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14-3-3 PROTEINS: Structure, Function, and Regulation

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■ **Abstract** The 14-3-3 proteins are a family of conserved regulatory molecules expressed in all eukaryotic cells. A striking feature of the 14-3-3 proteins is their ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors. This plethora of interacting proteins allows 14-3-3 to play important roles in a wide range of vital regulatory processes, such as mitogenic signal transduction, apoptotic cell death, and cell cycle control. In this review, we examine the structural basis for 14-3-3–ligand interactions, proposed functions of 14-3-3 in various signaling pathways, and emerging views of mechanisms that regulate 14-3-3 actions.

INTRODUCTION

The 14-3-3 protein was initially described as an acidic, abundant brain protein by Moore & Perez in 1967 (1). The name is derived from the combination of its fraction number on DEAE-cellulose chromatography and its migration position in the subsequent starch–gel electrophoresis. The unique terminology remains while the concept of 14-3-3 has evolved from a brain-specific protein to a family of ubiquitously expressed regulatory molecules of eukaryotic organisms. 14-3-3 has emerged as a group of multifunctional proteins that bind to and modulate the function of a wide array of cellular proteins. More than 50 signaling proteins have been reported as 14-3-3 ligands (see Annual Reviews' web site, www.AnnualReviews.org, Supplementary Table: 14-3-3–associated proteins, for a detailed list and references). This broad range of partners suggests for 14-3-3 a role as a general biochemical regulator, reminiscent of the well-defined regulatory protein calmodulin. Through interaction with its effector proteins, 14-3-3 participates in the regulation of diverse biological processes, including neuronal development, cell growth control, and viral and bacterial pathogenesis. This review focuses on recent developments in the understanding of the structural basis

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of 14-3-3–ligand interactions and on roles for 14-3-3 in three model systems. The functions of 14-3-3 in neuronal development (2), signal transduction (3–5), and plant biology (6, 7) have recently been reviewed elsewhere.

General Properties

14-3-3 is a family of highly homologous proteins encoded by separate genes. There are seven known mammalian 14-3-3 isoforms, named with Greek letters (β, ε, γ, η, σ, τ, ζ) after their elution profile on reversed phase high-performance liquid chromatography (8, 9). The species initially designated α and δ are actually the phosphorylated forms of β and ζ (10). The 14-3-3 proteins exist mainly as dimers with a monomeric molecular mass of approximately 30,000 and an acidic isoelectric point of 4–5.

14-3-3 proteins exhibit a remarkable degree of sequence conservation between species (11). For example, the *Saccharomyces cerevisiae* BMH1 and human ε isoforms are approximately 70% similar at the amino acid level. 14-3-3 proteins also share some basic biochemical properties, such as activation of the ExoS ADP-ribosyltransferase (12, 13) and of tryptophan hydroxylase (14). These similarities argue strongly for a high degree of functional conservation.

14-3-3 is abundant in the brain, comprising approximately 1% of its total soluble protein (15). It is now clear that 14-3-3 is also present in almost all tissues, including testes, liver, and heart (16). Within a eukaryotic cell, 14-3-3 is largely found in the cytoplasmic compartment. However, 14-3-3 proteins can also be detected at the plasma membrane and in intracellular organelles such as the nucleus and the Golgi apparatus (16–21). Like their high degree of conservation, the ubiquitous nature of 14-3-3 proteins may reflect their fundamental importance in eukaryotic biology. Indeed, recent research on 14-3-3 supports this view.

Rediscoveries

Since the initial discovery of 14-3-3, the history of 14-3-3 proteins has been full of rediscoveries. Characterization of a protein cofactor that activates tryptophan and tyrosine hydroxylases uncovered 14-3-3 (22) and led to the cloning of the first 14-3-3 gene (8). The availability of a 14-3-3 sequence set the stage for a flood of rediscoveries by investigators interested in a wide range of biological questions.

The initial introduction of 14-3-3 into some biological systems was based on functional studies aimed at identifying regulatory proteins. For instance, the isolation of inhibitors of protein kinase C (PKC) (23), the identification of stimulators of calcium-dependent exocytosis (24), and the cloning of a eukaryotic activator of the *Pseudomonas aeruginosa* ExoS ADP-ribosyltransferase (12) each resulted in the rediscovery of the 14-3-3 proteins. Similarly, 14-3-3 has been found as an activator of the 43-kDa inositol polyphosphate 5-phosphatase (5-phosphatase)

(25). It seems that 14-3-3 functions as an allosteric cofactor to affect the catalytic activity of some of its ligands.

Another major avenue of rediscovery of 14-3-3 accompanied technological advances in detecting protein-protein interactions, such as the yeast two-hybrid system. In recent years, many signal transduction pathways have been unveiled that control cell proliferation, differentiation, and apoptosis, but understanding the intricate mechanisms that regulate these pathways remains a daunting challenge (26). Many investigators search for clues by identifying proteins that interact with key signaling components. 14-3-3 proteins are easy prey for a variety of bait proteins in a large array of these screens. Such 14-3-3–associated proteins include receptors [e.g. glucocorticoid receptor (27) and insulin-like growth factor I receptor (IGFIR) (28, 29)], kinases [e.g. Raf-1 (17, 30–34), Bcr (35), and phosphatidylinositol 3 kinase (36)], phosphatases [e.g. Cdc25 (37) and PTPH1 (38)], docking molecules [e.g. insulin receptor substrate I (39) and $p130^{Cas}$ (18)], death regulators [e.g. Bad (40) and A20 (41)], and oncogene products [e.g. polyomavirus middle tumor antigen (MT) (42) and Bcr-Abl (35)]. These protein-protein interaction-based studies have dramatically expanded the range of 14-3-3–regulated events.

A third road to the rediscovery of 14-3-3 has been via genetic suppressor analysis. For instance, the 14-3-3 proteins Rad24 and Rad25 were isolated as suppressors that complement the radiation sensitivity of a *rad24* mutant in *Schizosaccharomyces pombe* (43). The *rad24* mutant is defective in the DNA damage checkpoint, linking 14-3-3 function to cell cycle control. In *S. cerevisiae,* expression of 14-3-3 homologs BMH1 or BMH2 complements the phenotype of the *CHC1* clathrin heavy-chain gene deletion, which supports a potential role for 14-3-3 in vesicular transport (44). Golgi localization and interaction with invariant chain p35 (Iip35) in the endoplasmic reticulum suggest a related purpose for 14-3-3 in mammalian cells (45). It is intriguing to note that BMH1 and BMH2 can also prevent rapamycin-mediated lethality in *S. cerevisiae* (46). Rapamycin, when combined with its immunophilin receptor FKBP12, interacts with Tor (target of rapamycin) to induce cell growth arrest, primarily via an effect on protein synthesis machinery. Although the role of 14-3-3 in this system is not clear, it stands as further proof that 14-3-3 is a significant component of many complex cellular processes.

The frequent isolation of 14-3-3 from many biochemical and genetic screens for different targets must reflect the physiological importance of 14-3-3 in diverse cellular pathways. Depending on its interaction with specific effectors, 14-3-3 participates in many vital regulatory processes, such as cell cycle control, survival signaling, cell adhesion, and neuronal plasticity. The trend of rediscovery of 14-3-3 proteins shows no sign of diminishing and in fact will likely become more common because of an increased understanding of the regulation of 14-3-3–ligand interactions.

14-3-3–LIGAND INTERACTIONS

The heterogeneity and sheer number of binding partners for 14-3-3 allows the prediction of some properties of the interaction. A natural conclusion that can be drawn is that 14-3-3 ligands share a common binding determinant that mediates their contact with 14-3-3. One such determinant is a specifically phosphorylated residue in 14-3-3 ligands. Several early observations guided the realization that phosphorylation of target proteins is the primary mechanism that controls 14-3-3 binding (47–49). In particular, S259 of Raf-1, a conserved phosphorylation site, was shown to be required for 14-3-3–Raf-1 interaction (48). Detailed dissection of the residues surrounding phosphorylated S259 led Muslin et al (49) to define a consensus 14-3-3 recognition motif, RSxpSxP, where x represents any amino acid and pS stands for phosphorylated serine. Screening degenerate phosphoserine-oriented peptide libraries against 14-3-3 revealed a similar sequence, in strong support of the above consensus motif (50). Indeed, numerous 14-3-3–associated proteins bind 14-3-3 through a phosphorylated serine site and contain this motif (Table 1).

