yellow sticks). The figure was prepared with PyMOL (https://pymol.org/2/).

Mechanism of 14-3-3-mediated inhibition of human death-associated protein kinase 2 (DAPK2)

The main function of death-associated protein kinases (DAPK) is to control various cellular processes, including membrane blebbing, apoptosis, and autophagy [75,76]. This family of Ca^{2+}/calmodulin (CaM) dependent Ser/Thr protein kinases includes five members that differ in their cellular localization and interactome [77].

DAPK2 is the smallest member and consist of an N-terminal kinase domain, followed by an autoinhibitory region (AID) and a Ca^{2+}/CaM domain (CBD) [78,79]. DAPK2 activity is controlled by autoinhibition, autophosphorylation, dimerization and interaction with 14-3-3 proteins *via* the unique C-terminal tail containing a C-terminal (mode III) 14-3-3 binding motif around Thr369 [80,81].

DAPK2 activation requires a protomeric form of the enzyme either capable of binding Ca^{2+}/CaM or with AID phosphorylated at Ser299 to prevent AID-induced autoinhibition from blocking the catalytic site [78,82]. Our structural analysis of the DAPK2:14-3-3γ complex suggested that 14-3-3 inhibits DAPK2 by directly interacting with the AID and CBD segments of DAPK2 and by stabilising DAPK2 dimerization, which obstructs access to the active site (Fig. 2C) [80,83]. As a result, complex formation destabilises the interaction between DAPK2 and Ca^{2+}/CaM and protects the DAPK2 inhibitory autophosphorylation site Ser318 located in CBD from dephosphorylation, further preventing Ca^{2+}/CaM binding.

We have also shown that the interaction between 14-3-3γ and the C-terminal 14-3-3 binding motif of DAPK2 can be stabilized by the diterpene glycoside phytotoxin fusicoccin A (FC-A). FC-A fills a gap in the interface between the 14-3-3 ligand binding groove and the 14-3-3 binding motif of the client protein [84]. Thus, our findings provide mechanistic insights into 14-3-3-mediated DAPK2 inhibition and highlight the potential of the DAPK2:14-3-3 complex as a target for anti-inflammatory therapies.

Differential regulation of Ca^{2+}/CaM-dependent protein kinase kinases 1 and 2 by 14-3-3 proteins

CaMKK1 and CaMKK2 play a key role in many physiological and pathological processes in the cell by phosphorylating and activating downstream protein kinases CaMKI, CaMKIV and protein kinase B, thereby triggering the phosphorylation of their downstream targets [85-87]. Both CaMKKs share a common domain structure consisting of a kinase domain, a regulatory region with an autoinhibitory segment (AID) and a binding region for the calcium/calmodulin complex (CBD) [88]. Both CaMKKs are partly inhibited by PKA-mediated phosphorylation at multiple sites bordering the kinase domain [89]. In addition, phosphorylation by PKA creates two motifs for 14-3-3 protein binding to phosphorylated CaMKKs [90-92]. The high-affinity 14-3-3 binding motif is located at the C-terminus containing Ser475 and Ser511 in CaMKK1 and CaMKK2, respectively [92,93]. The second motif, which binds to 14-3-3 proteins with lower affinity and further stabilises complex formation, is located at the N-terminus and contains Ser74 and Ser100 in CaMKK1 and CaMKK2, respectively.

Previous studies have suggested that 14-3-3 binding protects the inhibitory phosphorylation site located in the CBD of both CaMKKs from dephosphorylation, thereby maintaining CaMKKs in a partly inhibited state mediated by PKA [90,92,94]. However, complex formation differently affects the remaining catalytic activity of phosphorylated CaMKKs. While the activity of phosphorylated CaMKK1 is significantly reduced by 14-3-3 binding, the activity of phosphorylated CaMKK2 remains almost unchanged after complex formation [90-92,94], as explained in our recent structural characterization of CaMKK1:14-3-3 and CaMKK2:14-3-3 complexes by SAXS, H/D exchange coupled to MS, and fluorescence spectroscopy [95]. This study revealed that complex formation inhibits Ca^{2+}/CaM binding and affects the structure of kinase domains and autoinhibitory segments of both CaMKKs, but the CaMKK1:14-3-3γ complex has a more compact and rigid structure in which the active site of the kinase domain interacts with the last two C-terminal helices of the 14-3-3γ protein (Fig. 2D). This conformation explains the 14-3-3-mediated inhibition of CaMKK1. In contrast, in the CaMKK2:14-3-3 complex, the kinase domain interacts with 14-3-3γ differently, and the complex has a looser structure, resulting in negligible inhibition of CaMKK2 catalytic activity. Thus, Ca^{2+}/CaM binding inhibition and the interaction of the CaMKK1 active site with the C-terminal segment of the 14-3-3γ protein provide the structural basis for 14-3-3-mediated CaMKK1 inhibition.

We have also shown that the interaction between the N-terminal 14-3-3 binding motif of CaMKKs and 14-3-3 proteins can be stabilised by FC-A and other fusicoccanes [93]. Moreover, this study also demonstrated that fusicoccanes stabilise the complex between phosphorylated full-length CaMKK2 and 14-3-3γ and slows down CaMKK2 dephosphorylation, thus keeping it in its phosphorylation-mediated inhibited state. Therefore, targeting the 14-3-3 ligand binding groove by small-molecule compounds may offer

an alternative strategy to suppress CaMKK2 activity.

Conclusions

Studies focusing on 14-3-3-mediated regulation of the five different enzymes mentioned in this review, namely Nth1, Nedd4-2, caspase-2, DAPK2 and CaMKK1/2, show the variability of these protein-protein interactions. The common denominator of these complexes is their involvement in key signalling processes in the cell, such as apoptosis, ubiquitination, cell cycle regulation, cell stress response, or metabolic regulation. 14-3-3 proteins bind to unstructured regions of these multi-domain enzymes, and interactions responsible for the actual regulation of the activity of the bound enzymes often occur far from the phosphorylated motifs within the structured domains. Structural studies also demonstrate that targeting protein-protein interactions in these complexes may enable us to develop new strategies for modulating these physiologically important enzymes.

Conflict of Interest

There is no conflict of interest.

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