Unlocking the code of 14-3-3

Michele K. Dougherty and Deborah K. Morrison*

Laboratory of Protein Dynamics and Signaling, NCI-Frederick, Frederick, MD 21702, USA

*Author for correspondence (e-mail: dmorrison@ncifcrf.gov)

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Summary

One of the most striking 'rags to riches' stories in the protein world is that of 14-3-3, originally identified in 1967 as merely an abundant brain protein. The first clues that 14-3-3 would play an important role in cell biology came almost 25 years later when it was found to interact with various proto-oncogene proteins and signaling proteins. The subsequent identification of 14-3-3 as a phosphoserine/phosphothreonine-binding protein firmly established its importance in cell signaling. 14-3-3 family members are found in all eukaryotes – from plants to mammals – and more than 100 binding partners have been identified to date. The targets of 14-3-3 are found in all subcellular compartments and their functional diversity is overwhelming – they include transcription factors, biosynthetic enzymes, cytoskeletal proteins, signaling molecules, apoptosis factors and tumor suppressors. 14-3-3 binding can alter the localization, stability, phosphorylation state, activity and/or molecular interactions of a target protein. Recent studies now indicate that the serine/threonine protein phosphatases PP1 and PP2A are important regulators of 14-3-3 binding interactions, and demonstrate a role for 14-3-3 in controlling the translocation of certain proteins from the cytoplasmic and endoplasmic reticulum to the plasma membrane. New reports also link 14-3-3 to several neoplastic and neurological disorders, where it might contribute to the pathogenesis and progression of these diseases.

Key words: 14-3-3, Phosphoserine, Signal transduction, Cancer, Neurodegenerative disorders

Introduction

The 14-3-3 protein family was originally identified in 1967 by Moore and Perez during a systematic classification of brain proteins (Moore and Perez, 1967). In fact, the name '14-3-3' denotes the elution fraction containing these proteins following DEAE-cellulose chromatography and their migration position after subsequent starch gel electrophoresis. Besides their description as ~30 kDa acidic proteins, little else was revealed in this initial characterization. However, in the years since their discovery, the 14-3-3 proteins have risen to a position of importance in cell biology, having been shown to contribute to the regulation of such crucial cellular processes as metabolism, signal transduction, cell-cycle control, apoptosis, protein trafficking, transcription, stress responses and malignant transformation. Owing to the generation of new research tools, such as antibodies recognizing the 14-3-3-binding motif and also inhibitor peptides, together with the continued search for protein interactors, novel 14-3-3 targets are still being discovered. Moreover, the further characterization of known 14-3-3 interactions continues to provide insight into 14-3-3 function and regulation. Here, we briefly examine the basics of '14-3-3-ology' and then focus our discussion on recent studies examining the mechanisms that regulate 14-3-3 target binding, the expanding role of 14-3-3 in protein trafficking and the emerging importance of 14-3-3 in human disease. For a more thorough discussion of 14-3-3 isoforms, structure and binding properties, the reader is referred to several excellent reviews (Aitken, 2002; Fu et al., 2000; Tzivion and Avruch, 2002; van Hemert et al., 2001).

14-3-3: the basics

14-3-3 is a highly conserved, ubiquitously expressed protein family. In mammals, there are at least seven isoforms (β, γ, ε, σ, ζ, τ and η; the phosphorylated forms of β and γ initially being described as α and δ, respectively (Aitken et al., 1995), each encoded by a distinct gene. Up to 15 isoforms are present in plants and two isoforms have been identified in yeast, *Drosophila melanogaster* and *Caenorhabditis elegans* (Rosenquist et al., 2001; Wang and Shakes, 1996). 14-3-3 molecules form homo- and heterodimers (Chaudhri et al., 2003; Jones et al., 1995) that can interact with a wide variety of cellular proteins (Fig. 1). 14-3-3 associates with so many different molecules in large part because of its specific phosphoserine/phosphothreonine-binding activity (Muslin et al., 1996). Analyses of known 14-3-3-binding sites, together with the use of peptide libraries, have defined two high-affinity phosphorylation-dependent binding motifs that are recognized by all 14-3-3 isotypes: RSXpSXP (mode 1) and RXXXpSXP (mode 2), where pS represents phosphoserine (Rittinger et al., 1999; Yaffe et al., 1997). However, phosphorylation-dependent sites that diverge significantly from these motifs have been described (Aitken, 2002), and it should be noted that some 14-3-3 interactions are independent of phosphorylation. For example, binding of 14-3-3 to exoenzyme S, p190RhoGEF and the R18 peptide inhibitor does not require a phosphorylated residue (Henriksson et al., 2002; Masters et al., 1999; Petosa et al., 1998; Wang et al., 1999; Zhai et al., 2001).

Regardless of whether the interactions are dependent on phosphorylation or not, all targets appear to interact with the same binding domain on 14-3-3 (Wang et al., 1999). The

crystal structure of 14-3-3 dimers complexed with short peptides or native binding partners has revealed that each monomer contains an amphipathic groove that acts as a ligand-binding channel (Liu et al., 1995; Obsil et al., 2001; Rittinger et al., 1999; Xiao et al., 1995; Yaffe et al., 1997). Each dimer therefore contains two binding pockets and, as a result, can interact with two motifs simultaneously. These can be on a single target or on separate binding partners (Obsil et al., 2001; Rittinger et al., 1999; Yaffe et al., 1997).