The definition of 14-3-3 as a phosphoserine binding protein represents a major conceptual advancement in the study of 14-3-3 function. More important, it defines a novel paradigm by which phosphorylated serine, like phosphorylated tyrosine, can serve as a recognition signal for protein-protein interactions. Analogous to SH2 and PTB domain–containing proteins that bind phosphotyrosine motifs, 14-3-3 proteins represent a novel class of protein modules that recognize phosphoserine motifs (5). Thus, understanding the mechanisms that control 14-3-3–ligand interactions will provide insight into more general questions concerning the control of intracellular signal transduction.

14-3-3 Recognition Sequences

Phosphoserine-Mediated Interactions The prototype phosphorylated serine recognition motif, RSxpSxP, was deduced from a 15-mer Raf-1 peptide containing RQRS^{257}TS^{259}TP (49). This peptide, when phosphorylated on S259, directly binds 14-3-3ζ with an apparent K_d of 122 nM. The binding is site specific because the same peptide cannot interact efficiently with 14-3-3 when it is unphosphorylated or when phosphorylated at S257 or at S257 and S259 together. An Arg residue in the –3 or –4 position relative to the phosphoserine is also crucial for 14-3-3 association. The proline residue at +2 is important, but this position can tolerate other residues (51). Extensive screening of phosphoserine-oriented peptide libraries identified two alternative consensus motifs with one (mode 1) closely related to the RSxpSxP motif (50). However, the peptide library approach revealed some preference for certain amino acids in the –1, –2, and +1 positions. Accordingly, the 14-3-3 recognition motif is refined to R[S/Ar][+/Ar]pS[L/E/A/M]P, where Ar represents an aromatic residue and + indicates a basic residue. The second identified motif (mode 2) uses the optimal sequence Rx[Ar][+]pS[L/

TABLE 1 Ligands of 14-3-3 that are known to contain defined interaction motifs[a]

Ligand	Property	Sequence[b]	Effect of 14-3-3 binding[c]	References
RSxpSxP and related motifs				
Raf-1[d]	S/T kinase	RSTS^{259} TP, RSAS^{621} EP	Dual role: maintain both inactive and active conformations	49, 81
Bad[e]	Bcl-2 homolog	RHSS^{112} YP, RSRS^{136} AP	Cytoplasmic retention; inhibit proapoptotic function	40, 100
Cdc25C	Y/T phosphatase	RSPS^{216} MP	Cytoplasmic retention; block entry into M phase	53, 112, 113, 115, 118
ASK1	S/T kinase	RSIS^{967} LP	Inhibit proapoptotic function	78
Middle T antigen	Oncoprotein	RSHS^{257} YP	Promote tumors in certain tissues	135, 151
KSR[d]	S/T kinase	RSKS^{297} HE, RTES^{392} VP	—	152, 154
PTPH1	Y phosphatase	RSLS^{359} VE, RVDS^{853} EP	—	38
IRS-1	Docking protein	RSKS^{270} QS, HSRS^{374} IP, KSVS^{641} AP	—	39
Iip35	MHC-associated protein	RSRS^{8}CR	Allows exit from ER	45
FKHRL1	Transcription factor	RSCT^{32} WP, RAVS^{253} MD	Cytoplasmic retention; inhibit proapoptotic function	54
Slob	K^+ channel binding protein	RSNS^{54} AI, RSAS^{79} SE	Modulate voltage sensitivity of associated Slowpoke K^+ channels	153

(*continued*)

TABLE 1 (continued) Ligands of 14-3-3 that are known to contain defined interaction motifs[a]

Ligand	Property	Sequence[b]	Effect of 14-3-3 binding[c]	References
$Rx_{1-2}Sx_{2-3}S$ motifs				
Cbl	Adaptor protein	RHS^{619} LPFS^{623}, RLGS^{639} TFS^{642}	—	55
Keratin 18	Cytoskeletal component	RPVSSAAS^{33}	—	57
PKCμ	S/T kinase	RLS^{205} NVS^{208}, RTSS^{219} AELS^{223}	Inhibit kinase activity	56
Other phosphoserine motifs				
IGF-I receptor	Y kinase	*S*VPLDPSA *SSSS*^{1283} LP	—	28, 29
GP1bβ	Adhesion receptor	RLS^{166} LTDP	—	155–157
p53	Transcription factor	KGQSTS^{378} RH	Increase DNA binding	58
Nonphosphorylated motifs				
43 kDa 5-phosphatase	Lipid phosphatase	ELVLRSESEEKVV^{371}	Stimulate phosphatase activity	25
R18	Synthetic peptide	WLDLE^{14}		65, 71

[a]ASK1, apoptosis signal-regulating kinase 1; KSR, kinase suppressor of Ras; IRS-1, insulin receptor substrate I; MHC, major histocompatibility complex; ER, endoplasmic reticulum; PKC, protein kinase C.
[b]Residues implicated by mutation to be important for 14-3-3 binding are italicized.
[c]The effects of phosphorylation and 14-3-3 binding are not clearly distinguished for most of these ligands.
[d]Raf-1 and KSR also bind 14-3-3 via their cysteine rich domains.
[e]The role of Bad S^{112} in 14-3-3 binding is not clear.

E/A/M]P. It is important to note that synthetic peptides containing these motifs bind 14-3-3 with high affinities (50), which suggests that they may reflect the 14-3-3 binding determinant in proteins.

The prototype 14-3-3 recognition motif has been found in a number of 14-3-3–associated proteins, and its role in mediating 14-3-3 binding has been well established in several biological systems (Table 1). For instance, Raf-1 contains two such phosphoserine motifs encompassing S259 and S621 (49, 52). These Ser residues are phosphorylated in vivo, indicating their physiological significance (52). Unphosphorylatable mutants of Raf-1 that convert S259 and S621 to Ala exhibit diminished association with 14-3-3, which underscores the importance of these motifs for 14-3-3 binding (48). It is interesting to note that the defined 14-3-3 consensus motifs can have some predictive value, as several proteins that were anticipated to bind 14-3-3 based on these recognition sequences have now been experimentally verified, including Cdc25 (53) and Bad (40). However, the broad range of substitutions allowed in these motifs (Table 1), such as the use of phosphothreonine instead of phosphoserine in FKHRL1 (54), makes it clear that more work will be necessary to fully understand this mode of interaction between 14-3-3 and phosphoproteins.

Studies on the proto-oncogene product Cbl revealed another variation of the 14-3-3 binding motif, the Ser-rich motif (55). A consensus sequence was postulated: $Rx_{1-2}Sx_{2-3}S$, where x denotes any amino acid and where at least one of the Ser is phosphorylated. The Cbl protein contains two such motifs. Two Cbl-like motifs have also been identified in PKCμ (56). In contrast to Cbl, which requires both of its motifs to be intact for 14-3-3 binding, either one alone is sufficient for the 14-3-3 association with PKCμ. Depending on which Ser residue is phosphorylated, these motifs may be analogous to the RSxpSxP motif. On the other hand, a well-defined 14-3-3 binding site in keratin 18 appears to be novel (57). It is similar to the Cbl-like motif, but only S33 is required for 14-3-3 binding (Table 1). Another atypical phosphoserine epitope can be seen in the tumor suppressor p53. This is an interesting example because double phosphorylation in the 14-3-3 binding motif inhibits 14-3-3 association (58). Dephosphorylation of pS376 of p53 generates a functional motif at pS378 for 14-3-3 association (Table 1). In general, the necessity of phosphorylation for 14-3-3–ligand interaction permits control of these binding events by intracellular kinase/phosphatase signaling networks. The example provided by p53 should serve notice that this control can be a complex process.

Binding of Unphosphorylated Ligands It is clear that 14-3-3 primarily binds phosphorylated ligands. However, several observations have led to the notion that 14-3-3 is also capable of interacting with unphosphorylated ligands. For example, Raf-1 contains a third 14-3-3 binding site, the cysteine-rich domain (CRD) (residues 139–184) (48, 59). The CRD can directly bind 14-3-3 in vitro, although it is uncertain how the CRD participates in 14-3-3 binding in intact Raf-1 (60, 61). Other examples of unphosphorylated 14-3-3 ligands include mitochondrial tar-

geting signal sequences (62), the platelet GPIbα (63), the ExoS ADP-ribosyltransferase (12, 64), and 5-phosphatase (25). The interactions of 14-3-3 with unphosphorylated ligands are of high affinity, similar to those with phosphorylated proteins. Indeed, there are structural homologies between the two classes of ligands in some cases, such as 5-phosphatase, which displays an RSxSxP-like motif, RSESEE (25). However, unlike typical phosphoserine motifs, where phosphorylation is required for high-affinity binding, a nonphosphorylated 13-mer peptide containing the RSESEE motif interacts with 14-3-3 with a K_d of 92 nM (25). It is likely that the necessity of phosphorylation is overcome by the presence of multiple negatively charged Glu residues. In support of this notion, random selection of 14-3-3 binding peptides using phage display libraries has resulted in the isolation of several sequences with RSx_{1-3} E-like motifs (65). Because the interaction of 14-3-3 with unphosphorylated ligands can be inhibited by phosphoserine containing peptides (64), it is likely that both types of ligands employ a similar ligand binding site on 14-3-3.

