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Minireview

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14-3-3 Proteins: Active Cofactors in Cellular Regulation by Serine/Threonine Phosphorylation*

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The 14-3-3 proteins are a family of abundant, widely expressed 28–33-kDa acidic polypeptides that spontaneously self-assemble as dimers. The 14-3-3 proteins were first detected by virtue of their relatively high abundance in brain extracts and given their unique name (an unfailing source of curiosity) based on their fraction number on DEAE-cellulose chromatography and migration position on starch gel electrophoresis (1). One or another 14-3-3 isoforms was repeatedly rediscovered in association with proteins of biologic interest; however, the functions of these polypeptides remained speculative until the demonstration by Shaw and colleagues (2) that 14-3-3 proteins bind to phosphoserine-containing motifs in a sequence-specific manner. This insight enabled a systematic approach to understanding the functional roles of the 14-3-3 proteins and was followed by the demonstration that the 14-3-3 proteins participate in a wide range of biologic processes acting through a variety of regulatory mechanisms mediated mostly, and perhaps exclusively, through their binding to phosphoserine-containing sequence motifs in diverse partners (for review see Refs. 3–9). Other sequence-specific phosphoserine/phosphothreonine-binding domains have been subsequently defined with differing target specificity (Table I) such as WW domains, FHA domains, and WD40 and LRR domains of F-box proteins (reviewed in Refs. 10–12), complementing the previously defined phosphotyrosine-binding, SH_2 and PTB domains. This review seeks to summarize the features of the 14-3-3 proteins critical to the phosphoserine/phosphothreonine binding function and to illustrate through specific examples several ways in which a stereotyped binding function is deployed to regulate diverse biologic functions and signal transduction pathways. The reader is referred to recent excellent reviews on 14-3-3 proteins (6–9) and phosphoserine/phosphothreonine binding motifs in general (10–12).

14-3-3 Structure

14-3-3 proteins are expressed in all eukaryotic cells and are highly conserved in amino acid sequences from yeast to mammals. Seven isoforms encoded by seven distinct genes are identified in mammals, more than 10 isoforms are identified in plants, and two isoforms are identified in yeast, *Drosophila*, and *C. elegans*. Interestingly, the yeast 14-3-3s are functionally interchangeable with the plant and mammalian isoforms, indicating the high level of functional conservation of the gene products. The 14-3-3 proteins assemble as stable homo- and heterodimers (3, 6, 13, 14). All the 14-3-3 proteins appear to share similar tertiary structure, first defined for the τ (15) and ζ (16) isoforms (Fig. 1). Each polypeptide is organized into nine α-helices (A–I) in an antiparallel array, each separated by a short loop. The four N-terminal helices (A–D) lie in a planar array and create an extensive dimer surface with a central hole in the interface that is lined by polar and charged residues. The dimer contact residues are primarily hydrophobic with some uncharged polar residues and are highly conserved among all 14-3-3 isoforms, pointing to the likely basis for facile heterodimerization. Specifically, the 14-3-3ζ dimerization interface is formed by interaction of helix A (amino acids 3–17) of one 14-3-3 polypeptide with helices C (amino acids 39–68) and D (amino acids 75–107) of the opposite 14-3-3 and vice versa (6, 15, 16). Indeed, N-terminal deletions or substitution of critical amino acids in the predicted dimerization interface completely abolishes 14-3-3 dimerization, producing monomeric 14-3-3 forms (17–19). The five C-terminal helices (E–I) hang down in a palisade at nearly 90° from the extended planar surface created by the N-terminal four helices, so that the dimer, viewed from the side, looks like a symmetrical claw, whose inner concave channel is lined by highly invariant residues. Co-crystallization of 14-3-3 with a synthetic phosphopeptide of optimized sequence (see below) combined with mutational analysis demonstrates that the 14-3-3 phosphopeptide binding pocket is composed of residues from both the N-terminal and the C-terminal parts of the protein (15–17, 20–23). The phosphopeptide binds along a groove on the inner surface of the half-dimer formed by the C-terminal five helices with the phosphate group directed into a pocket formed by the N-terminal helices. Thus 14-3-3ζ mutations that abrogate phosphopeptide binding include Lys-49, Arg-56, and Arg-60 in the N-terminal region (21) and Val-176 and Leu at positions 216, 220, and 227 near the C terminus (22). In addition to these residues, 14-3-3-phosphopeptide co-crystal structures point to a role for Leu-120, Arg-127, Leu-172, Asn-173, Glu-180, Asn-224, and Trp-228 in formation of the phosphopeptide-binding groove (23). This work demonstrates directly that, as expected, each 14-3-3 half-dimer is capable of independently binding a phosphopeptide. Moreover, the phosphopeptide-binding grooves in each 14-3-3 half-dimer lie in antiparallel relationship within the dimer, as illustrated by the structure of a 14-3-3-serotonin *N*-acetyltransferase co-crystal, where the 14-3-3 dimer binds to a single polypeptide that is phosphorylated at two sites (24).