After the initial description of the mode 1 and 2 binding motifs, various targets, such as BAD, Raf-1 and Cbl, were found to have two clearly defined high-affinity 14-3-3-binding sites (Yaffe et al., 1997). Subsequently, other proteins have been shown to contact 14-3-3 through multiple sites. In particular, proteins that were previously identified to have one high-affinity binding motif have now been found to contain additional low-affinity sites that contribute to 14-3-3 binding – for example, the cell-cycle regulator Cdc25B (Giles et al., 2003). The high-affinity site has been proposed to act as a 'gatekeeper', recruiting the 14-3-3 dimer (Yaffe, 2002). Binding of a 14-3-3 monomer to this site could then allow other, low-affinity sites to engage the second monomer, stabilizing the overall target-14-3-3 dimer complex. In addition, given that a phosphopeptide containing two motifs binds 14-3-3 with a 30-fold greater affinity than a phosphopeptide containing one motif (Yaffe et al., 1997), multiple low-affinity sites might coordinately bind a 14-3-3 dimer, as has been proposed for 14-3-3 binding to the K_{ATP} channel Kir6.2 subunit (Yuan et al., 2003).

Historically, the greatest challenge for investigators studying 14-3-3 has been to define precisely the role of this protein in cell biology. One proposed model is that 14-3-3 acts as a 'molecular anvil' that causes conformational changes in the binding partner that can alter its enzymatic activity, or mask or reveal functional motifs that regulate its localization, activity, phosphorylation state and/or stability (Yaffe, 2002). Nonetheless, 14-3-3 also has adaptor functions, mediating interactions between two different binding partners – for example, Tau and glycogen synthase kinase 3β (GSK3β; Agarwal-Mawal et al., 2003) or the Ron receptor tyrosine kinase and α6β4/α3β1 integrin (Santoro et al., 2003a). Because of the dimeric nature of 14-3-3 and its ability to interact with so many diverse cellular proteins, one model or function will probably never be sufficient to define the role of this important protein family.

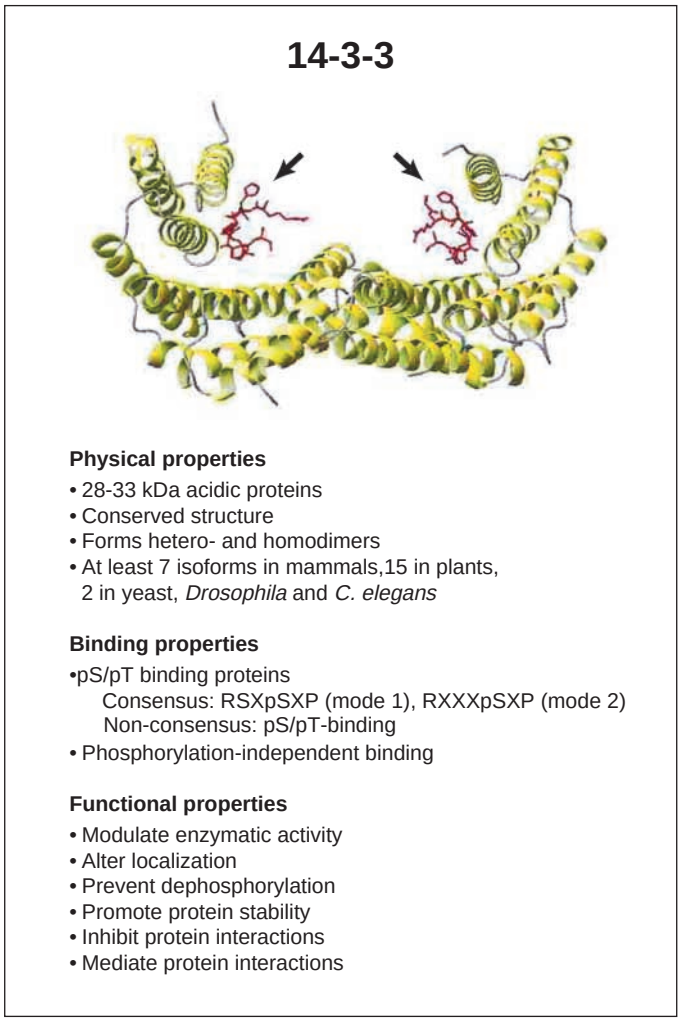

Fig. 1. Properties of a 14-3-3 dimer. The diagram shown is derived from the structure of a 14-3-3ζ dimer bound to the indicated (arrows) phosphoserine peptides (Protein Data Bank accession number 1QJB) (Rittinger et al., 1999).

14-3-3: regulation of target binding

14-3-3 binding is primarily phosphorylation dependent; therefore, 14-3-3 interactions are largely regulated by the kinases and phosphatases that modulate the phosphorylation state of the target protein. Defining the canonical mode 1 and mode 2 binding motifs has greatly facilitated the identification of many kinase regulators. The presence of arginine residues at the –3 and –4 positions in the consensus sequences make these motifs good targets for the basophilic AGC kinase family and the calcium/calmodulin-dependent kinase (CaMK) family (Pearson and Kemp, 1991). Indeed, members of these kinase families, including protein kinase A (PKA), protein kinase C (PKC), CaMKI, Chk1, Cds1/Chk2, Akt and p90Rsk, all phosphorylate sites that mediate 14-3-3 binding (for citations to primary literature, see references in Aitken, 2002; van Hemert et al., 2001). However, following the identification of sites that do not fit either consensus, members of other kinase families have also been implicated, including Cdk5 (Toyo-oka et al., 2003) and LIM kinase (Gohla and Bokoch, 2002).

