

## Minireview

## C-terminal binding: An expanded repertoire and function of 14-3-3 proteins

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**Abstract** Amino and carboxyl termini are unique positions in a polypeptide. They tend to be exposed in folded three dimensional structures. Diversity and functional significance of C-terminal sequences have been appreciated from studies of PDZ and PEX domains. Signaling 14-3-3 protein signaling by recognizing phosphorylated peptides plays a critical role in a variety of biological processes, including oncogenesis. The preferential binding of 14-3-3 to phosphorylated C-terminal sequences, mode III, provides a means of regulated binding and considerably expands the substrate repertoire of 14-3-3 interaction partners. © 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

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## 1. Introduction

Post-translational modifications of proteins are essential to their proper regulation, localization, and function. One of the most common types of post-translational modifications is phosphorylation of serine or threonine amino acids. Phosphorylation may affect a protein in a variety of ways including folding, stability, interactions, and activities (see review by Pawson and Scott [1]). A common mechanism to achieve these changes is through protein–protein interactions. The first protein with a property of preferentially recognizing phosphorylated target proteins is 14-3-3 [2]. The 14-3-3 proteins were originally identified from brain because of their abundance and unusual acidity [3]. In human, there are seven 14-3-3 isoforms ( $\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$ ,  $\tau$ ,  $\sigma$ ). They are highly homologous proteins, with approximately 50% amino acid identity, capable of forming either homo- or heterodimers. Evidence from both structural studies and sequence analyses support the notion that the primary function of 14-3-3 proteins lies in their preferential binding to phosphorylated substrates and their geometrically oriented bivalent binding sites formed by dimerization.

More than 300 proteins have been reported to interact with 14-3-3 and these proteins are known for their functions in a vari-

ety of biological processes [4,5]. The well-characterized 14-3-3 interactions thus far are mediated by two canonical internal binding motifs, mode I (RSX<sub>p</sub>SXP) and mode II (RXΦX<sub>p</sub>SXP) (Φ as an aromatic or aliphatic amino acid, X as any amino acid) (Fig. 1A). Their targeted sequences contain either phosphoserine or phosphothreonine [2,6]. As discussed in detail below, a number of earlier reports recognize the interactions between 14-3-3 and protein C-termini (see below). A crystallographic structure of 14-3-3 binding to C-terminus was also obtained [7]. With increasing evidence of specific and functional interactions between protein C-termini and 14-3-3, this characteristic binding is thus proposed as mode III [8]. Recent evidence further indicates that some mode III binding displays comparable binding affinity to that of modes I and II binding and they function independently [9]. Therefore, the mode III binding is unique and likely to represent a significant expansion of the 14-3-3 binding repertoire and thus functions.

## 2. C-terminal mode III binding

Hints of C-terminal binding to 14-3-3 were recognized from several lines of early evidence. First, the I $\beta$  subunit of glycoprotein complex Ib-IX-V [10], where a synthetic peptide containing only the C-terminal 15 residues from the I $\beta$  subunit was shown to bind to purified <sup>125</sup>I-labeled 14-3-3 $\zeta$ . Recombinant I $\beta$  subunit lacking the last 5 residues (<sup>606</sup>SGHSL-COOH) can no longer bind to 14-3-3. Intriguingly, the synthetic peptide used in the study was not phosphorylated. It was suggested as a non-canonical, phosphorylation-independent binding motif based on a cluster of serine residues. More definitively, the final four residues (GHSL-COOH) were sufficient for 14-3-3 $\zeta$  binding and mutation of any one residue abolishes the binding using a radioisotope affinity pull-down competition assay [11]. Recent evidence from an I $\beta$  pS609 specific antibody indicates that, while non-phosphorylated I $\beta$  may have some affinity for 14-3-3 $\zeta$ , in vivo I $\beta$  is primarily phosphorylated [12]. Furthermore, I $\beta$  with pS609 has a greater affinity for 14-3-3 $\zeta$  as demonstrated by competition with peptides corresponding to the last 15 aa of the I $\beta$  protein [12]. Regardless of the phosphorylation state of I $\beta$ , it is clear that the I $\beta$  C-terminus is not the sole 14-3-3 binding site in the Ib-IX-V complex [11,13].

