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14-3-3 Proteins in Neuronal Development and Function

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Abstract

The 14-3-3 proteins are small, cytosolic, evolutionarily conserved proteins expressed abundantly in the nervous system. Although they were discovered more than 30 yr ago, their function in the nervous system has remained enigmatic. Several recent studies have helped to clarify their biological function. Crystallographic investigations have revealed that 14-3-3 proteins exist as dimers and that they contain a specific region for binding to other proteins. The interacting proteins, in turn, contain a 14-3-3 binding motif; proteins that interact with 14-3-3 dimers include PKC and Raf, protein kinases with critical roles in neuronal signaling. These proteins are capable of activating Raf in vitro, and this role has been verified by in vivo studies in *Drosophila*. Most interestingly, mutations in the *Drosophila* 14-3-3 genes disrupt neuronal differentiation, synaptic plasticity, and behavioral plasticity, establishing a role for these proteins in the development and function of the nervous system.

Introduction

The number of publications on the 14-3-3 family of proteins has increased at a near exponential rate in the last few years! This reflects not only the involvement of 14-3-3 proteins in many fundamental cellular functions, but also, the renewed interest in deciphering their precise roles in these processes. This has not been an easy task, partly because of the diversity of functions, and the serendipitous ways in which these functions have been revealed. Since their discovery almost 30 yr ago, a plethora of seemingly unrelated functions ascribed to them have in several ways made these small molecules more of an oddity, than an subject of systematic study.

The first function attributed to 14-3-3 proteins was as a modest activator of tyrosine and tryptophan hydroxylases, the rate-limiting enzymes in the biosynthesis of catecholamine neurotransmitters and serotonin respectively (Ichimura et al., 1987; Makita et al., 1990). However, function as a direct activator seems unlikely since recombinant tyrosine hydroxylase is not activated by 14-3-3 (Sutherland et al., 1993). One or more 14-3-3 proteins were isolated as potent inhibitors of Protein kinase C

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(PKC) (Toker et al., 1990; Toker et al., 1992), but this finding was challenged by other studies suggesting activation of the kinase by these proteins (Isobe et al., 1992; Tanji et al., 1994). In addition, 14-3-3 was reported to possess phopholipase A_2 activity, but subsequent investigations failed to verify this finding (Aitken, 1995). Interestingly, one of the roles for 14-3-3 proteins that has remained unchallenged is their apparent involvement in exocytosis (Morgan and Burgoyne, 1992; Morgan and Burgoyne, 1992).

Interest in the 14-3-3 proteins was rekindled a few years ago principally because of a series of reports that strongly suggested their involvement in signal-transduction pathways and the cell cycle. Specifically, that 14-3-3 proteins bind and regulate the kinases PKC, Raf, and Bcr. Furthermore, some reports suggested roles for these proteins in cell growth and in checkpoint control for DNA damage prior to mitosis. These findings have led to a systematic examination of the properties of 14-3-3 proteins in an effort to understand their precise roles in these vital cellular functions. Though this investigation is far from complete, it has uncovered many of their properties and characteristics and provided insights into their mode of action. Importantly, the recent isolation and characterization of mutations in 14-3-3 genes of the fruitfly *Drosophila* melanogaster has elucidated some of the roles of 14-3-3 proteins at the organismal level, including the processes underlying behavior.

A Ubiquitous Family of Proteins

Proteins of the 14-3-3 family were first isolated as an apparent single molecular species in a systematic survey of bovine brain proteins. In fact, the name 14-3-3 reflects their migration position in the purification scheme with DEAE-cellulose chromatography followed by starch-gel electrophoresis (Moore and Perez, 1968). It was subsequently determined that homologous protein species from human brain could be resolved into multiple isoforms with similar molecular weight (Boston et al., 1982). Though highly abundant in the brain (up to 1% of soluble proteins), 14-3-3 isoforms were found at low levels in most human tissues examined (Boston et al., 1982).