The phosphorylation of some 14-3-3-binding motifs appears to be constitutive, whereas others are highly regulated, phosphorylation being mediated by kinases that are activated under specific conditions. In addition, some sites are substrates of multiple kinases, the enzyme involved depending on the cell type and conditions. For example, both Cdc25C-associated kinase 1 (C-TAK1) and the checkpoint kinases Chk1 and Cds1/Chk2 can phosphorylate Cdc25C on S216, which is a 14-3-3-docking site that regulates Cdc25C localization and function. However, C-TAK1 is a cytoplasmic kinase that phosphorylates this site in interphase (Peng et al., 1998), whereas the checkpoint kinases are nuclear enzymes that phosphorylate it following DNA damage (Peng et al., 1997; Sanchez et al., 1997).

An emerging theme from the study of kinase regulators is

that the effect of 14-3-3 on a particular process can, in some cases, be attributed primarily to the activity of a particular kinase or group of kinases. For example, Akt is a kinase that plays an important role in cell survival and, consequently, many of the anti-apoptotic effects of 14-3-3 involve binding to Akt substrates, such as BAD (Datta et al., 2000; del Peso et al., 1997), the forkhead transcription factor FKHRL1 (Brunet et al., 1999) and Yes-associated protein (YAP) (Basu et al., 2003). Similarly, by phosphorylating members of the Cdc25 phosphatase family, the checkpoint kinases Chk1 and Cds1/Chk2 contribute significantly to the inhibitory effect that 14-3-3 has on cell-cycle progression following DNA damage (Chen, M. S. et al., 2003; Peng et al., 1997; Sanchez et al., 1997).

14-3-3 interactions can also be regulated by phosphorylation of other residues within the consensus binding motifs. This mechanism of regulation is best characterized in the case of the p53 tumor suppressor and Cdc25C, in which phosphorylation of residues at the –2 position relative to the 14-3-3-binding phosphoserine residue prevents 14-3-3 association (Bulavin et al., 2003; Waterman et al., 1998). Because many 14-3-3 binding partners contain serine/threonine residues at the –2 position (Yaffe et al., 1997), phosphorylation of this residue might represent an under-appreciated regulatory mechanism.

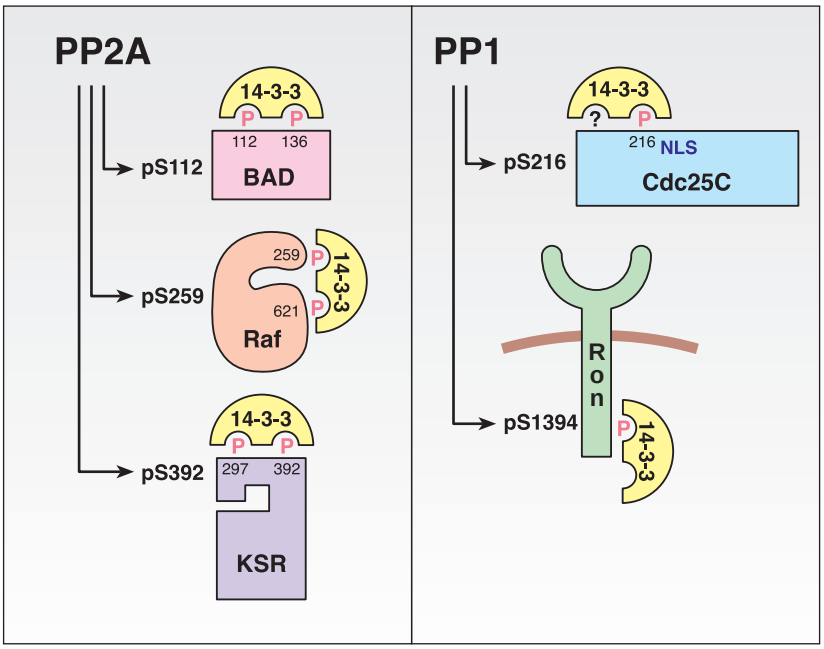

Fig. 2. Regulation of 14-3-3 target binding by protein phosphatases. PP2A directly interacts with BAD, Raf-1 and KSR1, and mediates the dephosphorylation of pS112 on BAD, pS259 on Raf-1 and pS392 on KSR1. PP1 binds Cdc25C and the Ron receptor tyrosine kinase, and dephosphorylates the pS216 site on Cdc25C [exposing a nuclear localization sequence (NLS)] and the pS1394 site on Ron.