Another early study recognizing a C-terminal 14-3-3 binding motif came from investigating the regulation of the plant plasma membrane H<sup>+</sup>-ATPase by 14-3-3. The consensus sequence

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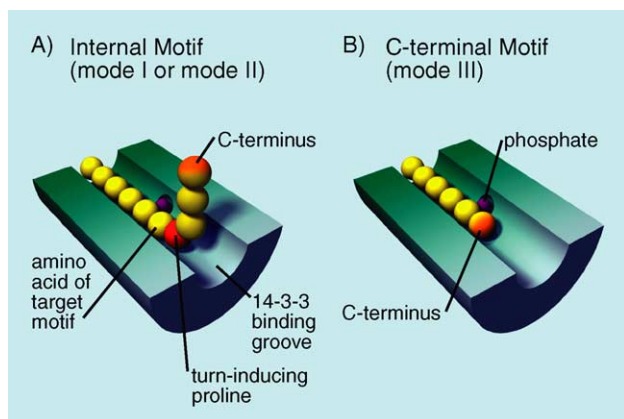


Fig. 1. Internal and C-terminal 14-3-3 binding motifs. (A) The internal mode I and mode II peptide motifs contain a turn inducing proline (red) that directs the peptide out of the 14-3-3 binding groove. (B) The C-terminal mode III peptide motif avoids continuing down the 14-3-3 binding groove by simple termination. The mode III diagram is based on homology modeling using the existing crystal structure of 14-3-3 [9].

for the C-terminal  $H^+$ -ATPase 14-3-3 binding motif is QQXYTV-COOH, a highly conserved motif among different plant species and isoforms [14]. Mutation of the Thr to Ala abolished the binding to 14-3-3, suggesting the potential phosphorylation. It is believed that the binding of 14-3-3 to plant plasma membrane  $H^+$ -ATPase activates the proton pump by preventing the function of a C-terminal autoinhibitory domain [15–17]. The weak interaction between 14-3-3 and  $H^+$ -ATPase could be potentiated by the binding of a fungal toxin, fusicocin, which leads to a tripartite complex [18–20]. The crystal structure for this tripartite complex provides a structural view of a C-terminal 14-3-3 binding motif and compatibility of phosphothreonine for the interaction [7]. In addition to the C-terminal QQXYpTV-COOH motif, an internal 14-3-3 binding site in  $H^+$ -ATPase has been reported. In fact, the binding of both C-terminus and additional upstream internal site(s) is required for the physiological regulation of plant plasma membrane  $H^+$ -ATPases by 14-3-3 [21,22].

An interaction between interleukin 9 receptor alpha chain (IL-9R $\alpha$ ) and 14-3-3 $\zeta$  was identified via a yeast two-hybrid screen. Truncation of the five C-terminal residues of IL-9R $\alpha$  abolished the interaction [23]. There are two phosphorylation sites at the C-terminus of IL-9R $\alpha$ . Phosphopeptides of either of two sites (MLLPSVLSKARSWpTF-COOH or MLLPSVLSKARpSWTF-COOH) were capable of 14-3-3 binding. While competition assays support the higher affinity for the phosphothreonine motif, it remains to be determined which residue or whether both residues are phosphorylated in cells [23].

Surface expression of two potassium channels, KCNK3 and KCNK9, requires an intact C-terminus, RRSpSV-COOH (KCNK3) and RRKpSV-COOH (KCNK9). Indeed, these motifs are capable of binding to 14-3-3 [24,25]. The requirement of phosphorylation for 14-3-3 binding was demonstrated using synthetic peptides as substrates. Additionally, truncation of the terminal valine eliminated the 14-3-3 binding. The ability of 14-3-3 binding is directly correlated with the surface expression.

