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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.**

Keywords: 14-3-3; Binding motif; Mode III; Cancer signaling; C-terminal; SWTY; Phosphorylation; Binding assay; Fluorescence anisotropy

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 (β, γ, ε, ζ, η, τ, σ). 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 variety 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_pSXP) and mode II ($RX\Phi X_pSXP$) (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 Ibα subunit of glycoprotein complex Ib-IX-V [10], where a synthetic peptide containing only the C-terminal 15 residues from the Ibα subunit was shown to bind to purified ^{125}I-labeled 14-3-3ζ. Recombinant Ibα subunit lacking the last 5 residues (^{606}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 14-3-3ζ binding and mutation of any one residue abolishes the binding using a radioisotope affinity pull-down competition assay [11]. Recent evidence from an Ibα pS609 specific antibody indicates that, while non-phosphorylated Ibα may have some affinity for 14-3-3ζ, in vivo Ibα is primarily phosphorylated [12]. Furthermore, Ibα with pS609 has a greater affinity for 14-3-3ζ as demonstrated by competition with peptides corresponding to the last 15 aa of the Ibα protein [12]. Regardless of the phosphorylation state of Ibα, it is clear that the Ibα 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^+-ATPase by 14-3-3. The consensus sequence for

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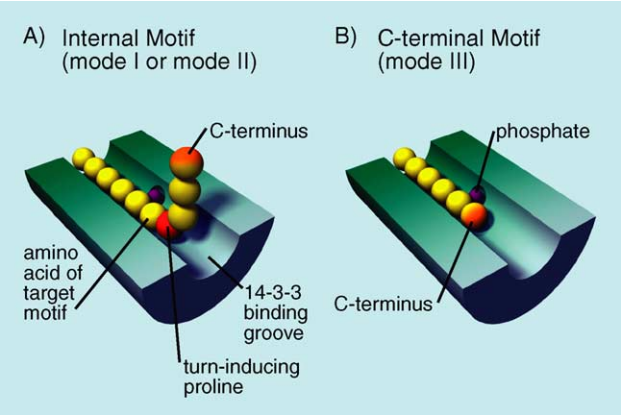

Fig. 1. Internal and C-terminal 14-3-3 binding motifs. (A) The internal mode I and mode II peptide motifs contain a turn inducing proline (red) that directs the peptide out of the 14-3-3 binding groove. (B) The C-terminal mode III peptide motif avoids continuing down the 14-3-3 binding groove by simple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

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, fusicoccin, 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-9Rα) and 14-3-3ζ was identified via a yeast two-hybrid screen. Truncation of the five C-terminal residues of IL-9Rα abolished the interaction [23]. There are two phosphorylation sites at the C-terminus of IL-9Rα. 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 μM, 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 $p27^{Kip1}$ [28]. The binding of 14-3-3 to the C-terminal motif RRRQpT-COOH causes a cytoplasmic localization of $p27^{Kip1}$, 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 $p27^{Kip1}$ 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 (oAANAT), the penultimate enzyme in melatonin synthesis, has two binding sites to 14-3-3ζ, 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 (^{202}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 oAANAT (RRNpSDR-COOH) and H^+-ATPase (QQXYpTV-COOH) proteins, a mode III consensus for 14-3-3 binding (pSX_{1-2}-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 oAANAT 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 oAANAT 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