Of equal importance are the phosphatases that dephosphorylate the 14-3-3 docking sites. Not surprisingly, the ubiquitously expressed serine/threonine protein phosphatases PP2A and PP1 found in all eukaryotic organisms are important players in this process (Fig. 2). PP2A is a heterotrimeric phosphatase composed of a dimeric core (structural A and catalytic C subunits) and a regulatory subunit (B-type subunits) (Janssens and Goris, 2001), whereas PP1 is a dimeric enzyme consisting of a catalytic and regulatory subunit (Cohen, 2002). Studies now indicate that PP2A contributes to the regulation of 14-3-3 binding to the pro-apoptotic agent BAD (Chiang et al., 2003) and to two components of the Ras signal transduction pathway: the Raf-1 kinase and the KSR1 scaffold (Ory et al., 2003). Raf-1 and KSR1 each contain two high-affinity phosphoserine sites that mediate binding to a 14-3-3 dimer (Cacace et al., 1999; Muslin et al., 1996). Both sites are highly phosphorylated in quiescent cells; however, the phosphorylation status of one site is reduced following Ras activation. Although prior studies had implicated PP2A in the dephosphorylation of Raf-1 (Abraham et al., 2000; Jaumot and Hancock, 2001), the precise mechanism triggering Raf-1 dephosphorylation and the role of PP2A in KSR1 regulation were unknown. Recently, Ory et al. showed by mass spectrometry that subunits of PP2A are associated with both KSR1 and Raf-1 protein complexes (Ory et al., 2003). Further characterization of these interactions revealed that, although binding of the PP2A dimeric core complex to Raf-1 and KSR1 is constitutive, the interaction with the regulatory B subunit increases dramatically following Ras pathway activation. The underlying mechanism(s) is unclear; however, incorporation of the B subunit into the complex should increase the catalytic activity of PP2A. Indeed, using phosphatase inhibitors, Ory and colleagues found that PP2A activity is required for the dephosphorylation of the Raf-1 and KSR1 14-3-3-binding sites following Ras activation (Ory et al., 2003).

BAD also binds 14-3-3 through multiple sites. Under survival conditions, BAD is phosphorylated on S112, S136 and S155 (Datta et al., 2000; Lizcano et al., 2000; Tan et al., 2000; Zha et al., 1996). S136 acts a gatekeeper site to recruit 14-3-3, whereas S112 helps stabilize the complex (Chiang et al., 2003; Datta et al., 2000). 14-3-3 binding retains BAD in the cytoplasm and appears to facilitate phosphorylation of the S155 site (Datta et al., 2000; Pastorino et al., 1999; Zha et al., 1996). S155 phosphorylation might contribute to 14-3-3 complex formation, but its primary effect is to prevent binding of BAD to the anti-apoptic proteins Bcl-2 and Bcl-X_L (Datta et al., 2000; Lizcano et al., 2000; Tan et al., 2000). Chiang et al. have found that, following apoptotic stimuli, PP2A associates with BAD and is the primary phosphatase that dephosphorylates the S112 site (Chiang et al., 2003). In addition, these studies revealed that S112 dephosphorylation increases the accessibility of both the S136 and S155 sites to other phosphatases, ultimately resulting in the release of 14-3-3 and binding of BAD to Bcl-X_L.

The PP1 phosphatase is also an important regulator of 14-3-3 interactions. Binding of 14-3-3 sequesters Cdc25C in the cytoplasm and, for Cdc25C to function during mitosis, 14-3-3 binding must be disrupted, exposing a nuclear localization motif that allows Cdc25C to translocate to the nucleus and activate the mitotic kinase Cdc2 (Graves et al., 2001; Kumagai and Dunphy, 1999; Yang et al., 1999). Elegant studies by Margolis et al. have shown that PP1 directly interacts with N-

terminus of *Xenopus laevis* Cdc25C through a PP1-binding motif and is the enzyme that dephosphorylates this important 14-3-3 docking site (Margolis et al., 2003). Interestingly, the authors also find that dephosphorylation of the binding site first requires 14-3-3 removal, a step that relies, at least in part, on the phosphorylation of Cdc25C by the Cdk2 kinase. These findings strikingly demonstrate the complexity that can exist in the regulation of 14-3-3 binding.

How broad a role PP1 and PP2A play in modulating 14-3-3 binding interactions is unknown; however, recent studies have implicated PP2A in the regulation of the 14-3-3ε–NUDEL interaction (Toyo-oka et al., 2003) and PP1 as a regulator of 14-3-3 binding to the Ron receptor tyrosine kinase (Santoro et al., 2003b). As more detailed analyses of 14-3-3 binding interactions are performed, the number of targets known to be regulated by these phosphatases will certainly grow.

14-3-3: a regulator of intracellular protein localization

One important means by which 14-3-3 regulates cellular processes is by modulating protein localization (Muslin and Xing, 2000) (Fig. 3). In most cases, 14-3-3 binding sequesters the target protein in a particular subcellular compartment, and the release of 14-3-3 then allows the target to relocate. This relocation is often due to the exposure of an intrinsic subcellular targeting sequence that was masked by the 14-3-3 dimer. This mechanism of regulation contributes to the nuclear retention of proteins such as human telomerase reverse transcriptase (TERT) (Seimiya et al., 2000), Txl-2 (Tang et al., 1998) and Chk1 (Jiang et al., 2003), and plays a crucial role in the cytoplasm-mitochondrion shuttling of BAD and BAX (Nomura et al., 2003; Zha et al., 1996) and in the cytoplasm-nucleus shuttling of proteins including Cdc25 (Kumagai and Dunphy, 1999; Yang et al., 1999), histone deacetylase (Grozinger and Schreiber, 2000), YAP (Basu et al., 2003) and the forkhead transcription factors (Brunet et al., 1999; Cahill et al., 2001).