Using a genetic screen of random peptide sequences, Shikano et al. recently identified a family of peptides, known as

SWTY, with a robust ability to override endoplasmic reticulum (ER) localization and confer surface expression. The SWTY peptide binds to 14-3-3 in a phosphorylation-dependent manner [26]. Furthermore, the dissociation constant ( $K_D$ ) of RGRSWpTY-COOH binding to 14-3-3 is 0.17  $\mu$ M, comparable to the values measured for mode I and II interactions [9,27]. The consensus sequence has allowed identification of native C-terminal 14-3-3 binding motifs through informatics. Their roles in interaction with 14-3-3 have been experimentally demonstrated [26].

The binding of 14-3-3 to protein C-termini also is implicated in subcellular localization of soluble proteins, exemplified by the cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> [28]. The binding of 14-3-3 to the C-terminal motif RRRQpT-COOH causes a cytoplasmic localization of p27<sup>Kip1</sup>, thereby preventing its activity in the nucleus. The evidence for phosphorylation of the terminal threonine came from pharmacological experiments manipulating upstream kinases or phosphatases, as well as from a p27<sup>Kip1</sup> T198A mutant.

Geometrically oriented, the two binding sites in a 14-3-3 dimer may constrain a targeted molecule in a certain conformation, providing a means to regulate an enzyme. Both structure and binding studies support that ovine arylalkylamin *N*-acetyltransferase (*o*AANAT), the penultimate enzyme in melatonin synthesis, has two binding sites to 14-3-3 $\zeta$ , and presumably, follows one enzyme per dimeric 14-3-3 stoichiometry [8,29]. The interesting aspect of this study came from initial recognition of 14-3-3 binding to an internal region (via mode II) and the coordinated phosphorylation under physiologically rhythmic cycles. The virtue of 14-3-3 binding causes considerable changes in enzymatic activities and stability, hence exerting the physiological function. The binding affinity to the C-terminal site (<sup>202</sup>RRNpSDR-COOH) appears to be very low, but detectable, as determined by radioisotope affinity pull-down assays [8]. The resultant non-saturated binary binding of 14-3-3 to these sites was suggested as a tuning mechanism for rhythmic enzyme activity that is coordinated with the daily cycle of melatonin production [8].

### 3. Consensus sequence for mode III

Based on the similarity between the C-terminal 14-3-3 binding motifs of the *o*AANAT (RRNpSDR-COOH) and  $H^+$ -ATPase (QQXYpTV-COOH) proteins, a mode III consensus for 14-3-3 binding (pSX<sub>1-2</sub>-COOH) has been proposed [8]. The focal points of this consensus are that the motif is at the C-terminus, and binding is phosphorylation-dependent. Two-site binding is also a common factor for both *o*AANAT and  $H^+$ -ATPase, for which the C-terminal interaction alone is of low affinity. The evidence of SWTY motif interaction with 14-3-3 demonstrates that mode III motifs are capable of single site binding similar to modes I and II. In fact, the mode III binding by SWTY motif has higher affinity compared to that of pS-Raf259 or pS-Raf621 peptides [27]. Amino acid selectivity upstream of the phosphorylated residue is conspicuously absent from the proposed mode III motif, presumably due to the discrepancy between the *o*AANAT and  $H^+$ -ATPase motifs. Upstream arginine residues are preferred for 14-3-3 binding as determined by random synthetic peptide library screening [6] and by random peptide selection in a cell-based genetic screen [26]. In a crystal structure with 14-3-3, a mode

II peptide displayed an arginine in the -4 position from pS (RLYPpSLPA) that was looped back to interact with the phosphate on the peptide [30]. Similarly, in a recent structure with a non-canonical peptide from histone H3 (RKpSTGGK), the -2 arginine also looped back to interact with the phosphate of the peptide [31]. These lines of evidence support the contribution of upstream arginines to 14-3-3 affinity. However, the arginine is absent in plant H<sup>+</sup>-ATPase, suggesting it is not an absolute requirement. For all three modes of 14-3-3 binding phosphorylation is a prerequisite, and arginines located upstream of the phosphorylated Ser/Thr are also important for recognition by a number of kinases [32]. It is possible that plant and animal differ significantly in kinase recognition. Alanine-scanning mutagenesis of the SWTY motif demonstrated the importance of the upstream arginines for phosphorylation-dependent 14-3-3 binding in HEK293 cells [26]. As more mode III motifs become available, it would be valuable to revisit the issue of upstream sequence requirements both in terms of binding per se and in terms of kinase recognition.