14-3-3 Target Binding

A number of early studies indicated that the binding of 14-3-3 required target protein phosphorylation, *e.g.* as in the binding of 14-3-3 to tryptophan and tyrosine hydroxylases (25, 26), Raf and Bcr (27), BAD (28), keratin K18 (29), and plant nitrate reductase (30). Building on these observations, Muslin *et al.* (2) used synthetic phosphopeptides based on the amino acid surrounding Ser-259 on c-Raf-1, a site of 14-3-3 binding, to define a specific sequence motif optimal for association with 14-3-3 proteins: R**S***X*p**S***X*P, where p**S** represents phosphoserine and *X* any amino acid. Substitution of amino acids surrounding the central phosphoserine (position 0) demonstrated that Arg at position −4 or −3, Ser at position −2, and Pro at position +2 were critical for high affinity association. Within this motif, phosphorylation of the serine at position −2 alone does not support 14-3-3 binding, and when the Ser at −2 is phosphorylated in addition to the Ser at position 0, phosphopeptide binding is completely abrogated. These studies did not identify a preference for specific amino acids −5, −1, +1, or +3; however, subsequent work by Yaffe *et al.* (31) and Rittinger *et al.* (23) using oriented phosphopeptide libraries provided evidence for two distinct 14-3-3 binding motifs: R**S***X*p**S***X*P (mode 1) and R*XXX*-p**S***X*P (mode 2). The binding of 14-3-3 to the mode1 motif is favored by aromatic or positively charged amino acid at position −1, whereas 14-3-3 binding to the mode 2 motif exhibits a preference for aromatic residues at position −2, positive residues at position −1, and Leu, Glu, Ala, or Met at position +1. The phosphorylation

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TABLE I
Binding preferences of phosphoserine/phosphothreonine-binding proteins and domains

Phosphoserine/ phosphothreonine-binding protein/domain	Binding preference	Ref.
14-3-3	RS*X*(p**S**/T)*XP* or R*XXX*(p**S**/T)*XP*	2, 31
WW	(p**S**/T)P	9–11
WD40	Dp**S**G*XX*p**S** or p**S***XXX*p**S**	9–11
FHA	PT*XX*(D/I)	9–11

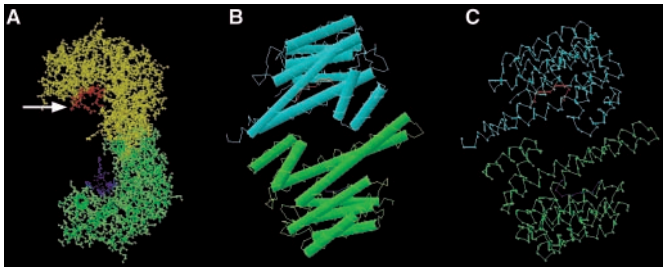

FIG. 1. **Several views of 14-3-3 structure.** The representations were adapted from the structure of 14-3-3 ζ bound to a phosphopeptide (Protein Data Bank accession number 1QJB (23)). *A*, a ball and stick representation of a 14-3-3ζ dimer bound to a mode 1 phosphopeptide (phosphopeptides are colored *red* (*upper part*, ARSHp**S**YPA) or *purple* (*bottom*, RSHp**S**YPA). *B* and *C*, secondary structure and neighbor representations of the structure in *A*, rotated 90° counterclockwise to provide a view through the peptide binding cavity indicated by the *arrow* in *A*.

of such motifs is specified largely by the Arg at −3 or −4 and is catalyzed predominantly by members of the AGC subfamilies of protein kinases, *e.g.* PKA,[1] PKB, PKC, etc.

