Minireview

How do 14-3-3 proteins work? – Gatekeeper phosphorylation and the molecular anvil hypothesis

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Received 8 November 2001; revised 19 November 2001; accepted 3 December 2001

First published online 27 December 2001

Edited by Gianni Cesareni and Mario Gimona

Abstract 14-3-3 proteins were the first signaling molecules to be identified as discrete phosphoserine/threonine binding modules. This family of proteins, which includes seven isotypes in human cells and up to 15 in plants, plays critical roles in cell signaling events that control progress through the cell cycle, transcriptional alterations in response to environmental cues, and programmed cell death. Despite over 30 years of research, distinct roles for most isotypes remain unknown. Though 14-3-3 proteins perform different functions for different ligands, general mechanisms of 14-3-3 action include changes in activity of bound ligands, altered association of bound ligands with other cellular components, and changes in intracellular localization of 14-3-3-bound cargo. We present a speculative model where binding of 14-3-3 to multiple sites on some ligands results in global ligand conformational changes that mediate their biological effects. For these multi-site ligands, one binding site is likely to function as a 'gatekeeper' whose phosphorylation is necessary for 14-3-3 binding but may not always be sufficient for full biological activity. If correct, then 14-3-3 may prove to be a bona fide phosphodependent signaling chaperone.

Key words: 14-3-3 Isotypes; Phosphoserine; Phosphothreonine; Cell signaling; Nucleocytoplasmic transport; Raf; Cdc25

1. Introduction

14-3-3 proteins continue to generate intense interest because of their roles in signal transduction pathways that control cell cycle checkpoints, MAP kinase activation, apoptosis and programs of gene expression. Historically, 14-3-3 proteins were identified as abundant polypeptides of unknown function in brain [1], and later re-discovered as activators of tryptophan and tyrosine hydroxylase [2,3] and inhibitors of PKCs [4]. Interest in 14-3-3 proteins grew when they were subsequently identified as molecules that co-associated with Raf and polyoma middle T antigen [5–8], and as molecules implicated in the DNA damage response of fission yeast [9]. Since then, over 100 proteins have been found to interact with 14-3-3, including various proteins kinases (PKCs, Raf family members, KSR, PCTAIRE, MEKK1, -2 and -3, Bcr, PKUα, ASK1), receptor proteins (glucocorticoid receptor, GpIb-IX, α2 adrenergic receptor, GABA receptor, insulin-like growth factor I receptor, IL-3/IL-5/GMCSF receptor βc chain), enzymes (tyrosine and tryptophan hydroxylase, nitrate reductase, serotonin *N*-acetyl transferase, PTPH1 tyrosine phosphatase) structural and cytoskeletal proteins (vimentin, keratins K8/K18, Tau, Kif1C), small G-proteins and their regulators (Rem, Rad, RGS3/7, p190RhoGEF [10]), scaffolding molecules (IRS-1, calmodulin, Grb2, poloma middle T, p130Cas, Cbl), proteins involved in cell cycle control (Cdc25 phosphatases, Chk1, Weel, p53, the catalytic subunit of human telomerase), proteins involved in transcriptional control of gene expression (TATA box binding proteins TBP and TFIIB, histone deacetylases 4,5, and 7, histone acetyl transferase 1, transcription factors NFAT, Msn2p and 4p, and forkhead family members, and the co-activators TAZ and YAP), and proteins involved in control of apoptosis (BAD, A20 and the p75NTR-associated cell death executor NADE) (See references [11–14] for citations to primary literature and additional details). Despite this plethora of known binding proteins, in many cases the function of 14-3-3 in these interactions has remained obscure. In this review, I will briefly summarize some of the recent biochemical, structural, and genetic data that is helping to elucidate the molecular basis of 14-3-3 function, and comment on some of the outstanding questions that need to be addressed. I present a model for how the 14-3-3 dimer might function through simultaneously engaging multiple phosphorylation sites on a single ligand, In this model, 14-3-3 stabilizes non-native conformations of bound ligands to promote their interactions with downstream targets, or facilitate their subsequent modification by kinases and phosphatases.