The first residue C-terminal to the phosphoserine or phosphothreonine (pS+1 position) has been represented as X (any residue) in all three modes of binding. Screening for optimal mode II binding peptides from random synthetic phosphopeptides shows that this position has high abundance for L/E/A/M [2,6]. To experimentally determine the selectivity at the pS+1 position in mode III binding, the SWTY motif was mutated and tested individually with the 20 different amino acids at that C-terminal position (RGRSWpTX-COOH) [9]. In this study, cell-based assays, which rely on kinase activity prior to 14-3-3 binding, led to the conclusion that, with the exception of proline, all amino acids are acceptable at the pS+1 position. Mutants with Glu, Asp, and Gly at this position have reduced surface expression. The relative order of function in the cell-based assay correlated with peptide affinity determined by synthetic phosphopeptides, suggesting that at least for SWTY motif, the kinase could not discriminate the C-terminal residues [9,27]. Noticeably, the preference in the SWTY mode III motif at the pS+1 position is different from that determined by random synthetic peptide screens that prefer L/E/A/M. It remains to be seen whether the determined selectivity profile at the pS+1 position will apply to other mode III motifs and how an additional amino acid downstream, as in the case of the oAANAT motif (RRNpSDR-COOH), may contribute to the selectivity (see Table 1).

The proline at the pS+2 position in modes I and II is thought to have an important contribution to 14-3-3 binding in that it causes a turn of the peptide out of the 14-3-3 binding groove, presumably preventing steric hindrance [30]. Mode III sequences avoid such hindrance by truncation, eliminating the need for a turn out of the groove (Fig. 1). In this case, the allowable number of amino acids following the phosphorylated residue is not well established, because few motifs have been identified thus far. From the genetic screen that identified SWTY, one residue seems to be preferable [9]. An elongated form of the SWTY motif, RGRSWpTYAAA-COOH failed to function either in a synthetic peptide binding assay or in the cell-based surface expression, suggesting that the presence of four amino acids following pT is not compatible with 14-3-3 binding [26]. However, the existing evidence could not rule out that residues at the pT + 3 and pT + 4 positions simply have a

Table 1  
C-terminal 14-3-3 binding motifs

Ib $\alpha$ subunit of glycoprotein complex Ib-IX-V	SIRYSGH $\mu$ SL-COOH <sup>a</sup> [12]
IL-9R $\alpha$ (2 alternative phosphorylation sites)	MLLPSVLSKARSW $\mu$ TF-COOH [23] MLLPSVLSKAR $\mu$ SWTF-COOH [23]
Plant plasma membrane H <sup>+</sup> -ATPase (consensus)	QQXY $\mu$ TV-COOH <sup>b</sup> [7,14,19,43]
KCNK3	RRSpSV-COOH [24,26]
KCNK9	RRK $\mu$ SV-COOH [24]
p27 <sup>Kip1</sup>	RRRQ $\mu$ T-COOH [28]
oAANAT	RRN $\mu$ SDR-COOH [8]
SWTY <sup>c</sup>	RGRSW $\mu$ TY-COOH [9,26]
GPR15	ARRRRKRSV $\mu$ SL-COOH <sup>d</sup> [26]

<sup>a</sup>The Ib $\alpha$  subunit C-terminus has also shown a phosphorylation-independent interaction with 14-3-3, for which the last 4 residues (GHSL-COOH) are sufficient [10,11].

<sup>b</sup>The X represents the diversity between the many homologs and orthologs in various plant species. Residues found in this position include, His, Ser, Ala, and Asn.

<sup>c</sup>SWTY is the name for the 7aa motif selected from a yeast random peptide screen.