Structural Basis

The 14-3-3 Monomer Contains a Conserved Amphipathic Groove The drive to comprehend the molecular mechanisms by which 14-3-3 interacts with its ligands led to the solution of the crystal structures of 14-3-3ζ (66) and 14-3-3τ (67), revealing strikingly similar dimeric structures. Each monomer consists of a bundle of nine α-helices organized in an antiparallel fashion [Figure 1*a* (see color insert)]. The molecule has a cup-like shape with a highly conserved, inner, concave surface and a variable outer surface. A striking feature of the concave surface is an amphipathic groove in each monomer [Figure 1*b* (see color insert)]. As revealed from the ζ structure (66), on one side of the groove, helices 3 and 5 present a cluster of charged and polar residues. On the other side of the groove, helices 7 and 9 present a patch of hydrophobic residues. It is interesting to note that these residues lining the concave surface of the groove are mostly conserved among different isoforms of the 14-3-3 family (50, 66, 67, 71). Because many 14-3-3 ligands bind well to all isoforms, it was thought that this conserved amphipathic groove could mediate the binding of 14-3-3 to its target proteins (66). It was further postulated that a basic cluster in the groove, consisting of K49, R56, and R127, may mediate the interaction of 14-3-3 with the phosphoamino acid in its ligands. This model has been unequivocally confirmed by both mutational analysis (68–70) and co-crystallization studies (50, 51, 71).

14-3-3ζ has been co-crystallized in complex with several peptide ligands, providing critical insights into the structural details of 14-3-3–ligand interactions (50, 51, 71). In the complexes, both phosphorylated and unphosphorylated 14-3-3 binding peptides lie in the conserved amphipathic groove [Figure 1*c*,*e* (see color insert)]. Instead of the α-helical structure originally proposed, 14-3-3 binding peptides adopt an extended conformation. These extended structures may have fewer steric constraints and greater conformational flexibility to sample different

residues in the groove for optimal association. This gives 14-3-3 great versatility in the recognition of a diverse range of ligand sequences.

The high-resolution model of 14-3-3ζ in complex with a mode 1 phosphoserine peptide derived from MT (MA*RSHpSYP*AKK) provides a structural explanation for the 14-3-3–phosphoserine motif interaction (50, 51). The phosphoserine contacts 14-3-3 by salt bridges to the side chains of K49, R56, and R127 in the basic cluster and a hydrogen bond to the hydroxyl group of Y128 (Figure 1*e*). In support of this interpretation, charge-reversal mutations K49E, R56E, and R127E drastically disrupt the interaction of 14-3-3ζ with Raf-1 and Bcr (68, 70; H Wang & H Fu, unpublished data). K120 in the charged face of the groove, together with N173 and N224, stabilize an extended ligand conformation by contacting backbone groups of the +1 and –1 residues, which may be important for positioning the phosphoserine to interact with the basic cluster of 14-3-3. All of these interactions were similar for a synthetic peptide based on the mode 2 binding motif (51), which suggests that diverse ligands use the basic cluster and its accessory residues to bind to 14-3-3. Outside of this cluster, there is considerable variability in 14-3-3–ligand connections, as assessed by comparison of the MT co-crystal structure with that of the mode 2 peptide. For example, E180 forms a hydrogen bond with the –2 Ser in the MT peptide but bonds with the –4 Arg in the mode 2 peptide (51). Similarly, several hydrophobic residues, including L172, L216, I217, and L220, are consistently involved in binding, but they interact with different parts of the two ligands. This type of flexible interaction may explain the diversity among 14-3-3 binding sequences from various ligands.

The conserved ligand binding groove is also involved in binding unphosphorylated ligands, such as R18 (Figure 1*c*,*e*). R18 is a peptide selected from a phage display library for its high affinity for 14-3-3 proteins (65). In the 14-3-3ζ–R18 complex, R18 assumes an extended conformation in the amphipathic groove, similar to phosphorylated peptides (71). Its core WLDLE sequence is located in the phosphoserine binding site with its two acidic residues, Asp and Glu, next to the basic cluster of 14-3-3. Two Leu of R18 interact with amino acids on the hydrophobic side of the 14-3-3 groove, including L172 and L220. Thus, R18 assumes a true amphipathic structure.

It has been reported that C-terminal fragments of 14-3-3 are capable of ligand binding (33, 72, 73). For example, the ''box-1'' region, which spans residues 171–213 of 14-3-3η, efficiently binds tryptophan hydroxylase, Raf-1, and Bcr (72, 74). Although several hydrophobic and polar residues that contribute to the ligand binding groove of 14-3-3 are located in these regions, the exact nature of the stable interaction of these regions with different proteins remains to be clarified.

The 14-3-3 Dimer Can Simultaneously Bind Two Ligands As seen in the various crystal structures, the N-terminal portion of 14-3-3 is involved in dimer formation (Figure 1) (66, 67). The dimer interface is formed by the packing of helix α1 from one monomer against α3 and α4 from the other, leaving a 6- to 8-Å hole

in the center. Several hydrophobic and polar residues are buried in the dimer interface, including L12, A16, V62, I65, and Y82. These residues are largely conserved among mammalian 14-3-3 isoforms, which raises the possibility that 14-3-3 proteins can form heterodimers between different isoforms (75).

The dimeric structure of the 14-3-3 protein allows it to bind two ligands simultaneously (Figure 1*c*,*d*). In the co-crystal structures of 14-3-3 with peptides, the ligand binding sites are located within the same concave surface, and each site is occupied (50, 71). The dimer is arranged such that the ligand binding groove runs in opposite directions in each monomer of the molecule (Figure 1*d*). Simultaneous binding of a 14-3-3 dimer to two protein ligands would have significant implications. In this regard, it is interesting to note that many 14-3-3 ligands, such as Raf-1 and Cbl, have multiple recognition motifs (Table 1). Dual recognition by 14-3-3 of two weakly interacting motifs, such as those found in Cbl, may promote a more stable interaction. In contrast, 14-3-3 may bind to two high-affinity sites, such as those found in Raf-1, perhaps to promote a regulatory conformational change in Raf-1. Alternatively, 14-3-3 may bring together two different signaling molecules to modulate each other's activity. Some evidence has been provided that 14-3-3 can mediate the association of Raf-1 with Bcr (76) or A20 (41), but the physiological significance of these complexes is unclear. It is plausible that this adaptor function of 14-3-3 exists only for specific ligand pairs. Yet another possible role for dimerization of 14-3-3 is in subcellular localization. For example, one monomer could function as a targeting unit by binding an anchored ligand while the other binds a cargo protein. Depending on the site of the anchored ligand, 14-3-3 may localize the cargo protein to distinct intracelluar compartments. The recently reported binding of 14-3-3 to CRM1 may serve such a purpose (51).

Although the exact function of 14-3-3 dimerization is not clear, the importance of this phenomenon is supported by data showing that several ligand binding–defective mutants of 14-3-3 act as dominant negative inhibitors in vivo (70, 77–79). The dimeric nature of 14-3-3 may hold the key to many critical roles of 14-3-3 in cells.

REGULATION OF INTRACELLULAR SIGNALING BY 14-3-3

By interacting with various regulatory proteins, 14-3-3 participates in diverse signal transduction pathways. Although the role of 14-3-3 in many cases remains elusive, some insights have been gained from recent investigations involving three 14-3-3 ligands, Raf-1, Bad, and Cdc25.