2. 14-3-3 proteins: genetics, structure and mechanism

The term 14-3-3 denotes a large family of ~30 kDa acidic proteins that exist primarily as homo- and heterodimers within all eukaryotic cells. Their unusual name refers to their elution position on DEAE–cellulose chromatography and gel electrophoresis during a systematic attempt at classifying bovine brain proteins [1]. In humans, there are seven distinct 14-3-3 genes denoted β, γ, ε, η, σ, τ (θ) and ζ (as well as a number of potential pseudogenes), while yeast and plants contain between 2 and 15 genes [15,16]. Despite this genetic diversity, there is a surprisingly large amount of sequence identity and conservation [15,17,18] between all the 14-3-3 isotypes

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0014-5793/02/$22.00
PII: S0014-5793(01)03288-4

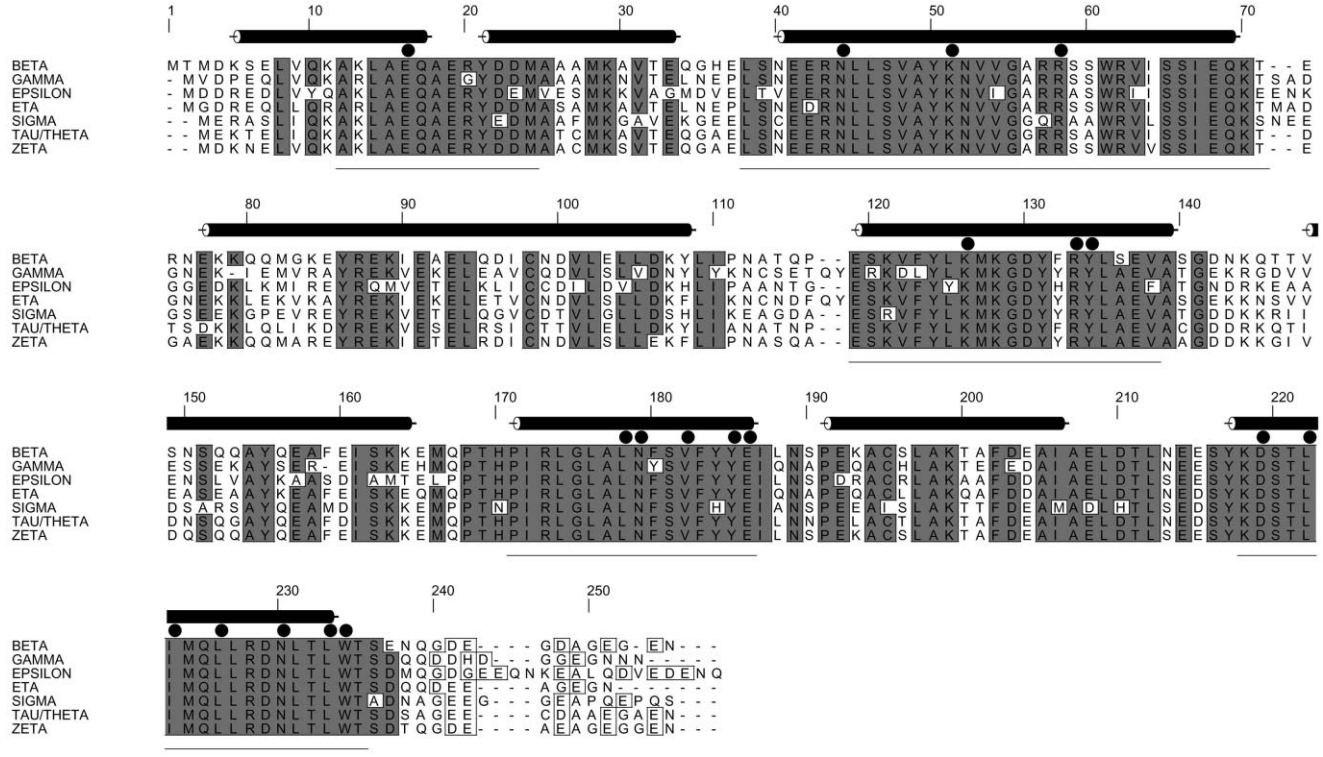

Fig. 1. Sequence alignment of human 14-3-3 isotypes. Residues conserved in at least six of the seven isotypes are shaded gray. The structure of 14-3-3 is indicated by helices above the alignment. Five conserved sequence blocks within the 14-3-3 family, as defined by Wang and Shakes [15], are indicated by a thin line below the alignment. Residues within the binding cleft that interact with peptide ligands or with the serotonin *N*-acetyl transferase molecule are indicated by filled circles. Acidic residues within the divergent C-termini are boxed.

(Fig. 1). Stringently conserved regions either form the dimer interface, or line the central ligand binding channel of the dimeric 14-3-3 molecule [17], including many of residues that form direct ligand contacts (Fig. 1).

Why then do we need so many isoforms? One possibility is that different 14-3-3 molecules form distinct populations of dimers with unique recognition motifs for ligands. Work by Aitken and colleagues, however, demonstrated that several different 14-3-3 isotypes are capable of forming mixed heterodimers in vitro, and heterodimerization in vivo was demonstrated for 14-3-3 ε and ζ in co-transfection experiments [19]. In addition, the optimal phosphopeptide motif selected by different 14-3-3 isotypes was extremely similar [20], and at least some 14-3-3 ligands such as Raf-1 [17,21], Cas [22] and Rem [23] appear to bind nearly equivalently to many, though not all, 14-3-3 isotypes. A subset of ligands, however, clearly bind to different 14-3-3 isotypes with significantly different affinities [24,25], and for most 14-3-3 binding ligands, a detailed analysis of 14-3-3 isotype specificity in vitro or in vivo has not been performed.