These motifs define the requirements for optimal binding of a single phosphoserine motif to a 14-3-3 protein, and many of the 14-3-3 partners identified thus far do contain either a mode 1 or mode 2 motif. Nevertheless, several well characterized proteins that interact with 14-3-3 in a phosphorylation-dependent manner do not contain either of these motifs, including *e.g.* the IGF-I receptor (32), IRS-1 (33, 34), vimentin (35), and Wee1 (36, 37); thus it is evident that some variation on the optimal motifs is tolerable for 14-3-3 binding. In fact, it may turn out that such "optimal" motifs defined through the use of short synthetic phosphopeptides occur in the minority of 14-3-3 partners. This possibility is raised by the finding that the majority of cellular ^{35}S-labeled Met polypeptides that were recovered in association with recombinant, wild-type 14-3-3 expressed in COS cells failed to associate with a recombinant mutant, monomeric 14-3-3 polypeptide. Thus, stable association of most cellular partners with 14-3-3 requires a 14-3-3 dimer (35).

Some 14-3-3 partners have been shown to bind monomeric and dimeric forms of 14-3-3 with similar efficiency (17–20, 38); however, the overall target protein binding profiles of monomeric and dimeric 14-3-3 forms differ greatly (35, 39). Thus, proteins such as c-Raf-1 that contain a high affinity 14-3-3 binding site, *e.g.* matching the mode 1/2 motifs, can bind monomeric 14-3-3 in a stable manner, whereas proteins that contain only low affinity 14-3-3 binding sites bind monomeric 14-3-3 weakly and achieve stable binding only with dimeric 14-3-3. The putative 14-3-3 partners such as Wee1 (36, 37), keratin K18 (40), Cbl (41) IGF-I receptor (33), IRS-1 (34), vimentin (35), and DAF-16 (39) have been shown to require more than one phosphorylation site for stable 14-3-3 binding, implying that a 14-3-3 dimer may be required to bind at two sites on these proteins to achieve a stable association; consistent with this view, these proteins all lack canonical high affinity 14-3-3 binding motifs. A plausible explanation for the dependence of target binding on the state of 14-3-3 dimerization is the demonstration that the creation of two identical 14-3-3 binding sites on a single synthetic phosphopeptide increases binding affinity for native 14-3-3 more than 30-fold over that of a phosphopeptide bearing a single motif (31).

The significance of 14-3-3 dimerization for target protein regulation has as yet been established in only a few instances. By

[1] The abbreviations used are: PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; IGF, insulin-like growth factor.

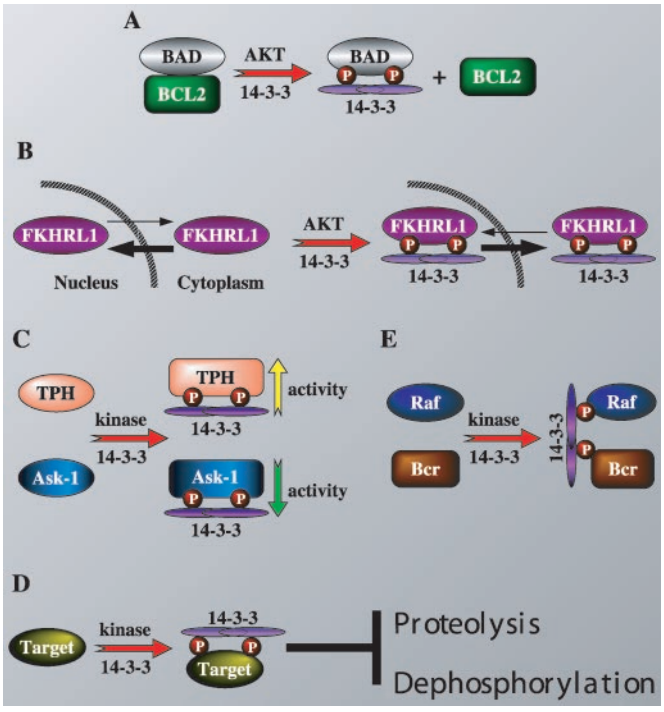

FIG. 2. **14-3-3 modes of action; illustrative examples.** See text for details. *TPH*, tryptophan hydroxylase.