Raf-1–Mediated Signal Transduction

Raf-1 is a Ser/Thr kinase that plays a pivotal role in the signal transduction pathway induced by growth factors (80, 81, and references therein). On activation, the small GTP binding protein Ras interacts directly with Raf-1 and recruits

Raf-1 to the plasma membrane. There, Raf-1 is activated by a poorly understood mechanism. Raf-1 then phosphorylates the kinase MEK, leading to stimulation of the mitogen-activated protein kinases, and ultimately to transcription of genes involved in cell division. How Raf-1 is activated is a question of central importance in the field of signal transduction. The identification of 14-3-3 as a Raf-1 binding protein added a new element to the regulatory machinery of Raf-1. Genetic analysis in yeast and *Drosophila* has now convincingly demonstrated a critical regulatory role of 14-3-3 in the Ras-Raf signaling pathway (2, 77, 82–84). Taken together, the available data support a dual role for 14-3-3 in Raf-1 activation: 14-3-3 maintains Raf-1 in an inactive state in the absence of activation signals but promotes Raf-1 activation and stabilizes its active conformation when such signals are received (48, 59, 81). This complex behavior may be explained in part by the existence of three regulated 14-3-3 interaction sites on Raf-1.

Raf-1 can be separated into two functional domains, an N-terminal inhibitory fragment and a C-terminal catalytic fragment (Figure 2). The N-terminal portion contains RBD (a Ras binding domain); CRD (a cysteine-rich domain), which can also bind Ras; and the phosphoserine-259 site for 14-3-3 interaction. Raf-CRD can bind 14-3-3 in addition to Ras and phosphatidylserine (85). The third site for

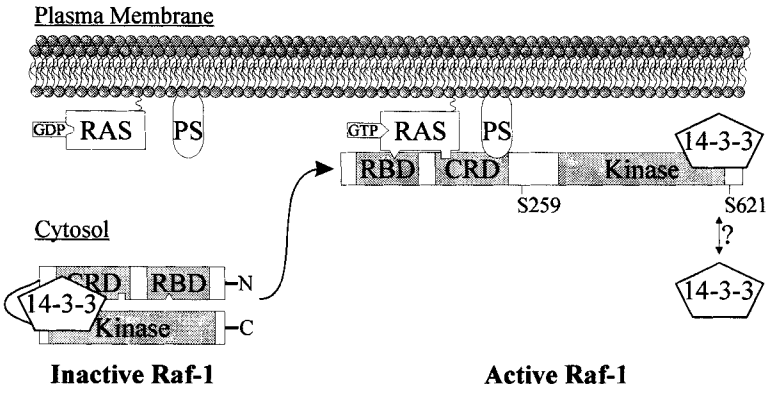

Figure 2 Dual role model of 14-3-3 in Raf-1 activation. In quiescent cells, 14-3-3 may function to keep Raf-1 in an inactive state by binding to pS259 and the cysteine-rich domain (CRD) (81, 85). pS621 may also be associated with 14-3-3. This 14-3-3-bound conformation of Raf-1 is inactive, but permissive for activation. In response to mitogenic signals, GTP-Ras associates with the Ras binding domain (RBD) and recruits Raf-1 to the plasma membrane. This event may lead to the contact of phosphatidylserine (PS) with the CRD and displacement of 14-3-3 from the CRD and pS259 sites of Raf-1. Removal of the inhibitory effect of 14-3-3 partially activates Raf-1, which can be further stimulated by other mechanisms. During activation, 14-3-3 may bind the pS621 site, maintaining its active conformation. An alternative model (90) is that on Ras binding, 14-3-3 is completely displaced from Raf-1. 14-3-3 is required for generating a Raf-1 conformation that is competent for activation, but it is no longer needed after Raf-1 is activated at the membrane.

14-3-3 binding, pS621, is located C-terminal to the kinase domain. Binding of 14-3-3 to CRD and pS259 sites in the N-terminal domain may negatively regulate Raf-1 function. (*a*) Mutations in Raf-1 that block the interaction of 14-3-3 with S259 stimulate Raf-1 kinase activity (48), potentiate mitogenic signal-induced Raf-1 activation (86), and activate Raf-mediated biological functions (48, 87). (*b*) Mutations in Raf-CRD that selectively decrease 14-3-3 binding enhance Raf-1 function (48, 59, 60). (*c*) 14-3-3 is displaced from the N-terminal domain by activated Ras, implying a requirement of 14-3-3 dissociation for Raf-1 activation (87, 88). These observations are the basis for the model that 14-3-3 is required to maintain an inactive conformation of Raf-1.

In contrast to the pS259 and CRD sites, the interaction of 14-3-3 with Raf-1 at the pS621 site may be required for Raf-1 activation (52, 70, 86). Tzivion et al (86) used a phosphoserine peptide to strip 14-3-3 away from epidermal growth factor–activated Raf-1 in vitro. Removing 14-3-3 from the Raf-1 complex leads to its inactivation, which suggests a strict requirement of 14-3-3 for Raf-1 activity. It is important to note that addition of recombinant 14-3-3ζ protein to the phosphopeptide-treated Raf-1 preparations significantly reactivates Raf-1. Thus, 14-3-3 may function as an essential cofactor for Raf-1 kinase activity (86). This conclusion was independently reached by Thorson et al (70) using a constitutively active, C-terminal fragment of Raf-1, CT-Raf. CT-Raf contains only one 14-3-3 binding site, pS621. They found that displacement of 14-3-3 from the CT-Raf complex with the detergent Empigen-BB reversibly abolishes CT-Raf kinase activity. These experiments suggest a requirement for the continuous presence of 14-3-3 for Raf-1 activity. Thorson et al (70) also postulate that 14-3-3 may be necessary for maintaining the phosphorylation state of S621 in vivo. This is consistent with an earlier demonstration that 14-3-3 protects Raf-1 from phosphatase treatment in vitro (89). These results together suggest that S621 is a site for positive regulation by phosphorylation and 14-3-3 binding.

A requirement of 14-3-3 for Raf-1 kinase activity is challenged by other reports (48, 61, 90). Subcellular fractionation studies show that 14-3-3 is bound to inactive Raf-1 in the cytosol but is totally displaced when Raf-1 is recruited to the plasma membrane, which suggests that activated Raf-1 does not bind 14-3-3 (90). This is consistent with the ability to isolate 14-3-3–Raf-1 complexes from extracts of quiescent, but not mitogen-stimulated, NIH 3T3 cells (34). However, such data do not preclude a positive role of 14-3-3 in Raf-1 activation. Roy et al (90) found that 14-3-3 potentiates the kinase activity of membrane-recruited Raf-1, even though such membrane-localized Raf-1 is not bound to 14-3-3. It is possible that 14-3-3 is required for Raf-1 recruitment to the plasma membrane and for inducing a conformation of Raf-1 competent for activation (90).

A major discrepancy has emerged from recent reports, concerning whether 14-3-3 must be continuously associated with Raf-1 for its catalytic activity. The different results reported may reflect the complexity of this biological system as well as experimental limitations to the faithful recapitulation in vitro of the activation mechanism of Raf-1. A critical examination of the phosphorylation status

of Raf-1 at S259 and S621 under different activation states will be essential for resolving the conflicting data, by clarifying which sites 14-3-3 binds in each state.

The dual role model of 14-3-3 in Raf-1 activation remains an attractive choice because this model explains most of the data in the literature (Figure 2) (48, 59, 81). 14-3-3 plays a negative role because it needs to be displaced at least from the N-terminal regulatory domain during Raf-1 activation. In a positive role, 14-3-3 may function as an allosteric cofactor to induce and maintain a conformation of Raf-1 competent for activation or required for activity. It is also possible that 14-3-3 may promote Raf-1 function through efficient coupling of Raf-1 to its downstream effectors. It is clear that more research must be done to clarify the detailed mechanism of how 14-3-3 regulates Raf-1 activation.

Bad and Cell Death Pathways

Apoptosis is a process of cell death that plays a critical role in normal development as well as in the pathophysiology of a variety of diseases, such as cancer (reviewed in 91). It is tightly regulated, and a pivotal component of this regulation is the Bcl-2 family of pro- and antiapoptotic proteins. 14-3-3 has been found to interact with a proapoptotic member of the Bcl-2 family, Bad, in a phosphoserine-dependent manner (40). 14-3-3 binding antagonizes the proapoptotic activity of Bad, providing a novel signal integration point for control of cell death.