There are distinct spatial and temporal patterns of 14-3-3 gene expression during development in both plants (cf. [26]) and animals, particularly in the rodent brain and heart [27–33], suggesting that there may be isotype-specific developmental roles. Intriguingly, in *Drosophila*, which normally expresses both 14-3-3 ε and ζ (also called Leonardo), complete loss of 14-3-3 ζ, despite normal amounts of ε, causes embryonic lethality [34], while single allelic mutations in ζ result in defects in memory and learning [35,36]. In other aspects, such as Ras-mediated MAP kinase activation, *Drosophila* 14-3-3 ε and ζ display at least partial redundancy [37].

Aside from development, isotype-specific expression of 14-3-3 proteins also appears to be a normal part of the cellular response to injury. One particular isotype found primarily in epithelial cells [38], 14-3-3 σ, is strongly upregulated in colorectal cancer cells following exposure to ionizing radiation and DNA-damaging agents [39], and appears to be essential for maintaining the G2/M checkpoint. The DNA damage-induced upregulation of 14-3-3 σ both in human colon carcinoma cells and in mouse ES cells occurs through a p53 and BRCA1-mediated increase in gene transcription [39,40]. Somatic cells lacking 14-3-3 σ, initiate, but are unable to maintain a G2/M arrest following DNA damage and die by mitotic catastrophe [41]. 14-3-3 σ, in contrast to other 14-3-3 isoforms, does not bind to one of the major targets of 14-3-3 that mediates the G2/M checkpoint, namely the dual-specificity phosphatase Cdc25C [42]. Normal checkpoint signaling, therefore, involves multiple isotype-specific functions and suggests that for σ, another binding partner, perhaps cyclinB-Cdc2 itself, is the G2/M checkpoint target [43]. A variety of clinical observations further suggest that 14-3-3 σ may function as a major tumor suppressor since it is frequently downregulated in breast and gastric cancer [44,45] as a result of methylation of the 14-3-3 σ promoter [46–48]. Curiously, 14-3-3 σ is upregulated in lung cancer [49], head and neck squamous cell carcinomas [50], and chemoresistant pancreatic adenocarcinoma cells [51]. Perhaps these increases reflect a compensatory response to alternative oncogenic mutations which cause genomic instability and initiate a DNA damage checkpoint in cells that still contain functional p53 alleles.