To date, 14-3-3 proteins have been identified in all vertebrates, invertebrates, plants, and fungi. An extraordinary feature of this protein family is the high sequence conservation among related isoforms from diverse species. Additionally, their primarily neuronal expression pattern appears conserved in all animal species examined (Ichimura et al., 1991; Aitken et al., 1992; Aitken, 1995; Skoulakis and Davis, 1996). Since the mammalian 14-3-3 isoforms were the first to be systematically isolated and analyzed, their sequences serve as canonical to other vertebrate and invertebrate family members. Seven unique isoforms elute at distinct positions after reverse-phase high-performance liquid chromatography (HPLC) of mammalian brain homogenates (Fig. 1). These isoforms are named according to their elution profile α, β, γ, δ, ε, ζ, and η (Ichimura et al., 1988; Aitken et al., 1992; Aitken, 1995). However, the α and δ proteins are phosphorylated forms of the β and ζ respectively (Aitken et al., 1995). Posttranslational modifications, such as acetylation (Martin et al., 1993) of certain 14-3-3s may account for putative new isoforms as suggested recently (Vincenz and Dixit, 1996). An additional source of heterogeneity within the 14-3-3 isoform pool which does not appear to be reflected by their HPLC profile, is use of alternative mini exons resulting in proteins with similar size and elution profile, but small stretches of unique primary sequence (Martin et al., 1993; Murakami et al., 1996; Kockel et al., 1997). Two additional unique isoforms are known. The θ or τ isoform, was initially isolated from T-cells (Nielsen, 1991), and the other named σ, found in skin and keratinocytes was called stratifin prior to its identification as a 14-3-3 isoform (Ichimura et al., 1988; Aitken et al., 1992). In Drosophila, there appear to be two 14-3-3 genes, one encodes a protein 88% identical to the mammalian ζ (D14-3-3ζ or Leonardo), the other an isoform 82% identical to the mam-

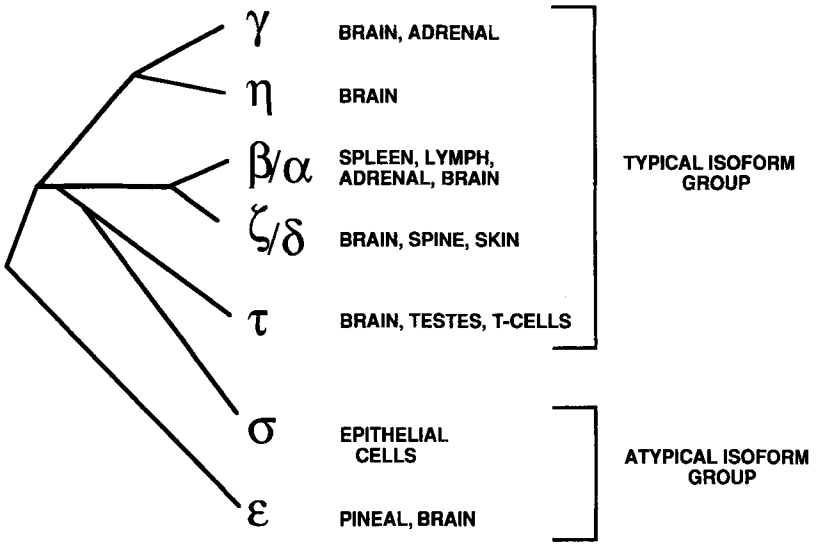

Fig. 1. The mammalian 14-3-3 protein family. The isoforms are arranged in order of similarity based on their amino-acid sequence and comprise two groups as shown. The tissue(s) in which each isoform is primarily expressed is indicated.

malian ε (D14-3-3ε) (Swanson and Ganguly, 1992; Skoulakis and Davis, 1996; Chang and Rubin, 1997). D14-3-3ζ was named Leonardo to reflect the multiple functions ascribed to these proteins.