Bad, like other Bcl-2 homologs, is capable of dimerizing with some of its family members, and it was discovered in a search for Bcl-2 binding proteins (92). The importance of Bad as a mediator of cell death has since become well established for several reasons. It is broadly expressed in human tissues (93), and its levels are dynamically regulated by apoptotic stimuli (94). Forced expression of Bad in the T cells of Bad transgenic mice leads to a dramatic reduction in the T cell population (94). Bad causes cell death by binding to and inhibiting the antiapoptotic effects of Bcl-X_L and Bcl-2 (92, 94, 95, 107, 108). 14-3-3 is involved in preventing the interaction of Bad with Bcl-X_L and Bcl-2 and, thus, Bad-induced cell death (40). Put briefly, when the binding of 14-3-3 to Bad is induced by phosphorylation, Bad is complexed with 14-3-3 in the cytosol, sequestered from mitochondrially localized Bcl-X_L/Bcl-2 and unable to induce apoptosis (Figure 3*a*). In this way, Bad is under the strict control of survival and death signal–driven kinases and phosphatases (40, 96–99).

Sequence analysis of murine Bad reveals two potential 14-3-3 phosphoserine motifs, RHSpS^{112}YP and RSRpS^{136}AP. It was suggestive when mapping of the in vivo phosphorylation sites of Bad showed only two residues: S112 and S136 (40). That S136 of Bad has an important role in 14-3-3 binding has been substantiated, whereas the role of S112 remains uncertain (40, 99, 100; H Yang, SC Masters & H Fu, unpublished results). The issue of which sites mediate 14-3-3–Bad complex formation is important because the phosphorylation of S112 and S136 is regulated by different signaling pathways.

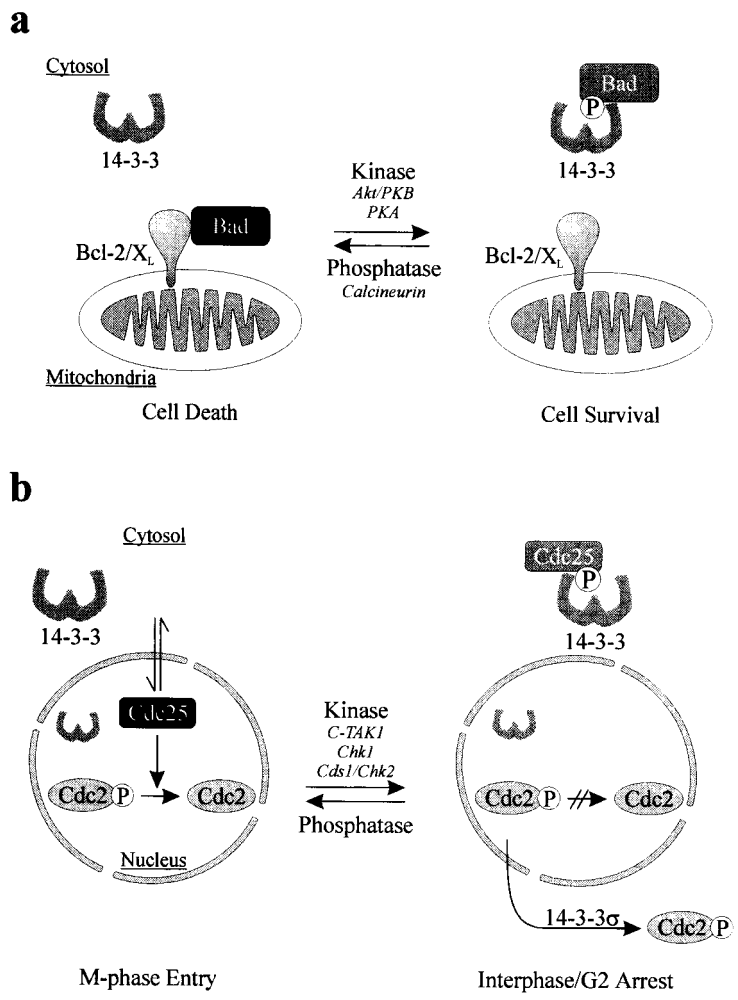

Figure 3 Parallel models for 14-3-3 effects on Bad and Cdc25. (*a*) 14-3-3 sequesters Bad from mitochondrial Bcl-X_L/Bcl-2. In its default state, Bad binds Bcl-X_L/Bcl-2 in the mitochondria, favoring the induction of apoptosis. Survival signals stimulate kinases, such as Akt/protein kinase B (PKB), leading to phosphorylation of Bad. Phosphorylated Bad is found in the cytosol bound to 14-3-3, where it is unable to induce death. Phosphatases such as calcineurin reverse this process. (*b*) 14-3-3 retains Cdc25 in the cytosol. During interphase or in response to DNA damage, Cdc25 can be phosphorylated by several kinases to create a 14-3-3 binding site. 14-3-3–bound Cdc25 is found in the cytosol where it cannot act on Cdc2, thus preventing mitosis. An unknown phosphatase is presumably involved in disrupting the Cdc25–14-3-3 interaction when mitosis is to be initiated. By an unknown mechanism, 14-3-3σ may sequester the Cdc2/cyclin B1 complex in the cytosol on DNA damage (110, 122a). PKA, Protein kinase A.

At least four kinases are capable of phosphorylating Bad in vitro, including protein kinase A (PKA) (40, 98), Akt/protein kinase B (96, 99), PKC (40), and Raf-1 (40, 101). However, of these, only PKA and Akt phosphorylate Bad at the S112 or S136 sites relevant to 14-3-3 interaction. Akt is a Ser/Thr kinase that is broadly involved in cell survival and differentiation signaling (reviewed in 102). It is located in a pathway downstream of phosphatidylinositol 3'-kinase and thus is activated in response to a multitude of prosurvival signals, including IGF-I, interleukin-3, and nerve growth factor. Akt signaling can also be stimulated in other ways, such as via Ca^{2+}/calmodulin-dependent protein kinase kinase (103). In any case, active Akt leads to the phosphorylation of S136 of Bad, to association of 14-3-3 with Bad, and to inhibition of Bad-induced cell death (99; SC Masters, SR Datta, ME Greenberg & H Fu, unpublished data). Phosphorylation of Bad by Akt at S136 directly links a general survival signaling pathway to a death promoter, one way that survival factors can inhibit cell death. The Akt effect on Bad may be cell-type specific, as several reports have raised questions regarding the relative importance of Akt as a Bad S136 kinase (98, 104–106). Bad S112 is phosphorylated by the mitochondrially localized pool of PKA (98). Phosphorylation of S112 by PKA appears to inhibit Bad proapoptotic activity. Whether or not the PKA/Bad system involves 14-3-3 awaits further testing.

Bad is the target for both anti- and proapoptotic signals. It can be dephosphorylated at S112 and S136 by the phosphatase calcineurin in response to Ca^{2+} influx (97). This dephosphorylation has been correlated with the dissociation of 14-3-3 from Bad, mitochondrial localization of Bad, and enhanced apoptotic cell death. Calcineurin completes a reversible regulatory system for Bad, and it emphasizes the importance of removing 14-3-3 for Bad proapoptotic activity. This work also highlights the necessity of determining the inactivating phosphatases for other 14-3-3 ligands.

Bad is thought to induce apoptosis by binding and inactivating Bcl-X_L/Bcl-2 (92, 107, 108). Thus, active, death-inducing Bad is found localized to the mitochondria. Because 14-3-3–bound, inactive Bad is found in the cytosol, it was proposed that 14-3-3 acts to sequester Bad away from its death effectors (Figure 3*a*) (40). Specific kinases and phosphatases thus dynamically regulate the phosphorylation of and 14-3-3 binding to Bad, which determines its proapoptotic function. Several other possible mechanisms can be devised. For example, phosphorylated Bad may be inactive, and binding of 14-3-3 could serve to protect Bad from the action of phosphatases, such as calcineurin (97). Alternately, 14-3-3 may block the Bcl-X_L/Bcl-2 interaction site on Bad, forcefully separating Bad from its effectors rather than stabilizing the cytosolic localization of free Bad protein. It should be noted that these mechanisms are not mutually exclusive. Dissecting the role of 14-3-3 in Bad-induced apoptosis may provide a model system for 14-3-3–ligand interactions in general.

Bad is not the only 14-3-3 ligand involved in apoptosis. Indeed, a large fraction of the known 14-3-3 binding proteins can directly or indirectly modulate cell-death pathways. For example, apoptosis signal-regulating kinase 1 (ASK1) is a

component of multiple death signaling pathways, including those activated by tumor necrosis factor α, Fas, and oxidative stress (109). 14-3-3 binding to ASK1 can suppress its proapoptotic activity, and an ASK1 mutant that cannot bind 14-3-3 has dramatically enhanced ability to kill (78). Besides Bad, Akt has several other substrates that are involved in apoptosis, and 14-3-3 also targets some of these, such as the Forkhead transcription factor FKHRL1 (54). When phosphorylated by Akt, FKHRL1 becomes a 14-3-3 ligand and is no longer able to induce cell death. A few of the other 14-3-3 ligands that are involved in the cell death or survival process include MEKK1 (19), A20 (41), IGFIR (28, 29), phosphatidylinositol 3'-kinase (36), and Raf-1 (101). The hypothesis that 14-3-3 plays a key role in the regulation of cell fate determination is strongly supported by this large list of ligands. It is possible that 14-3-3 serves as a general survival factor by enhancing prosurvival signaling while suppressing proapoptotic pathways.