Increased levels of 14-3-3 γ have been observed in rat carotid arteries following balloon angioplasty injury [52,53], and

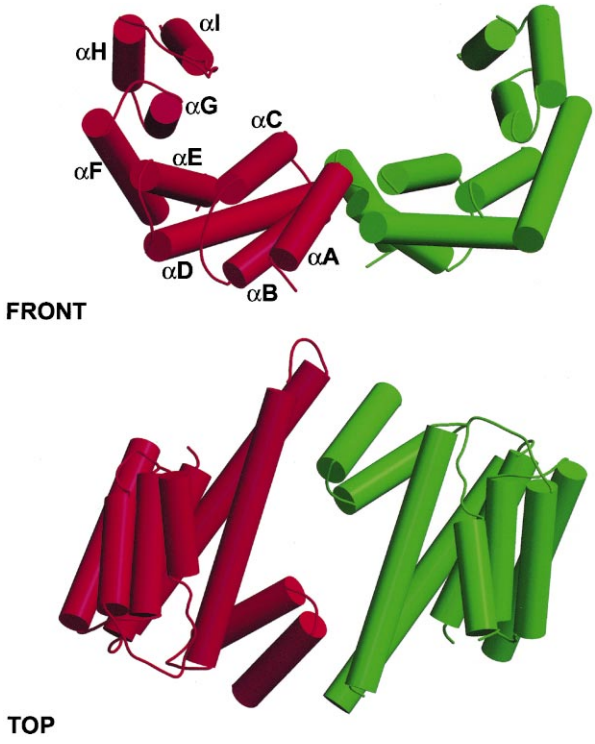

Fig. 2. The structure of 14-3-3 in two orthogonal views. Helices αA–αI are shown as cylinders.

increased mRNA for ζ and τ have been observed in rat motoneurons following axotomy [54]. Taken together, these findings in cell injury, coupled with the developmental differences in 14-3-3 expression patterns, suggest that the balance between homo- and heterodimerization, as well as isotype restriction in heterodimer formation may be regulated in vivo. Additional experimental data is clearly needed to further address this issue.

How is 14-3-3 ligand binding controlled? The initial observation that 14-3-3 binding might require ligand phosphorylation emerged from work on tryptophan hydroxylase [55], an enzyme involved in neurotransmitter biosynthesis, and Raf, the upstream activator of the classical MAP kinase pathway [56]. Pioneering investigation of the 14-3-3 binding sites on Raf [57], together with oriented peptide library screening on all mammalian 14-3-3s [20], led to the identification of two optimal phosphoserine/threonine-containing motifs, RSXpSXP and RXXXpSXP, that are recognized by all 14-3-3 isotypes. In these consensus sequences pS denotes both phosphoserine and phosphothreonine, and there is a moderate to strong preference for particular amino acids over others in the X positions [20]. Well over half of the 14-3-3 binding proteins identified to date use phosphorylated sequences which are reasonably close matches to the optimal 14-3-3 consensus motifs. The motifs are clearly not absolute, however, since the presence of a non-phosphorylated S immediately following R within the first motif, and a P two positions C-terminal to the phosphorylated S or T in both motifs, though strongly favored, is not required for peptide and protein binding to 14-3-3 [58]. Furthermore, there are several examples of proteins and peptides containing dramatic variations from these motifs, including some that do not even require phosphorylation for binding such as exoenzyme S [59], A20 [24], the platelet glycoprotein IB–IX–V complex [60,61] and a 14-3-3 binding peptide, R18, obtained by phage display [62]. Atypical phosphorylated and non-phosphorylated sequences, including YpTV [63], KGQSTpSRG [64] and GHSL [60,61], directly mediate 14-3-3 binding for these ligands, though most likely other parts of the proteins also aid in the 14-3-3 interaction.