example, although monomeric and dimeric 14-3-3 polypeptides bind Raf equally well during co-expression in COS7 cells, only the dimeric form supports Raf kinase activity *in vivo* or enables the reactivation of Raf kinase after displacement of 14-3-3 from c-Raf-1 *in vitro* (19, 35). Moreover, analysis of the structure of co-crystallized 14-3-3 and the enzyme serotonin *N*-acetyltransferase indicates that simultaneous binding of a dimeric 14-3-3 to two sites on the enzyme is required to create the conformation that results in enhanced catalytic activity (24). Indirect evidence for the importance of dimerization to 14-3-3 function derives from studies that demonstrate the ability of 14-3-3 mutations that disrupt target protein binding to function as dominant negative forms, presumably by sequestering endogenous 14-3-3 into dimer forms in which only one monomer is capable of target binding (42, 43).

Finally, it should be noted that 14-3-3 can bind well to a variety of nonphosphorylated proteins such as exoenzyme S (44) and Cdc25B (45) as well as to nonphosphorylated synthetic or recombinant peptides (46). Interestingly, the residues in the 14-3-3 N-terminal segment that mediate phosphopeptide binding also mediate the binding to the nonphosphorylated peptides, and these moieties compete with each other for binding to 14-3-3 (46). Selection of 14-3-3 partners by phage display yielded sequences related to mode 1 and mode 2 save for the presence of multiple acidic amino acids, *e.g.* RS$X_{(1-4)}$E. A similar sequence (RSESEE) is present in the candidate 14-3-3 partner, inositol 5-phosphatase (47). In addition, 14-3-3 proteins have been observed to interact with the nonphosphorylated Cys-His-rich (zinc finger) domain of Raf (48) and with a leucine zipper structure situated upstream of the Cys-His-rich (PHD) domain of several plant homeodomain proteins (49). The latter interaction appears to involve a potential leucine zipper motif in 14-3-3 helix D and is thus distinct from the phosphopeptide-binding groove. Whether nonphosphorylated polypeptides are among the physiologic partners of 14-3-3 *in vivo* remains to be established.

Modes of Regulation through 14-3-3 Binding: Several May Occur with a Single Partner

The mechanisms by which 14-3-3 binding participates in the regulation of target protein function can be assembled into five general categories (Fig. 2). In Fig. 2*A*, binding of 14-3-3 can alter the ability of the target protein to interact with other partners. A well studied example is the binding of 14-3-3 to BAD. In response to ligands such as IGF-1, interleukin-3, insulin, and others, a variety of kinases, *e.g.* AKT, PAK, RSK1, and PKA, are activated

and catalyze the phosphorylation of BAD on Ser-112, -136, and/or -155 (28, 50–54). These phosphorylations cooperatively mediate 14-3-3 binding, which interferes with the ability of BAD to bind and inhibit Bcl2. The net outcome of 14-3-3 binding to BAD is an inhibition of apoptosis and the promotion of cell survival. This regulatory mechanism can be counteracted by activators of protein phosphatase 2A (55) and/or calcineurin (56, 57). Other instances wherein 14-3-3 binding serves to interfere with target protein interaction include 14-3-3 binding to Cbl, which attenuates Cbl tyrosine phosphorylation and diminishes its ability to recruit downstream effectors (58); 14-3-3 binding to IRS-1 inhibits the ability of IRS-1 to bind phosphatidylinositol 3-kinase (59).