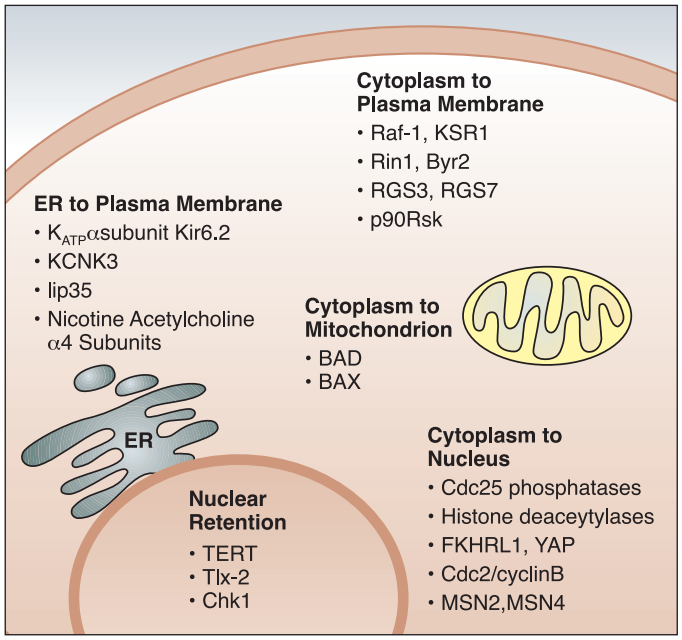

Fig. 3. 14-3-3 is a regulator of protein localization. Indicated are proteins whose subcellular localizations are regulated by 14-3-3 binding. See text for details.

More recently, it has become apparent that this regulatory mechanism also applies to proteins that shuttle from the cytoplasm to the plasma membrane, particularly proteins that are involved in Ras and heterotrimeric G-protein signaling. Heterotrimeric G-proteins and members of the Ras family are membrane-bound; however, many of their effectors and regulators are localized to the cytoplasm and, in order to exert their effects, these molecules must translocate to the cell surface. Various components of these pathways interact with 14-3-3, and more-detailed analyses of these interactions have revealed that, when 14-3-3 binding is disrupted, many of these molecules exhibit increased levels of association with the plasma membrane. This enhanced membrane localization has been observed for the Ras pathway components Raf-1 (Dhillon et al., 2002), Rin1 (Wang et al., 2002), KSR1 (Ory et al., 2003) and p90 Rsk (Cavet et al., 2003), and the G-protein regulators RGS3 (Niu et al., 2002) and RGS7 (Benzing et al., 2000). Interestingly, it is the release of 14-3-3 from only one of the two 14-3-3-binding sites in Raf-1 and KSR1 that occurs following Ras activation and is involved in membrane localization (Ory et al., 2003). As described above, the binding of the 14-3-3 dimer appears to mask regions on the target protein that are required for translocation. In the cases of Raf-1, Rin1 and the two RGS proteins, 14-3-3 binding interferes with the ability of these proteins to contact the membrane-bound GTPases (Benzing et al., 2000; Light et al., 2002; Niu et al., 2002; Wang et al., 2002). In the case of KSR1, 14-3-3 binding probably conceals the cysteine-rich C1 domain that is involved in membrane binding (Zhou et al., 2002). 14-3-3 also plays a role in the Ras-dependent translocation of the Byr2 MAPKKK in *Schizosaccharomyces pombe* (Ozoe et al., 2002), which indicates that regulation of Ras/G-protein signaling is a broadly conserved function of 14-3-3.

14-3-3 has also been implicated in the endoplasmic reticulum (ER)-to-plasma-membrane trafficking of certain multimeric complexes, including the KCNK and K_{ATP} potassium channels (O'Kelly et al., 2002; Yuan et al., 2003). During forward transport, a trafficking checkpoint prevents the release of unassembled or incomplete forms of these complexes from the ER. Certain complex subunits contain ER-retention motifs that bind to COP1 proteins, which in turn mediate the recycling back to the ER (Ellgaard and Helenius, 2003; Ma and Jan, 2002). Yuan et al. and O'Kelly et al. have shown that 14-3-3 interacts with components of various multimeric membrane complexes and that this association interferes with the binding of COP1 to the ER-retention motifs (Yuan et al., 2003; O'Kelly et al., 2002). Interestingly, the mechanism regulating 14-3-3 binding appears to be different for various complexes. In the case of KCNK3, the major histocompatibility complex (MHC) class II molecule-associated invariant chain lip35 and the nicotinic acetycholine α4 subunit, 14-3-3 binding is dependent on phosphorylation and, as a result, relies on the activity of a kinase to generate the binding site (O'Kelly et al., 2002). In contrast, binding of 14-3-3 to the K_{ATP} channel Kir6.2 subunit is independent of phosphorylation and occurs only with multimeric complexes

Table 1. Involvement of 14-3-3 in human diseases

	Isoform	Proposed function and comments
Neurological disorders		
Alzheimer's disease	ζ (β in vitro)	Found in NFTs; binds Tau; interaction with Tau might facilitate aberrant Tau phosphorylation
Parkinson's disease	ε, γ, ζ, θ	Found in Lewy bodies; binds α-synuclein; association with α-synuclein might sequester 14-3-3 and inhibit its anti-apoptotic function
Spinocerebellar ataxia type 1	ε, ζ, η, γ, β	Binds ataxin-1; 14-3-3 binding might stabilize and slow the degredation of pathogenic polyglutamine forms of ataxin-1
Miller-Dieker syndrome	ε	Gene encoding 14-3-3ε is deleted in MDS; loss might result in dephosphorylation of NUDEL, mislocalization of the LIS1 complex and reduced cytoplasmic dynein function
Creutzfeldt-Jakob disease	β, γ, ε, η	No function indicated but found in patients' CSF
Neoplastic disorders		
Epithelial cancers (breast, gastric, others)	σ	4-3-3σ levels are reduced/absent due to promoter methylation; binds Cdks and p53; participates in G2/M checkpoint control
Tuberous sclerosis complex	β, γ, ζ (others?)	Binds TSC2; relieves inhibiton of mTOR signaling by negatively regulating TSC1/TSC2 complex function