The X-ray structures of 14-3-3 τ and ζ in the absence of bound ligand showed that the molecule was a cup-shaped dimer [65,66] (Fig. 2). Each monomer contains nine α-helices (denoted αA–αI) with the dimer interface formed from helices αA, αC and αD. Helices αE–αI form the side walls and roof of a $35\times35\times20$ Å central channel that forms the binding clefts for peptide and protein ligands. Maximal isotype divergence occurs in an acidic-rich stretch at the extreme C-terminus following helix αI (Fig. 1), a region of the protein which, unfortunately, is not seen in any of the X-ray structures. The structures of several ligand-bound 14-3-3 complexes have now been solved, including 14-3-3 ζ bound to peptides representing both of the phosphoserine consensus motifs [17,20], a peptide corresponding to the Ser-259 binding site in Raf-1, and the non-phosphorylated R18 peptide [67]. The peptides in all cases occupy similar positions, nestled within an amphipathic groove where the central channel meets the side walls [17,20,67], and are held in a highly extended conformation by multiple contacts between the main chain amides and side chains of residues in helices αE, αG and αI. The phosphopeptide phosphate forms ionic and hydrogen bonds with three absolutely conserved basic residues, Lys-49, Arg-56, and Arg-127, along with Tyr-128 (Fig. 3) which forms a solitary basic pocket on this otherwise negatively charged molecule, explaining why for many ligands, phosphorylation is the critical switch that regulates their 14-3-3 binding. Recently, the structure of 14-3-3 ζ bound to a bona fide protein ligand, the serotonin *N*-acetyl transferase molecule in complex with a bisubstrate analog, was solved [68]. In this structure, the 14-3-3 binding portion of the enzyme displays a very similar conformation to that seen in isolated phosphopeptide:14-3-3 complexes, but in addition, 14-3-3 appeared to stabilize the conformation of an adjacent region in the enzyme, causing enhanced substrate binding and product formation.

This structure, therefore, provides the first glimpse at atomic resolution of one of the general mechanisms of 14-3-3 action, namely the (1) direct regulation of catalytic activity of the bound protein. The other equally important general

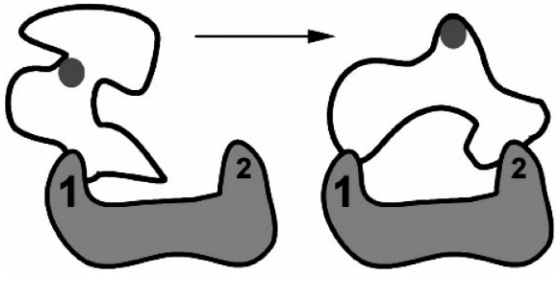

Fig. 3. A model for 14-3-3-dependent conformational change upon multi-site binding. 14-3-3 binding relies initially upon interaction of a gatekeeper residue with one monomeric subunit (1). Binding of one or more weaker secondary sites (2) facilitates ligand conformations that is not favorable in the unbound state, exposing one or more regions of the protein (shaded circle) that are inaccessible in the free or monomer-bound form.

mechanisms that underlie 14-3-3 function include (2) regulating interactions between the bound protein and other molecules within the cell through sequestation or modification, and (3) controlling the subcellular localization of bound ligands.

A major clue to understanding the mechanistic basis of 14-3-3 function seems to be an appreciation of its dimeric nature, with its two ligand binding clefts running in opposite directions. A ligand molecule containing multiple 14-3-3 binding motifs might therefore be able to simultaneously engage both monomeric subunits within a single dimer. Intriguingly, Raf and FKHRL family members contain at least two 14-3-3 binding sites, as do the 14-3-3 binding proteins CBL [69], BAD [69], and yeast forms of Cdc25C [70]. A synthetic phosphopeptide with two 14-3-3 consensus motifs binds over 30-fold more tightly than the same peptide containing only a single motif [20], and individual 14-3-3 binding sites located at the N- and C-termini of serotonin *N*-acetyl transferase allow a single molecule to interact simultaneously with both subunits in a 14-3-3 dimer through this type of bidentate interaction [68]. Furthermore, point mutations that disrupt the 14-3-3 dimer significantly impair 14-3-3's ability to cooperate in Raf signaling [71], as well as its ability to modulate signaling by DAF-16, the *Caenorhabditis elegans* homologue of the forkhead transcription factor FKHRL [72]. Thus, in the model shown in Fig. 3, 14-3-3 regulates signaling events through causing conformational changes in its bound ligands by virtue of multiple interacting sites.