Cdc25 and Cell Cycle Control

The requirement of Rad24 and Rad25 for G2-checkpoint control in *S. pombe* links 14-3-3 to the cell cycle machinery (43). Genetic and biochemical studies in yeast, frog, and human cells have now defined a specific 14-3-3 role in cell cycle control. Mounting evidence has indicated that one major effector of 14-3-3 in this system is the phosphatase Cdc25.

Cdc25 is a major cell cycle regulator that dephosphorylates and activates the protein kinase Cdc2 to trigger entry into mitosis (reviewed in 111). Inhibition of Cdc2 dephosphorylation is pivotal for blocking mitosis in response to damaged or unreplicated DNA. Thus it is not surprising that Cdc25, a key activator of Cdc2, is highly regulated. During interphase, human Cdc25C is predominantly phosphorylated at S216 (53). Conversely, this site is not phosphorylated during mitosis, which suggests that phosphorylation of S216 negatively regulates Cdc25C function. Peng et al (53) have found that phosphorylation of S216 generates a 14-3-3 binding motif (Table 1) and leads to Cdc25–14-3-3 association. As expected, substitution of S216 with Ala abrogates the Cdc25C–14-3-3 interaction. 14-3-3 may be required to maintain an inactive state of Cdc25 because expression of S216A Cdc25 accelerates mitotic entry and allows cells to escape the G2-checkpoint arrest induced by DNA damage signals (53). An analogous mechanism may operate in *Xenopus* oocytes (112, 113) and in fission yeast (114, 115, 115a). Thus, it is conceivable that the regulation of Cdc25–14-3-3 complex formation is a critical, conserved part of the cell cycle machinery. However, 14-3-3 binding does not affect the catalytic activity of Cdc25 (37, 53), which suggests that 14-3-3 plays an indirect role in the inhibition of Cdc25 function.

The mitosis-inducing function of Cdc25 requires its entry into the nucleus where its substrate Cdc2 is located. Notably, Cdc25 contains a putative bipartite nuclear localization sequence near S216 (116) as well as a nuclear export sequence (NES) (113, 117). It is possible that 14-3-3 binding regulates the shuttling of Cdc25 between the cytosol and nucleus (Figure 3*b*). Recent data support

this hypothesis. Elimination of a 14-3-3 homologue, Rad24, in *S. pombe* causes nuclear accumulation of Cdc25 (115). Expression of 14-3-3ε in a *Xenopus* tissue culture system causes the localization of Cdc25 in the cytosol whereas a mutant Cdc25 that cannot bind 14-3-3 is exclusively nuclear (117). Similarly, human Cdc25C is retained in the cytoplasm during interphase, under the control of 14-3-3 (118). Thus, 14-3-3 binding correlates well with cytoplasmic localization of Cdc25 (Figure 3*b*). To explain the role of 14-3-3 in Cdc25 localization, two alternative models have been proposed. Lopez-Girona et al (115) suggested that 14-3-3 is actively excluded from the nucleus. Mutations in a putative NES in Rad24, equivalent to residues I217 and L221 in helix 9 of 14-3-3ζ, impair nuclear export of Rad24 as well as the DNA damage-induced nuclear depletion of Cdc25. Thus, Rad24 may function as ''an attachable nuclear export signal'' that promotes the nuclear export of associated Cdc25 (115). In support of the NES model, Rittinger et al (51) report the interaction of 14-3-3ζ with CRM1, a component of the nuclear export machinery, in vitro and suggest that hydrophobic residues in helix 9 of 14-3-3ζ play a role in both ligand binding and nuclear export. Another possible explanation is that mutations in the putative NES sequence of Rad24 disrupted the 14-3-3 interaction with Cdc25, rendering 14-3-3 inactive (69, 115a, 117). Examination in the *Xenopus* system suggests an alternative model: 14-3-3 inhibits Cdc25 function primarily by attenuating its nuclear import, in part through blockage of the Cdc25-importin α interaction (113, 117). In contrast to fission yeast, 14-3-3 binding is neither necessary nor sufficient for Cdc25 nuclear export in *Xenopus.* However, the above models may not be mutually exclusive because 14-3-3 may cooperate with the NES and nuclear localization sequence of Cdc25 in controlling the dynamic shuttling of Cdc25.

The Cdc25–14-3-3 interaction is regulated by specific phosphorylation in response to cellular signals. Several kinases that phosphorylate S216 of Cdc25C have been described, including C-TAK1 (119), Chk1 (53, 120, 121, 121a), and Cds1/Chk2 (121a, 150). Chk1 is activated in response to DNA damage (122). Both Chk1 and Cds1/Chk2 are found in the nucleus and may induce Cdc25 phosphorylation and 14-3-3 binding in response to activation signals, such as DNA damage. Therefore, these kinases can indirectly act to localize Cdc25 in the cytoplasm, preventing entry into mitosis.

Another major event during mitotic entry is the accumulation of activated Cdc2 in the nucleus. In addition to its importance in the control of Cdc25, 14-3-3 may play a critical role in Cdc2 localization (110, 122a). Following DNA damage signals, 14-3-3σ is dramatically up-regulated by a p53-dependent mechanism (125). The induction of 14-3-3σ appears to enhance the cytoplasmic localization of Cdc2, thus preventing Cdc2 from entering the nucleus and initiating mitosis (122a). 14-3-3σ–deficient cells exhibited nuclear localization of Cdc2 and failed to maintain G2 arrest on DNA damage. Thus, using analogous mechanisms, 14-3-3 isoforms may ensure DNA damage-induced cell cycle arrest by simultaneously sequestering Cdc25 and Cdc2 in the cytoplasm.

14-3-3 has been found to interact with other proteins involved in the control of the cell cycle, such as Chk1 (123), Wee1 (122a, 124), and p53 (58). Interaction of 14-3-3 with multiple cell cycle regulators suggests that 14-3-3 may help coordinate cell cycle progression.

REGULATION OF 14-3-3

The interaction of 14-3-3 with its ligands is under tight control. Besides phosphorylation of target proteins, the status of 14-3-3 itself is also a critical determinant of this control. Mechanisms that regulate 14-3-3 may include isoform specificity, posttranslational modifications, and expression levels in cells.

The presence of seven 14-3-3 isoforms in mammalian cells suggests a possible role for isoform-specific interactions with different targets. However, structural studies have not supported this conclusion. The key residues of 14-3-3 involved in ligand binding are conserved among different isoforms (50, 51, 66, 71), which suggests a lack of isoform selectivity for ligands that dock in this binding groove (i.e. most 14-3-3 ligands). In support of this notion, different 14-3-3 isoforms bind phosphoserine peptides with similar affinities and select similar phosphoserine motifs from oriented peptide libraries (49, 50). Also, distinct 14-3-3 isoforms interact equally well with the intact proteins Raf-1, Bad, and ExoS (13, 51; RR Subramanian & H Fu, unpublished data). On the other hand, it is possible that variable residues in and near the ligand binding groove of 14-3-3 may contribute to certain ligand preferences. The selective interaction of A20 with 14-3-3η suggests that 14-3-3 isoforms may bind differentially to some ligands (41). Among the mammalian isoforms, 14-3-3σ has been shown to bind the least well to $p130^{Cas}$ and B-Raf in vitro (18, 51). Further, 14-3-3σ is capable of inducing an isoform-specific biological effect. Overexpression of 14-3-3σ caused a G2 cell cycle arrest in human colorectal cancer cells, whereas 14-3-3β expression did not (125). The 14-3-3σ effect may be a consequence of its preferential interaction with the Cdc2/cyclin B1 complex (122a). One difficulty in assessing isoform specificity is the lack of knowledge of how dramatic a difference in affinity is necessary to create a biologically relevant effect. Thus, the small differences between isoforms observed in vitro may indeed be biologically relevant.