The primary sequence of 14-3-3 isoforms is remarkably similar, but the family can be divided into two major groups (Fig. 1). Most forms, except the ε and σ, are classed into the typical group. The ε and σ isoforms form the more divergent, atypical group In fact, the mammalian ε isoform is more closely related to the yeast and plant isoforms than members of the mammalian typical group. This property, along with the ubiquitous presence of 14-3-3s in all eukaryotes examined, underscores the evolutionary age of these proteins that apparently evolved prior to the separation of plants and animals (Wang and Shakes, 1996). It is interesting that one of each of the typical and atypical isoforms are present in *Drosophila*, which may reflect functional differences among the two groups. Family members within each group can be distinguished on the basis of unique sequences interspersed among the conserved, often invariant, domains characteristic of the 14-3-3 proteins (Aitken et al., 1992; Martin et al., 1993; Wang and Shakes, 1996). The β and ζ isoforms are unique for the possession of the sequence SPEK in their carboxy-terminal half, which is a putative phosphorylation site by a cyclin-dependent kinase (Aitken et al., 1995). Interestingly, though the Leonardo protein bears a conservative substitution in this motif (SPDK), there is no evidence for a δ-like phosphorylated isoform, at least in adult animals (Skoulakis and Davis, 1996).

Structural and Functional Domains

The 14-3-3 proteins are small, with molecular masses around 30 kD. Their isoelectric points of 4.5–5 reflect the abundance of acidic residues especially at their core conserved domains. The crystal structures of two typical mammalian isoforms, ζ and τ, have been resolved and provide valuable insights as to the mode of action of these molecules (Liu et al., 1995; Xiao et al., 1995). All 14-3-3 isoforms form dimers, with both homodimers and heterodimers detected in brain and other tissues

(Jones et al., 1995). Dimerization appears to involve primarily hydrophobic interactions mediated by highly conserved residues within the variable amino-termini of the molecules (Liu et al., 1995). This suggests that all 14-3-3 isoforms may be able to heterodimerize, and that heterodimer formation may be regulated by their tissue and subcellular distributions. Each subunit of the dimer is composed of nine antiparallel α-helices, perpendicular to the axis of dyad symmetry, which form a palisade around a central negatively charged groove comprised mostly of invariant amino acids (Fig. 2A). This provides a binding surface conserved throughout evolution and suggests recognition of common features in target proteins. The amino-terminal helix mediates dimerization and the conserved residues in this interface are in support of the observed heterodimer formation. The remainder of this unusual structural arrangement can be subdivided into two domains of three-helix bundles each, both contributing to the formation of the acidic pocket (Liu et al., 1995; Xiao et al., 1995). The importance of the evolutionarily conserved residues in the binding interface is illustrated by mutations in two conserved amino acids at the binding domain of the *Drosophila* 14-3-3 ε gene (Chang and Rubin, 1997). Though subtle (phenylalanine to tyrosine and tyrosine to phenylalanine), these changes result in strong "dominant negative" phenotypes.

Binding of Bcr, PKC, and Raf-1 is thought to involve the basic zinc-finger (Fig. 2B III) motif present in the regulatory domains of these kinases (Freed et al., 1994; Fu et al., 1994; Reuther et al., 1994; Robinson et al., 1994; Aitken, 1995). In support of the crystallographic data, deletion analysis of the ζ isoform suggested that helices 1-4 are required for efficient dimerization. Although helices 8 and 9 are capable of binding Raf-1, only full length protein, capable of dimerization and complete acidic groove formation appears able to bind efficiently to active kinase (Luo et al., 1995). Additional motifs may be involved in PKC binding. The first, located in helix 3, resembles a PKC phosphorylation site (pseudosubstrate domain). In addition, helix 5 contains a domain homologous to annexin, a PKC binding protein. This domain may also mediate binding to this kinase. Depending on the particular composition of a 14-3-3 dimer and the binding motifs on the target(s), association of 14-3-3 proteins with them may be selective, but also have drastically different consequences. This idea is consistent with the differential activation of PKC isozymes by 14-3-3 ζ homodimers and conversely, inhibition of the kinase by homodimers of the phosphorylated form of the protein, 14-3-3 δ (Acs et al., 1995; Aitken et al., 1995).