(Yuan et al., 2003). Yuan and colleagues propose that each individual Kir6.2 subunit contains a phosphorylation-independent low-affinity binding site and that 14-3-3 can only bind with high avidity when multi-subunit complexes are assembled. 14-3-3 would thus act as a sensor recognizing only correctly assembled complexes. Despite differences in the mechanism, both studies propose that 14-3-3 binding is crucial for overriding ER retention and for the forward transport of the complex. Important questions that remain to be addressed are whether 14-3-3 accompanies the complexes to the plasma membrane and whether 14-3-3 serves an additional function at the membrane. In addition, further investigation will be needed to determine whether this is a general mechanism for regulating ER export, given that numerous proteins contain both ER-retention signals and 14-3-3-binding motifs.

14-3-3: the cancer connection

A role for 14-3-3 in human cancer is not unexpected given the interaction of these proteins with components of both signal transduction and cell-cycle regulatory pathways (Table 1). The most compelling evidence that 14-3-3 is involved in neoplasia comes from studies of 14-3-3σ. 14-3-3σ is unique among the 14-3-3 family members in that it is expressed primarily in epithelial cells (Leffers et al., 1993) and appears to have isoform-specific functions (Chan et al., 1999). 14-3-3σ expression is coordinately upregulated by p53 and BRCA1 and contributes to the DNA-damage cell-cycle checkpoint mediated by these proteins (Aprelikova et al., 2001; Chan et al., 1999; Chan et al., 2000; Hermeking et al., 1997). Unlike other 14-3-3 family members, which inhibit cell-cycle progression by interacting with the Cdc25 phosphatases, 14-3-3σ appears to induce a G2/M arrest by binding to Cdc2–cyclin-B complexes and sequestering them in the cytoplasm (Chan et al., 1999; Laronga et al., 2000). 14-3-3σ also interacts with p53 as part of a positive-feedback loop (Waterman et al., 1998). Yang et al. have found that binding of 14-3-3σ protects p53 from Mdm2-mediated ubiquitylation, thereby stabilizing its levels (Yang et al., 2003). 14-3-3σ binding also promotes p53 tetramerization, resulting in increased transcriptional activity.

Strikingly, 14-3-3σ protein levels are significantly reduced or negligible in various transformed cell lines and primary tumors of epithelial origin, including most breast and gastric cancers and hepatocellular carcinomas (Iwata et al., 2000; Melis and White, 1999; Nacht et al., 1999; Ostergaard et al., 1997; Prasad et al., 1992; Vercoutter-Edouart et al., 2001). This loss of expression is due to methylation of the 14-3-3σ promoter (Ferguson et al., 2000; Iwata et al., 2000; Suzuki et al., 2000; Umbricht et al., 2001). Moreover, the expression level of 14-3-3σ, in some cases, correlates with disease stage. For example, 14-3-3σ is detected in hyperplastic breast tissue, but its level decreases in tissues that exhibit atypical hyperplasia and it is absent in cancerous tissue (Umbricht et al., 2001). In addition, Dellambra et al. have shown that downregulation of 14-3-3σ constitutes a single step that can immortalize primary epithelial cells (Dellambra et al., 2000). 14-3-3σ thus acts as a tumor suppressor, and loss of its function may be a crucial event in the progression of certain human cancers.

Tuberous sclerosis complex

14-3-3 proteins interact with TSC2, one of two tumor suppressors (TSC1 and TSC2) whose mutation is implicated in the genetic disorder known as tuberous sclerosis complex (TSC). In TSC, the formation of hamartomas (benign tumor-like growths) in numerous organs can result in mental retardation, seizures and autism (Young and Povey, 1998). In addition, mice lacking one allele of either *TSC1* or *TSC2* are highly predisposed to renal and other carcinomas (Kobayashi et al., 2001; Onda et al., 1999). TSC1 and TSC2 form a heterodimeric complex that negatively regulates cell growth by inhibiting protein synthesis through the mTOR pathway (reviewed by Manning and Cantley, 2003). Numerous studies have shown that 14-3-3 binds to TSC2 in a phosphorylation-dependent manner and inhibits the function of the TSC1-TSC2 complex (Li et al., 2003; Liu et al., 2002; Nellist et al., 2002; Shumway et al., 2003). The exact mechanism by which 14-3-3 binding inactivates this complex is unknown. However, because TSC2 regulates mTOR signaling by acting as a GAP for the Rheb GTPase (Castro et al., 2003; Garami et al., 2003; Inoki et al., 2003; Tee et al., 2003; Zhang et al., 2003), it will be important to determine whether 14-3-3 binding inhibits either the GAP activity of TSC2 or the interaction of the TSC1-TSC2 complex with Rheb.