In Fig. 2*B*, binding to 14-3-3 can modify the cytoplasmic/nuclear partition of the protein partner by increasing nuclear export rate, decreasing nuclear import, or both (7). Proteins shown to be subject to this mode of regulation include Cdc25 (60–62), telomerase (63), protein kinase U-α (42), histone deacetylase (64), and the insulin-regulated forkhead domain transcription factors, FKHRL1 (65) and DAF-16 (39). Thus insulin activates phosphatidylinositol 3-kinase and AKT; the latter phosphorylates DAF-16 on Thr-54 and Ser-242 and -314, enabling the binding of 14-3-3, which promotes the export of DAF-16 from the nucleus (39). In Fig. 2*C*, the binding of 14-3-3 can either inhibit or augment the intrinsic catalytic activity of the target protein. Phosphorylation of tryptophan and tyrosine hydroxylases by calmodulin kinase II does not alter the catalytic activity of these enzymes, but the subsequent binding of 14-3-3 increases their activity severalfold (25). Interestingly, phosphorylation of either hydroxylase by PKA results in activation directly without a requirement for 14-3-3 binding. The binding of 14-3-3 to p53 enhances its DNA binding (66), and 14-3-3 increases the activity of serotonin *N*-acetyltransferase (24), whereas the binding of 14-3-3 inhibits ASK-1 kinase activity (67, 68), plant nitrate reductase activity (30), and DAF-16 DNA binding (39). In the latter example, AKT-catalyzed phosphorylation of DAF-16 residues 54 and 314 enables 14-3-3 binding, which inhibits DAF-16 DNA binding; the inhibition requires concurrent phosphorylation at both sites on DAF-16 and binding to a dimeric 14-3-3 (39). In Fig. 2*D*, the binding of 14-3-3 can protect the target protein from proteolysis and/or dephosphorylation. Examples include the protection by 14-3-3 of Raf (69, 70), histone (71), and BAD (55) from dephosphorylation, as well as plant nitrate reductase (72) and several other partners in *Arabidopsis* (73) from proteolysis. In Fig. 2*E*, a 14-3-3 protein can also serve as a phosphorylation-dependent adapter/scaffold to bridge two targets. Such 14-3-3-dependent ligation has been demonstrated between Raf and Bcr (74) Raf and A20 (75), and Raf and PKC (76).

Thus, the dimeric structure of the native 14-3-3 protein and the requirement for target Ser/Thr phosphorylation enable the 14-3-3 proteins to serve more varied and dynamic roles than those provided by conventional "scaffold" proteins (*e.g.* IRS-1). The binding of 14-3-3 generally contributes an active regulatory input to the target protein and often serves more than one function, *e.g.* binding of c-Raf-1 to a 14-3-3 dimer contributes to the maintenance of an inactive Raf conformation in resting cells, reduces Ser-259 and Ser-621 dephosphorylation, and decreases the rate of Raf turnover. Moreover, maintenance of the activated state of c-Raf-1 following cell stimulation also requires the continued binding to a dimeric 14-3-3 (19, 35, 69, 70, 77, 78).

14-3-3 Regulation

Most studies of the role of 14-3-3 in cellular regulation have focused on changes in target protein phosphorylation as the initiating regulatory event, viewing the 14-3-3 polypeptide as the passive element in the interaction. Nevertheless, several potential modes of 14-3-3 regulation merit consideration, including isoform-specific expression, subcellular localization, and differential target binding specificity. Although the 14-3-3 proteins are quite abundant, it is unclear whether *in vivo* there exists an excess of total 14-3-3 polypeptides in comparison to total potential binding sites. Treatment of COS cells with the Ser/Thr phosphatase inhibitor calyculin A leads to a transient increase in total endogenous ^{35}S-labeled Met polypeptides associated with overexpressed, recombinant 14-3-3 followed rapidly by the displacement of nearly all 14-3-3 partners by increasing levels of phosphovimentin (35). Apparently the phosphorylation-induced disassembly of intermediate filaments results in a pool of soluble phosphovimentin that is sufficient to displace essentially all other cellular 14-3-3 partners. The phosphovimentin-induced displacement of 14-3-3 from Raf is accompanied by deactivation of Raf. This specific deactivation as well as the general displacement of cellular polypeptides from 14-3-3 can be overcome by simply increasing the level of recombinant 14-3-3 expression to accommodate the additional phosphovimentin generated by calyculin A inhibition of protein phosphatases (35). Hyperphosphorylation of vimentin and keratin K18 and disaggregation of intermediate filaments, such as occurs after calyculin A treatment, does occur transiently during mitosis; however, whether such global alterations in the association of 14-3-3 with its targets actually occur in the normal course of cell physiology is unknown (9).