An additional motif on the target proteins that appears to mediate 14-3-3 binding has been recently identified. This is the short peptide Arg-Ser-x-Ser-x-Pro (where x = any amino acid). Binding by most typical 14-3-3 isoforms occurs only when the second serine in the motif is phosphorylated (Fig. 2B). The high affinity (K_d = 122 n*M*) of most 14-3-3 homodimers towards this motif has been measured on short peptides encompassing the phosphoserines at positions 259 and 621 of Raf-1 (Muslin et al., 1996). In addition to Raf-1, this motif is found in two of the PKC isozymes, Bcr, and many other proteins thought to bind 14-3-3, such as the *Polyoma* middle T antigen and the CDC25 phosphatase (Muslin et al., 1996). A mutation preventing phosphorylation of the serine in this motif abrogates binding of 14-3-3 proteins on human CDC25C, which renders the phosphatase constitutively active with catastrophic consequences on mitotic and G_2 checkpoint control (Peng et al., 1997). Variants of this sequence are found in a number of proteins, notably the kinase suppressor of ras (KSR), shown to form an oligomeric complex with 14-3-3 and Raf-1 in vertebrate cells (Xing et al., 1997) and the protooncogene product Cbl, shown to increase its association with 14-3-3 proteins upon phosphorylation of the conserved Serine in the motif (Liu et al., 1997).

The 14-3-3 dimers then, may bind on multiple locations of a target protein, or alternatively, may bind multiple target proteins via different motifs and hold them in multiprotein complexes (Fig. 2B). Indeed, in transfected-tis-