It seems most likely that isoform-specific cellular effects are determined by different posttranslational modifications of 14-3-3 isoforms and by isoform-specific 14-3-3 levels in various subcellular compartments. One intriguing possibility is that 14-3-3 isoforms interact in vivo to form heterodimers. Such molecules would amplify dramatically the implications of any isoform specificity, either intrinsic or due to external regulation.

Phosphorylation of 14-3-3 appears to modulate the function of 14-3-3 isoforms. Three phosphorylation sites have been determined in 14-3-3ζ: S58 (126), S184 (10), and T232 (127). S184 lies within a proline-directed kinase consensus sequence, S^{184}PEK, and is phosphorylated in 14-3-3ζ as well as in β in brain

tissues. Such phosphorylation gives rise to the species initially designated as the δ and α isoforms, respectively (10). In the crystal structure of ζ, S184 is a surface residue located at the N terminus of helix 8, near the top of the ligand binding groove (Figure 1*b*) (66). This position implies a possible role of phosphorylation in regulating ligand binding. Consistent with this idea, the phosphorylated forms of β and ζ show increased potency in the inhibition of PKC in vitro (128). The kinase that phosphorylates S184 has not been identified (127). 14-3-3ζ, but not 14-3-3β, is also phosphorylated at T232 in HEK293 cells (127). T232 resides in the C-terminal loop of 14-3-3, which has been implicated in regulating ligand binding (66). In fact, 14-3-3ζ phosphorylated at T232 is devoid of Raf-1 association, which suggests that this modification negatively affects ligand binding (127). Dubois et al (127) have identified casein kinase Iα as a T232 kinase. Among mammalian isoforms, only 14-3-3τ and 14-3-3ζ have a phosphorylation site at the corresponding 232 position, and casein kinase Iα can indeed phosphorylate S232 of 14-3-3τ in vitro. Thus, 14-3-3ζ– and 14-3-3τ–induced ligand interactions may in part be controlled by casein kinase Iα activity. The third site, S58 of 14-3-3ζ, is phosphorylated by sphingosine-dependent protein kinase 1 (SDK1) (126, 129). SDK1 phosphorylates 14-3-3ζ as well as 14-3-3β (S60) and 14-3-3η (S59), but not 14-3-3τ or 14-3-3σ. This phosphorylation is stimulated in response to sphingosine, which is generated after treatment of cells with mitogens such as platelet-derived growth factor and IGF-I (130). S58 is facing away from the ligand binding groove, buried in the dimer interface (Figure 1*b*). Such positioning implies a potential role of S58 phosphorylation in dimer formation or dissociation. Because dimerization of 14-3-3 is thought to be important for its function, modulation of 14-3-3 dimerization by SDK1 may be a key target of sphingosine in cells. In addition to Ser/Thr phosphorylation, 14-3-3 isoforms can be phosphorylated on tyrosine residues, for example by Bcr-Abl (35) and by IGFIR (29).

The interaction of 14-3-3 with ligands can be influenced not only by its modification but also by fluctuation of 14-3-3 levels in cells. It appears that the amount of 14-3-3, or at least of specific isoforms, is limiting in cells despite the relative abundance of 14-3-3. For instance, overexpression of 14-3-3 isoforms enhances the specific activity of Raf-1 in HeLa and COS cells (70, 90) and inhibits PKC activity in Jurkat T cells (56, 131). One role of the multiple isoforms of 14-3-3 may be to allow regulation of the total 14-3-3 pool via unique transcriptional controls for each isoform. Thus, regulation of 14-3-3 expression can serve as an effective mechanism for controlling 14-3-3 functions. In human colorectal cancer cells, 14-3-3σ is dramatically induced by DNA damaging agents in a p53-dependent manner, leading to G2 arrest (125). 14-3-3γ is induced by serum and platelet-derived growth factor in vascular smooth muscle cells (132) whereas 14-3-3ε is down-regulated during differentiation of mesenchyme cells (133). The dynamic expression patterns of various 14-3-3 isoforms during mouse embryogenesis and neuronal development underscore the importance of each 14-3-3 in mediating cellular processes (133, 134). Perhaps the temporal and spatial expres-

sion patterns of 14-3-3 isoforms control the interaction of 14-3-3 with its specific effectors, enabling the activation or suppression of particular signaling pathways.

14-3-3 AND DISEASES

Although 14-3-3 has not been directly linked to a specific disease, it has been implicated in a variety of pathological processes. A large number of 14-3-3 ligands are proto-oncogene or oncogene products, which suggests the participation of 14-3-3 in mitogenic signal transduction as well as neoplastic transformation. Indeed, the tumor profile of mice infected with polyomavirus expressing a 14-3-3 binding–defective mutant MT showed a striking deficiency in the induction of salivary gland tumors (135). Another possible connection of 14-3-3 to tumorigenesis is its involvement in regulating cell survival, for example through its interaction with IGFIR. The IGFIR plays an important role in controlling normal cell survival as well as in tumorigenesis (136). The major site for 14-3-3 interaction is located within a Ser quartet critical for IGFIR-mediated cell transformation (28, 29, 137), implying that 14-3-3 participates in this process. Significantly elevated levels of both the IGFIR (138) and 14-3-3 proteins (139) have been detected in all major types of lung cancer. On the other hand, the 14-3-3ε gene is found in a region with frequent loss of heterozygosity in several cancers, which suggests that some 14-3-3 isoforms may be important for suppression of tumorigenesis (140). Taken together, these data argue that 14-3-3 may be involved in the development of human cancer.

The abundance of 14-3-3 proteins in brain tissues points to a critical role of 14-3-3 in neuronal function. There are some indications that 14-3-3 is involved in several neurological disorders. 14-3-3ε is located in a chromosomal region, 17p13.3, that contains genes implicated in isolated lissencephaly sequence (ILS) and Miller-Dieker syndrome (MDS) (141, 142, and references therein). ILS is a brain malformation malady marked by disorganization of the cortical layers. MDS causes malformations similar to those of ILS, as well as additional abnormalities, and is associated with larger deletions of 17p13.3. The 14-3-3ε sequence is deleted in some MDS patients, and this loss may contribute to the development of MDS phenotypes (141). 14-3-3 proteins are also found in the neurofibrillary tangles seen in patients with Alzheimer's disease (143). A possible genetic association of a 14-3-3η polymorphism with early onset schizophrenia has been reported (144). Hsich et al (145) noted that 14-3-3 proteins are specifically detected in cerebrospinal fluid from patients with Creutzfeldt-Jakob disease (CJD) and related transmissible spongiform encephalopathies. This observation has allowed the use of 14-3-3 as a biochemical marker for a premortem diagnostic test for CJD and related diseases (145, 146). It is not known whether 14-3-3 is involved in the pathogenesis of CJD, or whether the presence of 14-3-3 in cerebrospinal fluid is simply the consequence of neuronal cell death in CJD brains.

14-3-3 ANTAGONISTS

Development of 14-3-3 antagonists is important not only for functional analysis of 14-3-3 proteins but also for potential therapeutic interventions against diseases involving 14-3-3 malfunction. Two types of 14-3-3 antagonists have been described, phosphoserine-motif based and nonphosphorylated peptides.

Several phosphoserine-motif–containing peptides have been characterized by kinetic studies and crystallographic analysis. The pS-Raf-259 peptide exhibits high affinity for multiple isoforms of 14-3-3 with K_d values of 120–140 nM (49). This phosphopeptide has been used in a number of systems for probing the function of 14-3-3 association. In particular, it has been extensively used in the establishment of a role of 14-3-3 in Raf-1–mediated functions. This peptide is broadly capable of abolishing 14-3-3–ligand interactions in vitro, including those with ASK1 (78) and the α2 adrenergic receptor i3 loop (147). pS-Raf-259 specifically docks in the conserved amphipathic groove of 14-3-3ζ, which explains the potent inhibitory effect of this peptide on the interaction of 14-3-3 with various ligands. It is expected that the vast majority of phosphopeptides derived from the 14-3-3 binding motifs of natural ligands will be effective 14-3-3 antagonists. Phosphopeptides from insulin receptor substrate-1 and IGFIR can disrupt the interaction of 14-3-3 with these two ligands (28, 39). An interesting enhancement for phosphopeptide antagonists was described by Yaffe et al (50), where coupling two 14-3-3 binding sequences via a flexible linker led to a ~30-fold increase in affinity for 14-3-3.