For most proteins, it seems likely that there is a single dominant site that functions as a 'gatekeeper'. If the site is absent or not phosphorylated, then the secondary sites are too weak to promote a stable 14-3-3 interaction. In contrast, once the gatekeeper site is phosphorylated and bound to one monomer in the 14-3-3 dimer, the secondary sites are able to interact with the other monomeric subunit by virtue of their high local concentration induced by its proximity. Mapping these secondary sites, if they exist, becomes quite challenging, since mutations are likely to have only a minor effect on total ligand:14-3-3 binding, which is what one usually measures. The significance of potential secondary sites will only be revealed when the 14-3-3 binding assay directly measures the effects of binding on ligand function.

The 14-3-3 molecule appears to be extraordinarily rigid due to a distributed series of interactions between the α-helices. There is a negligible amount of movement between the free and peptide-bound forms of 14-3-3 [17], and the average RMS deviation among 426 Cα carbons in the serotonin *N*-acetyl transferase-bound form of 14-3-3 and the peptide-bound form barely exceeds 1 Å [68]. Thus, 14-3-3 behaves in essence like a molecular anvil, deforming its bound ligands while itself undergoing only minimal structural alterations. What function might these conformational changes in the ligand accomplish? In the case of serotonin *N*-acetyl transferase, and presumably exoenzyme S, 14-3-3 binding deforms the catalytic residues so as to promote substrate binding and product formation, perhaps through inducing a conformation that stabilizes the transition state of the enzyme:substrate complex. For other proteins, 14-3-3-mediated conformational changes might facilitate their interaction with other proteins, leading to enhanced post-translational modifications such as phosphorylation, or alternatively, might facilitate subcellular relocalization by deforming and/or masking a nuclear localization signal (NLS) or nuclear export signal (NES) [73], or perhaps through facilitating interactions between cryptic NES/NLS sequences and nuclear importers or exporters.

There are a few examples where this type of tandem 14-3-3 binding has been clearly shown to be functionally important, most prominently for Raf, although the exact role of 14-3-3 in catalytic regulation of Raf continues to be controversial. All Raf isoforms contain two 14-3-3 binding sites, one located in the regulatory region and one located within the catalytic portion of the molecule. For c-Raf-1, binding of 14-3-3 to the regulatory region appears to suppress the basal catalytic activity, but maintains the inactive form near the plasma membrane in a form that is readily re-activatable [74] when the N-terminal segment is displaced from 14-3-3 through dephosphorylation and binding to Ras [75,76]. In addition, binding of the C-terminal part of c-Raf-1 is required for maintenance of catalytic activity [77]. Thus, only 14-3-3 dimers, which can interact simultaneously with both the N- and C-terminal portions of Raf, in contrast to monomeric 14-3-3 subunits, are capable of facilitating Raf activation [71]. If 14-3-3 binding to the N- and C-termini did not induce a general conformational change, and instead had only local effects on the isolated catalytic and regulatory domains, then it is difficult to understand why monomeric 14-3-3 would not function equally well. The role of 14-3-3 proteins in regulation of A-Raf and B-Raf is less well understood, but as with c-Raf-1, 14-3-3 proteins may play an additional role in regulating their interaction with other upstream or downstream effector molecules [78–82].

It seems certain that there will be a number of 14-3-3 ligands where this type of tandem binding does not occur or is irrelevant to their function. In those cases, 14-3-3 might function as a molecular adaptor, coupling independent 14-3-3 binding molecules in a single complex as has been proposed for complexes between BCR and Raf [83], PKCζ and Raf [79], and A20 and Raf [24]. Obviously, the model shown in Fig. 3 remains speculative, and definitive proof will require the crystal structure of additional 14-3-3-ligands, both free and in complex with 14-3-3.

Acknowledgements: I thank Justine Stehn and Jeong-Ho Hong for critical review of the manuscript. This work was supported by NIH Grant GM-60594 and a Career Development Award from the Burroughs-Wellcome Fund.

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