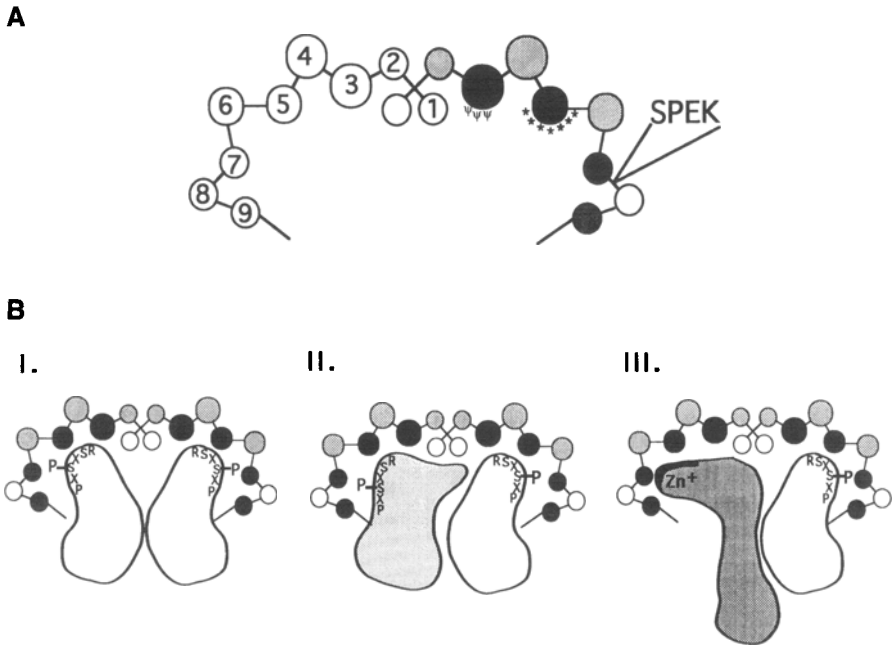

Fig. 2. Structure of a 14-3-3 dimer and binding to target proteins. **(A)** The structure of a 14-3-3 dimer viewed from above. The nine antiparallel α helices are numbered starting with the amino-terminus proximal helix 1 in the monomer on the left. The abundance of acidic residues is indicated by shading in the monomer on the right with black helixes indicating highly acidic areas, whereas helixes with rare acidic residues are depicted as open circles (modified from Xiao et al.). The PKC pseudosubstrate domain in helix 3 is indicated by the ψ's, and the annexin-like domain in helix 5 is represented by the stars. The location and amino-acid sequence of the putative in vivo phosphorylation site between helixes 7 and 8 is depicted. **(B)** Binding of 14-3-3 dimers to target proteins. Binding via the phosphoserine in the R-S-x-S-x-P motif to: I; two molecules of the same target protein, an interaction that may mediate oligomerization or autophosphorylation of target proteins. II. Binding via the phosphoserine motif to two different target proteins, an interaction that may mediate multiprotein complex formation and potential modification (i.e., phosphorylation). Similarly, binding to two identical or different molecules may be mediated by two zinc-finger motifs. III. Binding to two different target proteins via the phosphoserine motif and a zinc-finger motif. Such interactions may also mediate multiprotein complex formation and modification of target proteins.

sue culture cells, Raf-1 binds to Bcr in a 14-3-3 dependent manner, resulting in the formation of tight complexes that contain all three molecular species (Brasselman and McCormick, 1995). Similarly, 14-3-3 molecules mediate the interaction between A20, a zinc-finger-containing inhibitor of tumor necrosis factor-induced apoptosis and Raf-1 (Vincenz and Dixit, 1996) and the complex between KSR and Raf-1 (Xing et al., 1997). Thus, 14-3-3 dimers can act as a novel type of adapter, capable of spatial coordination of different proteins and possibly in the case of kinases, coordinate modulation of their activities. It is intriguing that 14-3-3 may bind to Raf and PKC simultaneously, especially in light of the possibility that PKC phosphorylation is requisite for Raf activation.

In summary, the unique structural properties of 14-3-3 proteins and the apparent capacity to selectively bind multiple targets suggest that their precise role will be dictated by composition of the dimers and the types of target proteins present in a given cell type. Therefore, investigation of the functions of these proteins

in vitro or in cultured cells may not reflect the native, or even appropriate environment for a given homo or heterodimer. However, recent studies in *Drosophila*, facilitated by the apparent simplicity of the system with a single isoform of each 14-3-3 group, and the sophisticated genetics have provided evidence for physiological roles for this protein family. These in vivo studies are in good agreement with the biochemical or tissue-culture results and establish roles for 14-3-3 proteins in the development and plasticity of the nervous system.

Neuronal Expression of 14-3-3 Genes

Two main points can be made from expression studies of the mammalian 14-3-3 genes. First, with the exception of σ, all other 14-3-3 isoforms are primarily, but not exclusively expressed in neurons. Second, their developmental expression pattern is complex.

In humans and other mammalian species, the proteins are found ubiquitously at low levels but are abundant in most areas of the central nervous system (CNS). It is noteworthy that isoforms of the conserved group are highly enriched in areas of the brain of particular importance to neuroplasticity and higher brain functions such as the hippocampus, neocortex, olfactory bulb, thalamus, and the cerebellum (Boston et al., 1982; Boston et al., 1982; Ichimura et al., 1988; Ichimura et al., 1991; Watanabe et al., 1991; Watanabe et al., 1993; Rosenboom et al., 1994; Watanabe et al., 1994). There is a large degree of overlap in expression patterns among the isoforms but it is unclear whether they are coexpressed in the same cells. For example, though the ζ isoform is present at high levels, β, γ , η and τ isoforms are also expressed in the gray matter of the rat brain. Conversely the τ form is the only one found in the white matter (Watanabe et al., 1991; Watanabe et al., 1993; Watanabe et al., 1993; Watanabe et al., 1994). Variation in abundance is also observed among brain areas. In comparison to rest of the brain, the β, γ, and η isoforms are enriched in the Purkinje cells of the cerebellum (Watanabe et al., 1991). The atypical ε isoform is highly enriched in the pineal gland, and significant amounts are present in the retina (Rosenboom et al., 1994). The variations in distribution and abundance of 14-3-3 isoforms may reflect functional differences or engagement of distinct signal-transduction pathways by different groups of cells. Furthermore, within a particular cell, homodimers and heterodimers among these isoforms could be involved in distinct neuronal functions.