The development of unphosphorylated 14-3-3 antagonists may permit efficient delivery or expression in cells. The R18 peptide is such an antagonist. R18 is one of a set of peptides isolated from phage display libraries (65). This peptide exhibits a high affinity for many isoforms of 14-3-3 with estimated K_d values of 70–90 nM, similar to those of the 14-3-3 binding phosphopeptides. The binding of R18 to 14-3-3 appears to be specific because R18 recognizes only 14-3-3 proteins in total cell lysates despite the presence of many other proteins. Functionally, the R18 peptide can abolish the association of 14-3-3 with both phosphorylated and unphosphorylated protein ligands, including Raf-1, ASK1, and ExoS (64, 65, 78). The potent inhibitory effect of R18 on 14-3-3–ligand interactions can be explained by the localization of R18 in the conserved amphipathic groove of 14-3-3 (71). Thus, R18 is likely able to block the interaction of 14-3-3 with most or all of its ligands and may serve as a general antagonist of 14-3-3 proteins. Peptides from phosphorylation-independent 14-3-3 ligands, such as the RSESEE-containing sequence of 5-phosphatase (25), may also prove to be effective 14-3-3 antagonists.

CONCLUDING REMARKS

Through protein-protein interactions, 14-3-3 carries out multiple functions. (*a*) In a broad sense, it can act as an allosteric cofactor to modulate the catalytic activity

or conformational state of its effectors, such as PKCμ, ExoS, and 5-phosphatase. (*b*) 14-3-3 may function as steric regulator to prevent the interaction of its ligands with other cellular components, leading to altered intracellular localization or complex formation. Disruption of Bad/Bcl-X_L interaction by 14-3-3 could offer such an example. (*c*) The 14-3-3 dimer can simultaneously bind two ligands, which may allow 14-3-3 to operate as an adaptor/scaffold protein to induce protein-protein associations. 14-3-3 has been reported to bring Bcr and Raf-1 together (76). Any of these molecular mechanisms could be used to derive the most notable common effect of 14-3-3 binding: sequestration of effector proteins in the cytosol. 14-3-3 maintains a cytosolic localization of Bad, Cdc25, and inactive Raf-1 to achieve its inhibitory function, although the biochemical details may differ in each case. Given the broad participation of 14-3-3 in diverse physiological processes, dissection of the biochemical mechanisms by which 14-3-3 governs its effector pathways is of central importance for understanding intracellular signal transduction.

14-3-3 may represent a novel class of phosphoserine binding modules, which is reminiscent of the docking of phosphorylated Tyr by SH2 and PTB domains. The presence of a 14-3-3–like tertiary structure in protein phosphatase 5 (148) lends hope that proteins containing a ''14-3-3 module'' may yet be discovered. Such molecules would expand dramatically the scope of Ser/Thr kinase-regulated events.

The requirement of phosphorylation for 14-3-3 binding subjects 14-3-3–ligand interactions to the control of specific kinases and phosphatases, and thus to specific signaling pathways. Therefore, phosphorylation of a particular 14-3-3 recognition motif on a target protein often serves as a point of cross talk between different pathways. For example, Akt-mediated survival signals and calcineurin-mediated death signals compete to control the phosphorylation of S136 of Bad, and the resulting 14-3-3 inhibition of Bad proapoptotic activity (96, 97, 99). Thus, identifying upstream kinases and phosphatases that modify 14-3-3 recognition sites will be necessary for understanding the dynamic regulation of 14-3-3 actions.

The concept that 14-3-3 lies at points of cross talk between different cell signaling pathways takes on a new life when combined with the knowledge that the amount of 14-3-3 in the cell can be limiting. There is a multitude of 14-3-3 targets in cells that are modified by environmental signals through the actions of kinases and phosphatases. However, it is possible that not all phosphorylated 14-3-3 ligands will be bound, depending on the amount of 14-3-3 present and the relative strength of the signals. Therefore, 14-3-3 could act as a signal integrator, amplifying strong signals and filtering out weaker conflicting ones to achieve a meaningful, coordinated biological output, such as cell death or survival. When other factors such as heterodimerization, subcellular localization, and differential expression of 14-3-3 isoforms are taken into account, it is apparent that this model could produce rich, complex behaviors. As additional proteins and pathways become identified as 14-3-3 targets, a new challenge will emerge, that of determining an integrated model of 14-3-3–mediated signaling.

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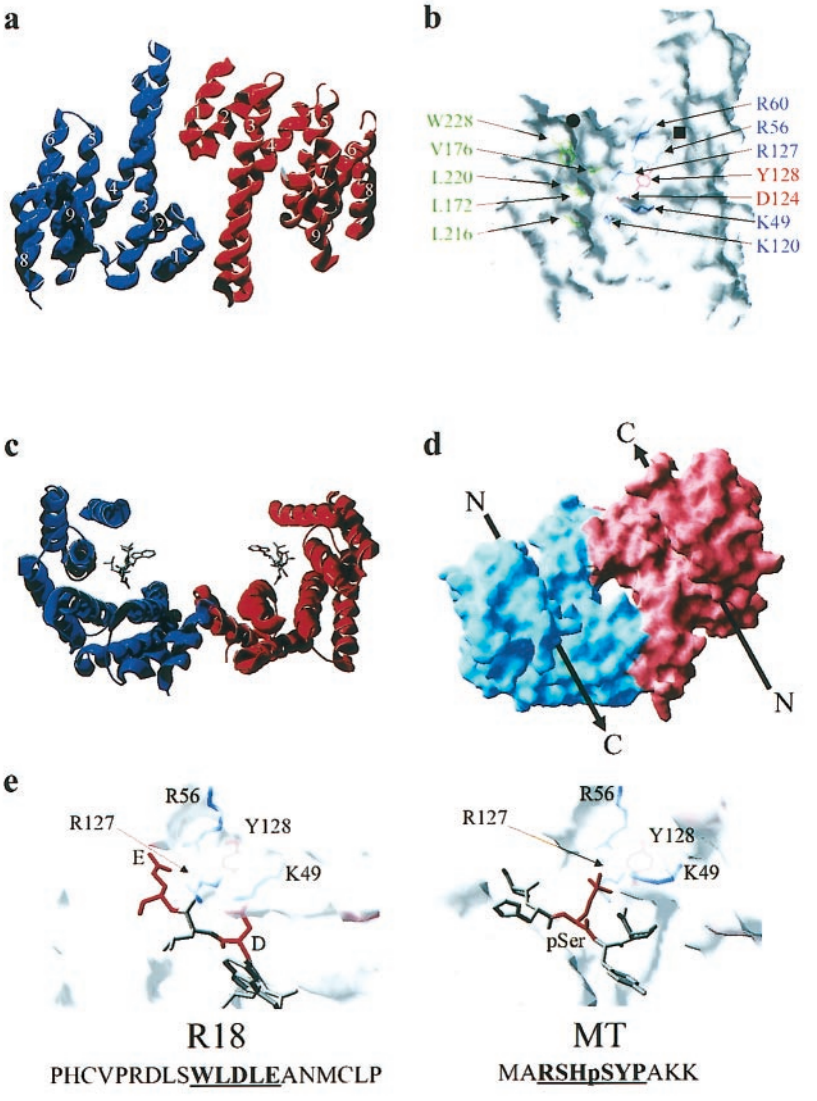

Figure 1 Crystal structural model of 14-3-3ζ. (*a*) The 14-3-3ζ (PDB ID 1A3o) dimer. Each monomer (*red* or *blue*) is composed of nine α-helices (*numbered*). (*b*) A surface representation of a 14-3-3ζ monomer. Selected hydrophobic (*green*), acidic (*red*), and basic (*blue*) residues are displayed to illustrate the amphipathic nature of the groove. Phosphorylation sites are marked (S58, *square*; S184, *circle*). (*c*) 14-3-3ζ crystallized with R18 (PDB ID 1A38), rotated 90° relative to (*a*). This peptide is localized in the amphipathic groove of 14-3-3. In this view, its position is indistinguishable from that of phosphoserine peptides. (d) Solvent accessible surface of 14-3-3ζ with bound peptides (*represented by arrows*). Dimerization forces bound ligands to adopt opposite orientations. (*Continued on next page.*)

(e) Surface representation of 14-3-3ζ crystallized with R18 or a phosphopeptide derived from middle tumor antigen (MT) (PDB ID 14PS). The peptide sequences are listed and a segment is visible in the crystal structure (*bold underline*). R18 and MT occupy similar positions in the 14-3-3 groove, and the two acidic residues of R18 (*red*) act in a homologous fashion as the phosphoserine of MT to contact the basic cluster. This figure was created using Swiss-PdbViewer 3.5β4 (149), POV-Ray 3.1, and Corel Draw 8.