To the extent that overexpression of 14-3-3 *per se* does not increase basal or epidermal growth factor-stimulated specific activity of endogenous or recombinant, overexpressed c-Raf-1 in COS (35) or NIH3T3 cells (79), we infer that 14-3-3 abundance is not ordinarily limiting for Raf activation in these cell backgrounds. Raf, however, binds 14-3-3 (at two sites each) with very high affinity. The relative sufficiency of 14-3-3 binding sites in comparison with the cellular levels of physiologic phosphoprotein partners that bind 14-3-3 with lesser affinity/avidity than does Raf remains to be determined. Even with regard to Raf, microinjection of 14-3-3 into *Xenopus* oocytes has been shown to increase Raf kinase activity (80), suggesting that 14-3-3 abundance in this background may be limiting for Raf activation.

Most reports suggest that the various 14-3-3 isoforms exhibit similar binding specificities. Considerable overlap specificity would be anticipated from structure, as the residues lining the phosphopeptide-binding groove of the various 14-3-3 isoforms are highly conserved. Nevertheless, differences in the abilities of 14-3-3 isoforms to bind synthetic peptides and proteins have been reported (31, 75, 76, 81). In addition, several examples of isoform-specific biologic responses are known. Overexpression of 14-3-3σ, for example, can elicit G_2 arrest in colorectal carcinoma cells, a response that is not observed on comparable overexpression of 14-3-3β. Whether this is because of differences in binding specificity, subcellular localization, or another property is unknown; however, the selective increase in the expression of the 14-3-3σ isoform in response to DNA damage (a p53-dependent response) points to the physiologic significance of this difference in response to 14-3-3σ and -β (82–86). Although most isoforms other than σ are ubiquitously expressed, 14-3-3θ expression is also subject to stimulus-dependent regulation (87). In view of the existence of seven, independently regulated 14-3-3 genes, their differential expression during development, and the considerable tendency toward heterodimerization in addition to homodimerization, it is clear that small differences in binding specificity among isoforms, when combined with heterodimerization and differential regulation of the level of expression of individual 14-3-3 isoforms, can combine to generate a potentially robust regulatory apparatus (88).

Phosphorylation of 14-3-3 has been suggested as another regulatory mechanism (89), and several kinases are reported to phosphorylate 14-3-3, *e.g.* a sphingosine-dependent protein kinase (SDK1) (90, 91), casein kinase I (92) and PKCs (14, 76). The role of these phosphorylations in the physiologic regulation of 14-3-3 function remains to be determined.

Localization of 14-3-3 to cytoplasm, nucleus, various membranes, and cytoskeletal and centrosome structures have been reported (reviewed in Ref. 9); however, apart from cytoplasmic/nuclear partition the significance of such differential localization for 14-3-3 function remains unknown. The pathophysiologic importance of changes in 14-3-3 expression and localization in conditions such as cancer and neurodegenerative diseases is unresolved (9).

Conclusions and Perspectives

The past 5 years have witnessed a plethora of reports describing the interaction of 14-3-3 with over 70 proteins, both *in vivo* and *in vitro.* These are likely to represent only a fraction of the physiological 14-3-3 partners. It is clear that any Ser/Thr phosphorylation event wherein an Arg residue is present at position −3 or −4 cannot be considered functionally silent unless the possibility of an interaction with 14-3-3 has been specifically excluded. Among the

most pressing questions regarding 14-3-3 function are the delineation of the isoform specificity of 14-3-3 binding, the impact of homo- and heterodimerization on the range and reversibility of target binding, the regulatory significance of developmental and tissue-specific 14-3-3 expression, and the role of 14-3-3 phosphorylation in the physiologic regulation of 14-3-3 function. Among the tools useful for probing the involvement of 14-3-3 in a regulatory event are the overexpression of 14-3-3 mutants deficient in phosphopeptide binding (43) and the competitive displacement of 14-3-3 *in vitro,* using sequence-specific synthetic phosphopeptides (19). In addition, genetic approaches in yeast (93) and invertebrates (4) as well as gene knockout and RNAi-mediated inhibition of specific isoforms in mammalian cell culture (83) should provide important insight into 14-3-3 specificity and redundancy.

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