This preferential expression of 14-3-3 isoforms in the nervous system is characteristic of the *Drosophila* ζ isoform, Leonardo, as well (Fig. 3). In fact, the gene was isolated because of its preferential expression in a specific area of the brain, the mushroom bodies, centers for learning and memory in insects (Davis, 1996). Like its mammalian homolog, Leonardo is also expressed ubiquitously at low levels (Skoulakis and Davis, 1996). The expression pattern of D14-3-3ε is not known. However, it is likely that similar to its mammalian counterpart, the *Drosophila* 14-3-3ε is expressed in the retina, since mutations in the gene perturb development of retinal cells (Chang and Rubin, 1997). The preferential expression of mammalian and *Drosophila* 14-3-3 isoforms in the nervous system is consistent with their roles in signal-transduction pathways, essential processes for nervous-system function.

Involvement of 14-3-3 proteins in fundamental cellular activities is reflected in the highly patterned regulation of their expression during development of the mammalian CNS. The genes for the β, γ, and η isoforms are expressed in a dynamic and complex pattern. Some populations of neurons exhibit high levels of mRNA early in development with a subsequent decrease, other neurons show the converse expression pattern, and yet others maintain low levels of expression throughout development (Watanabe et al., 1993; Watanabe et al., 1993; Watanabe et al., 1994). In mature neurons, 14-3-3 isoforms appear to be enriched in the axons