14-3-3: the neural connection

14-3-3 was discovered during a systematic characterization of

brain proteins; thus, it seems fitting that 14-3-3 molecules are now being identified as important contributors to neuronal development and certain neuropathologies (Table 1). Brain tissue contains the highest concentration of 14-3-3 proteins, 14-3-3 representing ~1% of total soluble brain protein. This high expression provides one possible explanation for the appearance of 14-3-3 isoforms in the cerebral spinal fluid (CSF) of humans and animals with certain neurodegenerative diseases, such as scrapie, bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD) (Baxter et al., 2002; Hsich et al., 1996). Leakage of proteins as a consequence of progressive neuronal loss has been hypothesized to account for the presence of 14-3-3 in the CSF of CJD patients (Hsich et al., 1996). However, not all 14-3-3 isotypes expressed in normal neurons have been detected in the CSF of CJD patients (Wiltfang et al., 1999), which has led investigators to suggest that some isoforms might be prevented from entering the CSF or that the presence of 14-3-3 is not due solely to protein leakage (Berg et al., 2003). Currently, there is no direct evidence that 14-3-3 is involved in the etiology of CJD; however, because a definitive diagnosis of this disease has historically required post-mortem analysis (Kretzschmar et al., 1996), considerable effort is being made to validate the presence of various 14-3-3 isoforms in CSF as a reliable biomarker for CJD (Green, 2002).

Alzheimer's and Parkinson's disease

In contrast to CJD, evidence is accumulating that 14-3-3 might contribute to both Alzheimer's disease (AD) and Parkinson's disease (PD). 14-3-3 proteins have been identified in the characteristic pathological lesions associated with each disease, namely neurofibrillary tangles (NFTs) for AD (Layfield et al., 1996) and Lewy bodies for PD (Kawamoto et al., 2002; Ubl et al., 2002). NFTs in AD brain tissue are composed of paired helical filaments containing hyperphosphorylated Tau (Lee et al., 1991; Morishima-Kawashima et al., 1995). The hyperphosphorylation of this microtubule-binding protein is thought to inhibit its normal function, causing microtubule instability and, ultimately, neurodegeneration (Lee et al., 2001). Tau can be phosphorylated by numerous kinases, including GSK3β (Reynolds et al., 2000), and Agarwal-Mawal et al. have shown that 14-3-3 facilitates Tau phosphoryation by acting as an adaptor that directs GSK3β to Tau (Agarwal-Mawal et al., 2003). 14-3-3 binding might also alter the conformation of Tau, rendering it more susceptible to phosphorylation (Hashiguchi et al., 2000), and protect the hyperphosphosphorylated form from dephosphorylation (Agarwal-Mawal et al., 2003), both of which would further promote NFT formation.

PD and diffuse Lewy body disease (DLBD) are neurological disorders characterized by the presence of intracellular inclusions known as Lewy bodies. A major component of Lewy bodies is α-synuclein (Spillantini et al., 1997), a protein expressed primarily in neurons that has been proposed to have chaperone activities (Ostrerova et al., 1999) and to be involved in vesicle transport (Lotharius et al., 2002) and the regulation of dopamine neurotransmission (Perez et al., 2002). 14-3-3 interacts with α-synuclein (Ostrerova et al., 1999) and colocalizes with it in Lewy bodies (Kawamoto et al., 2002; Ubl et al., 2002). Also observed in PD is a selective loss of dopaminergic neurons in the substantia nigra, a brain region where α-synuclein- and 14-3-3-containing Lewy bodies are prevalent. Xu et al. have found that overexpression of α-synuclein in cultured dopaminergic neurons results in the accumulation of soluble α-synuclein–14-3-3 complexes (Xu et al., 2002). These cells also exhibit an increased sensitivity to endogenous dopamine and apoptose as a result of dopamine toxicity. The authors speculate that overexpression of α-synuclein induces apoptosis in part by sequestering 14-3-3 and preventing it from inhibiting the proapoptotic function of proteins such as BAD, FKHRL1, ASK1 and YAP. Although further studies are needed to clarify the functional significance of the α-synuclein–14-3-3 interaction (e.g. by examining whether 14-3-3 binding has any effect on α-synuclein activity), these provocative observations suggest that 14-3-3 is a contributing factor in the pathology of PD.

Spinocerebellar ataxia type 1

Recent studies have also implicated 14-3-3 in the pathogenesis of the neurodegenerative disease spinocerebellar ataxia type 1 (SCA1). SCA1 belongs to the 'polyglutamine' class of diseases that includes Huntington's disease and is caused by the expansion of glutamine-encoding CAG trinucleotide repeats in the coding sequence of ataxin-1. The normal physiological function of ataxin-1 is unclear; however, it localizes primarily to the nuclei of neuronal cells (Servadio et al., 1995) and has been reported to bind RNA in vitro (Yue et al., 2001). Previous studies have shown that the neurotoxicity observed in SCA1 is associated with the accumulation of mutant polyglutamine ataxin-1 (Banfi et al., 1994), and recent reports indicate that 14-3-3 binding contributes to this process (Chen, H. K. et al., 2003; Emamian et al., 2003). Emamian et al. have found that ataxin-1 is phosphorylated on S776 and that pathogenic proteins mutated at this site have a reduced ability to form aggregates and are less toxic when expressed in transgenic mice, suggesting that phosphorylation of this site is crucial in SCA1 pathology (Emamian et al., 2003).