<sup>d</sup>The length of this motif is not yet refined by mutagenesis studies, the 10aa length is functional in a reporter construct.

limited number of compatible amino acids or that an alanine residue(s) adversely affects the binding.

#### 4. Affinity and valency of interactions

The affinity of 14-3-3 interactions is crucial to its function, especially in distinguishing phosphorylated from non-phosphorylated forms. For the SWTY motif, the difference in affinity is more than 100-fold [9]. In several well-characterized interactions, bivalent binding via dimeric 14-3-3 proteins involves two different binding sites. In some enzymes, the binding and catalysis involves two distinct sites. The initial binding of targeted substrates greatly facilitates the subsequent recognition and catalysis [33]. Because many enzymes such as kinases and phosphatases are promiscuous in substrate recognition, the binding confers the specificity of catalysis. This has also motivated the notion of step-wise engagement for 14-3-3 interaction [6].

Precise determination of binding constants is critical for full understanding of 14-3-3 function for several reasons. First, there is considerable diversity of binding proteins or sequences suggesting an adaptor function for 14-3-3 (Fig. 2A) [34]. Many well-characterized interactions, such as Raf-14-3-3 [6] and AN-NAT-14-3-3 [8], involve binding of one 14-3-3 dimer to two different sites (Fig. 2B). Second, a number of examples are proposed where 14-3-3 binding competes against or masks a target from other interactions (see review by Bridges and Moorhead, [35]). Recently the direct interaction of 14-3-3 with the nuclear localization sequence (NLS) of FoxO4 protein was demonstrated using fluorophore-labeled FoxO4 NLS [36]. Furthermore, Yuan et al. have shown stoichiometric binding of 14-3-3 to tetrameric substrate and a lack of effective binding to monomeric substrate is critical for ER localization activity of RKR motif [37]. Therefore, relative affinity is critical for the physiological outcome.

Methods for measuring 14-3-3 affinity vary considerably, making comparisons of different findings difficult. Some techniques utilize certain specific characteristics, like 14-3-3 binding-regulated enzyme activity [29]. Other techniques include quantitative affinity precipitation [2] and surface plasmon reso-