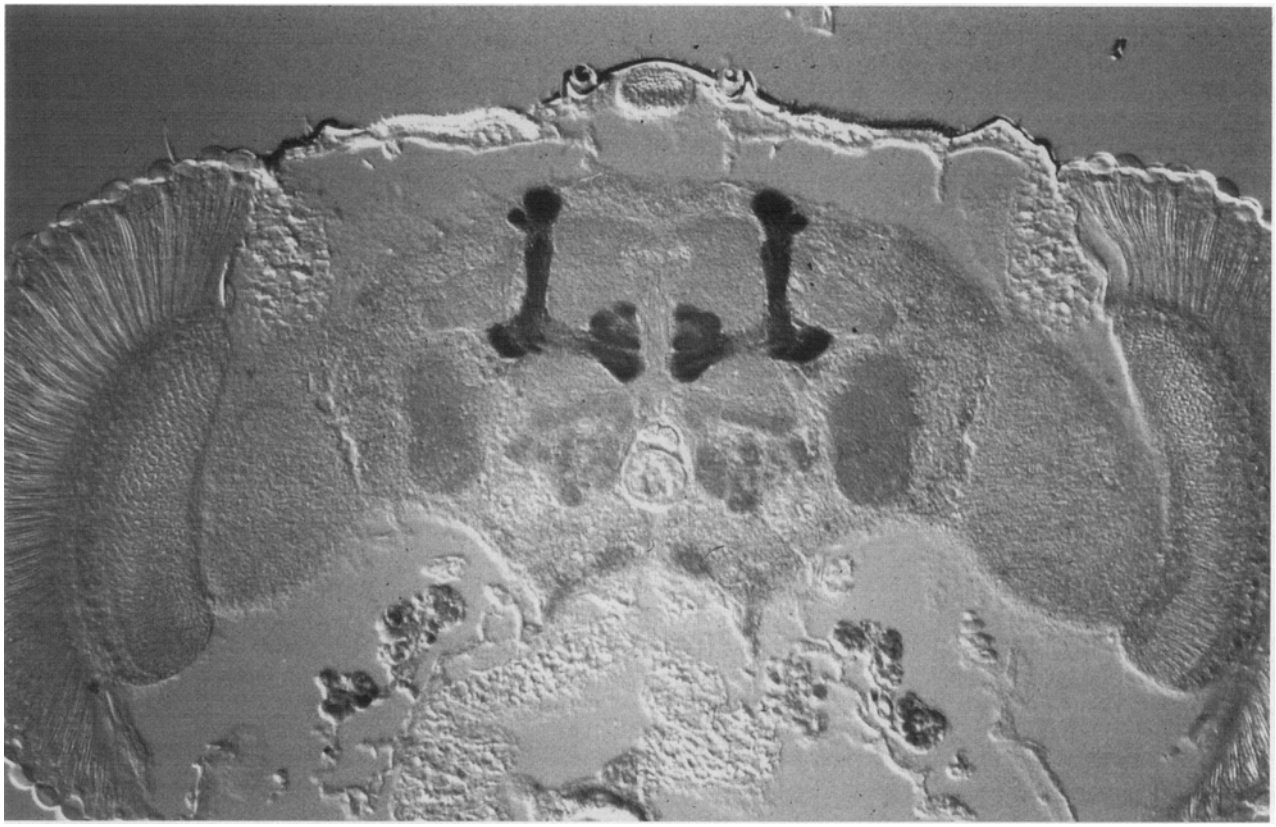

Fig. 3. Expression of Leonardo (D14-3-3ζ) in the mushroom bodies of the adult *Drosophila* brain. A 5-micron frontal section in the anterior of an adult *Drosophila* head challenged with anti-Leonardo antibody. The Leonardo protein is abundant in mushroom body neurons shown here at the level of their axonal projections (lobes), and much reduced in other neuropil areas.

(Erickson and Moore, 1980; Boston et al., 1982; Layfield et al., 1996). Similarly, the *Drosophila* Leonardo gene is expressed throughout the embryo, but is highly enriched in the developing CNS and PNS. The protein is progressively partitioned to the embryonic CNS and the motor neurons where by the end of development, is barely detectable in the axons but highly enriched in the neuromuscular synapses (Broadie et al., 1997). Mutations in the gene that abolish its expression do not precipitate gross aberrations in the CNS or the motor neurons. This suggests that Leonardo is not involved in the development of the nervous system per se, but rather, that the dynamic expression pattern reflects the functional maturation of the nervous system (Broadie et al., 1997). By analogy, it is likely that the elaborate expression patterns of the mammalian isoforms reflect functional maturation of different neuronal populations in the embryonic CNS. It is interesting to note that 14-3-3 proteins also appear highly enriched in areas of massive death of adult neurons caused by pathological reasons. Neurofibrillary tangles of Alzheimer's disease brains, as well as the cerebrospinal fluid from humans and animals with spongiform encephalitis contain elevated amounts of 14-3-3 proteins that may serve as markers for these diseases (Hsich et al., 1996; Layfield et al., 1996).

14-3-3 Proteins as Regulators of Raf Kinase

The Ras/Raf/MAP kinase cascade is a transduction pathway that links signals at the cell surface to changes in gene expression in the nucleus (Fig. 4). Ras acts a switch such that when active, promotes the membrane association of the serine /threonine kinase Raf. Activation of Raf at the membrane by an as yet unknown mechanism results in phosphorylation and activation of MAP kinase kinase (MEK), which then activates by phosphorylation the MAP kinase. MAP kinase in turn, is known to activate transcription factors in the nucleus. This pathway is evolutionarily conserved and known to be operant in neurons (Davis, 1993; Schlessinger, 1993; Morrison, 1995; Wassarman et al., 1995).