Subsequent work by Chen et al. has gone on to demonstrate that S776 is a 14-3-3 docking site and that binding of 14-3-3 to this site stabilizes ataxin-1 and slows its degradation (Chen, H. K. et al., 2003). Significantly, although both wild-type and mutant ataxin-1 proteins interact with 14-3-3, the mutant proteins have an increased affinity for 14-3-3 that correlates with the length of the polyglutamine expansion, which indicates a gain-of-function activity for the disease-causing protein. Further evidence validating the role of 14-3-3 in SCA1 pathology comes from studies using a *Drosophila* model of SCA1. Here, overexpression of both 14-3-3 and pathogenic ataxin-1 induces a more acute neurodegenerative phenotype than is observed in flies expressing pathogenic ataxin-1 alone (Chen, H. K. et al., 2003). Genetic studies in *Drosophila* further indicate that phosphorylation of ataxin-1 might require the phosphoinositide 3-kinase/Akt signaling pathway and might be mediated by Akt (Chen, H. K. et al., 2003). Other polyglutamine-containing proteins, such as ataxin-2, ataxin-7, atrophin-1 and the α1A subunit of voltage-gated calcium channels, contain potential 14-3-3-binding motifs, and further investigation will be needed to determine whether 14-3-3 also contributes to the pathogenesis associated with these proteins.

Miller-Dieker syndrome

Studies characterizing Miller-Dieker syndrome (MDS) have recently uncovered a role for 14-3-3ε in neuronal development. MDS and the related isolated lissencephaly sequence (ILS) are diseases characterized by classical lissencephaly (smooth brain), a neuronal migration defect that results in mental retardation and epilepsy (Dobyns et al., 1993). MDS and ILS patients have hemizygous deletions in human chromosome 17p13.3, which results in the loss of sequences encoding LIS1 (Dobyns et al., 1993; Reiner et al., 1995), a protein involved in dynein motor function (Leventer et al., 2001). However, in MDS, the deletion is larger and is accompanied by a more severe lissencephaly with additional clinical symptoms, such as craniofacial defects (Chong et al., 1997; Dobyns et al., 1991). Studies aimed at identifying the genotypic difference between ILS and MDS have now revealed that the gene encoding 14-3-3ε is always deleted in MDS but not in ILS, suggesting that deletion of *14-3-3ε* contributes to the severity of MDS (Cardoso et al., 2003). Supporting this model, mice lacking 14-3-3ε have developmental and neuronal migration defects similar to those observed in *LIS1* knockouts, and mice that lack one copy of both *LIS1* and *14-3-3ε* have a more severe migration defect than do mice that lack one copy of either gene alone (Toyo-oka et al., 2003).

Toyo-oka and colleagues have found that loss of 14-3-3ε may affect neuronal migration by altering the function of the LIS1 complex and hence dynein motor function (Toyo-oka et al., 2003). Specifically, they found that 14-3-3ε interacts with NUDEL, a known binding partner of LIS1, and that the interaction with 14-3-3ε protects Cdk5-phosphorylated NUDEL from dephosphorylation. Maintaining the phosphorylation status of NUDEL is crucial for the correct localization and function of the LIS1-NUDEL complex. Thus, in MDS, the LIS1 complex appears to receive a double hit. First, because there is less LIS1 protein, fewer LIS1-NUDEL complexes form. Then, because of the reduction in 14-3-3ε levels, the complexes that do form are more susceptible to phosphatase attack, which results in their mislocalization and decreased cytoplasmic dynein function. These studies clearly demonstrate that NUDEL is a crucial target of 14-3-3ε; however, whether there are other 14-3-3ε binding partners that contribute to the severity of MDS is unclear. These studies also provide strong evidence that 14-3-3ε has isoform-specific functions, in that none of the other 14-3-3 isotypes present in the brain appears to compensate for the migrational defects observed following loss of 14-3-3ε.

Conclusion and future directions

Although we have made significant progress in discovering 14-3-3 targets and elucidating the molecular mechanisms that underlie 14-3-3 function and regulation, numerous questions remain. In particular, one area of 14-3-3 biology that remains unclear concerns the role of monomeric 14-3-3 proteins. 14-3-3 molecules naturally form dimers, but phosphorylation of the dimer interface has been shown to promote the generation of 14-3-3 monomers (Hamaguchi et al., 2003; Powell et al., 2003; Woodcock et al., 2003). 14-3-3 monomers can bind target proteins, but how often monomers are formed in vivo and whether they have specific in vivo functions are unknown.

Another question is why cells need many isoforms of a protein that are so similar. Moreover, is the functional redundancy of certain 14-3-3 isotypes crucial for the cell? And do all 14-3-3 family members have isoform-specific functions or is this a property of only certain isotypes? The study of 14-3-3 proteins in human disease has proven invaluable for uncovering isoform-specific functions of 14-3-3 proteins. Two such examples are the involvement of 14-3-3σ in epithelial cancers and 14-3-3ε in MDS. As our knowledge of the isoform-specific functions of 14-3-3 family members increases, it might become possible to consider therapeutic approaches that target the activity of one isoform in certain disease contexts without interfering with the activities of the remaining isoforms. Nonetheless, even if direct therapeutic applications are not realized, it is possible that information regarding the expression and/or activity of various 14-3-3 isoforms might prove useful in the staging and prognostic evaluation, as well as the selection of treatment, for certain cancers and neurological diseases.

Looking back on the initial discovery of 14-3-3, it now seems fitting that these proteins received a generic name that has no suggestion of a specific function or activity. Indeed, over three decades of research have now revealed that many diverse, yet essential, cellular processes rely upon or are influenced by the 14-3-3 proteins. Recent work has highlighted the involvement of these molecules in human disease, most notably in cancer and neurological syndromes, and it is likely that other disease links will continue to emerge. Similarly, biochemical studies continue to elucidate the mechanisms by which 14-3-3 influences the activity of its binding partners and how these interactions are regulated. Future research will undoubtedly provide additional insights into the functions of 14-3-3 and uncover more of the secrets of this fascinating protein family.

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