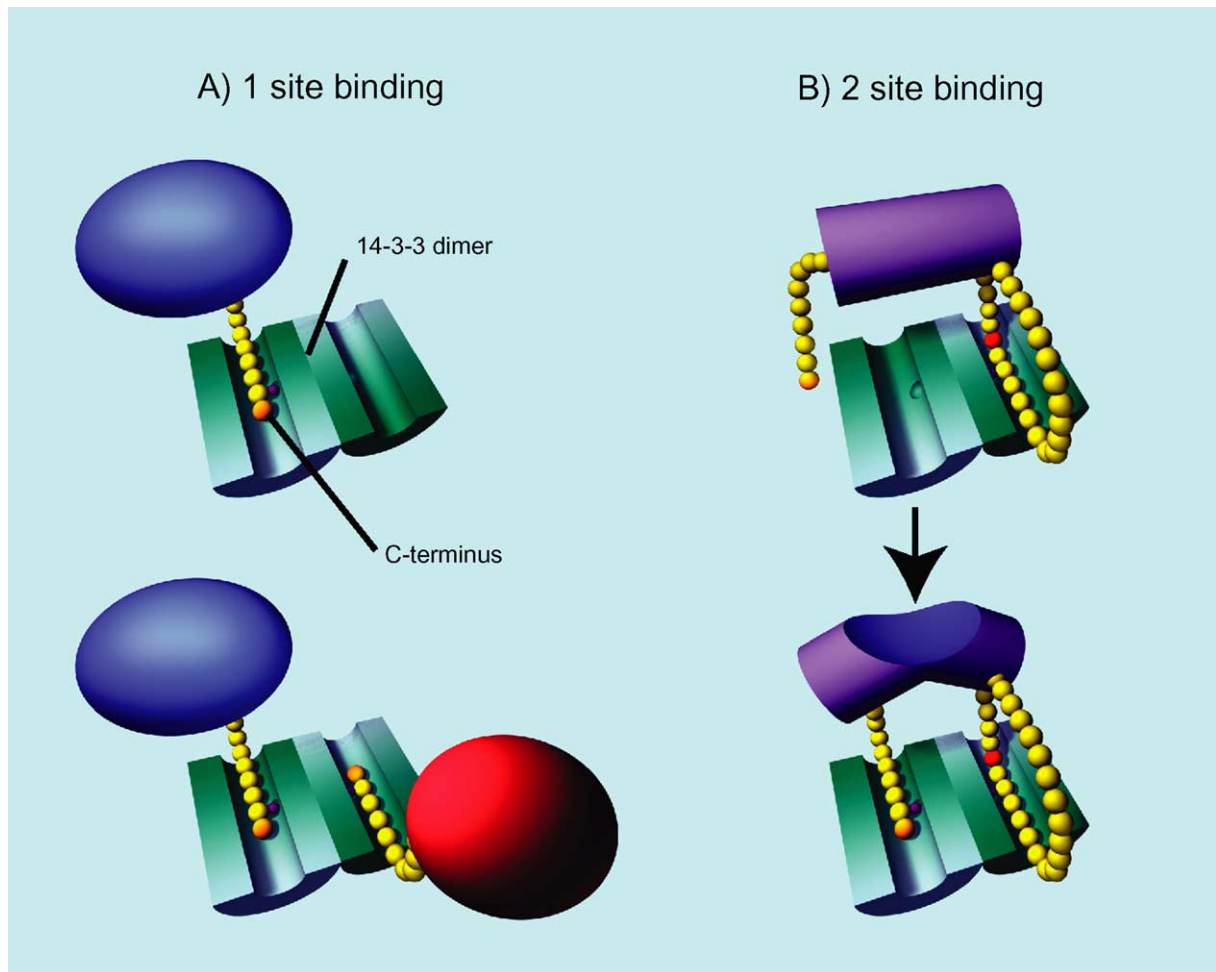


Fig. 2. 14-3-3 may bind to proteins that contain one or two target sites. (A) Binding to a protein with one target site may mask the protein from other interactions (upper). Multiple proteins with single target sites may be colocalized by binding to the same 14-3-3 dimer (lower). (B) A protein with two target sites may demonstrate cooperative binding to 14-3-3. In this case functional consequences may include a conformational change (activating or inactivating) or masking of other interactions.

nance which involves immobilization of 14-3-3 or target to a solid phase [38,39]. While solid phase (heterogenous) assays provide a useful assessment of relative affinities, immobilization of binding partners on a surface often leads to overestimation of binding affinities as seen in both 14-3-3 and PDZ binding [31,40]. These measurements are not compatible to that obtained by microcalorimetry, a preferred method, because it is both solution-based (homogenous) and label-free. The caveat to the current microcalorimetry technique is that it requires considerable amounts of materials. Recently, a fluorescence polarization method was reported using fluorophore-labeled peptide (FAM-RGRSWpTY-COOH) to enable solution-based measurement of affinities with small quantities of materials [9,27]. The combination of a solution-based format, mix-and-read capability, and the minimal material requirement affords its general applicability, including high throughput screens.

## 5. Conclusion

C-terminal 14-3-3 binding motifs have become a recognized group with a distinct mode of interaction. They interact with

the same ligand-binding groove of 14-3-3 as do the canonical mode I and mode II motifs [9]. This interaction requires phosphorylation, contrasting with C-terminal recognition by PDZ domain or PEX domain [41]. Existing mode III peptides have a general consensus of p(S/T)X<sub>1-2</sub>-COOH. For the mode III binding sites found in animals, upstream arginine residues are frequently present, presumably contributing to both 14-3-3 interaction and kinase recognition. Although this is a highly degenerate motif, the restricted C-terminal location enables bioinformatic identification of more mode III peptides from protein databases [26,42]. As more mode III 14-3-3 binding proteins continue to be identified, new insights into the general mechanisms of action may be discovered, especially in the context of the signaling events and kinases that recognize specific C-terminal sequences and confer 14-3-3 interactions.

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