A number of typical 14-3-3 isoforms interact physically with Raf as initially shown by yeast two-hybrid screens designed to identify proteins that interact with the kinase (Fantl et al., 1994; Freed et al., 1994; Fu et al., 1994; Irie et al., 1994). This interaction seems to be mediated by the basic zinc-finger and phosphorylated Arg-Ser-x-Ser*-x-Pro (Ser* = phosphoserine) motif in the regulatory domain of the protein, as discussed above. These observations suggested that 14-3-3 proteins might be factor X, the unknown protein hypothesized to be required along with Ras for activation of membrane-associated Raf (Morrison, 1995; Morrison and Cutler, 1997). Indeed, some experiments suggested that the kinase can be activated by overexpression of 14-3-3 proteins, either in the yeast two-hybrid system or *Xenopus* oocytes (Fantl et al., 1994; Irie et al., 1994; Li et al., 1995). However, the role for 14-3-3 proteins as stimulators of Raf has been challenged by other results obtained either in vitro, or by overexpression in cultured cells that failed to produce consistent Raf activation (Fu et al., 1994; Michaud et al., 1995; Suen et al., 1995). It seems likely then, that interaction between Raf and 14-3-3 is not sufficient to activate the kinase. However, it is possible that 14-3-3 dimers may bring Raf and its putative activa-

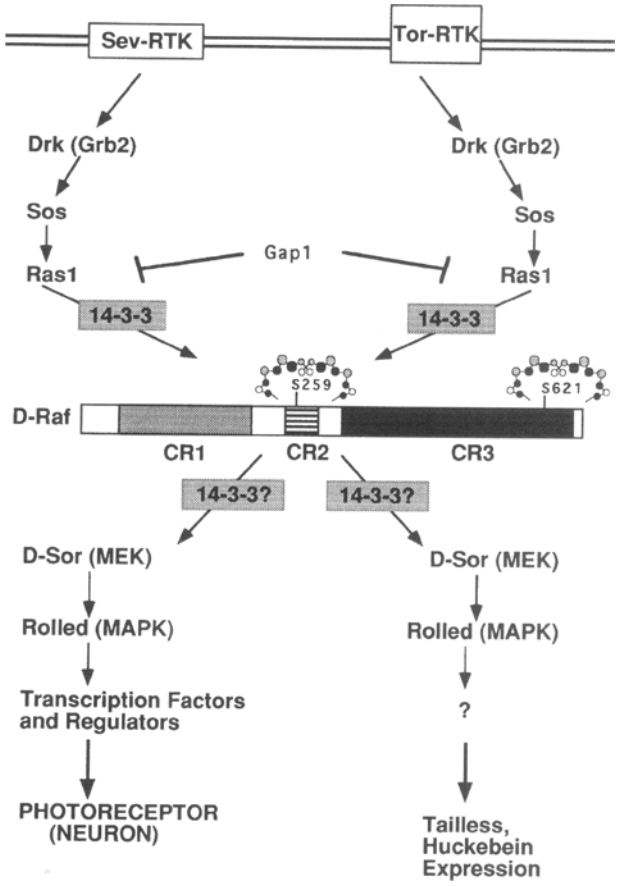

Fig. 4. The Ras/Raf-signaling cascade in *Drosophila*. The cascade operant in neuronal differentiation in the *Drosophila* eye is shown on the left and the cascade regulating cell fates along the antero-posterior embryonic axis is shown on the right. The cascade components are shown in the order in which they function. Sev-RTK is the seven-less-receptor tyrosine kinase that receives the extracellular signal. Conversely, the torso-receptor tyrosine kinase (Tor-RTK) is the signal receptor for embryonic terminal differentiation. Drk(Grb2) is an SH3-SH2-SH3 adaptor molecule which links the RTK with Sos. Sos, a guanine nucleotide-releasing factor activates Ras by promoting the exchange of GDP for GTP. GTP bound Ras1 remains active until its intrinsic GTPase activity—which is stimulated by Gap1by promoting the inactive GDP bound state—inactivates it. Active Ras1 along with 14-3-3 and other yet unknown factors are responsible for activation of the Raf1 kinase. The 14-3-3 proteins are depicted inside boxes to suggest that although the proteins are participants in the pathway, they themselves are not signal-

tor into proximity, or hold them in a complex by simultaneously binding to both. The necessity of 14-3-3 proteins for Raf activation has been demonstrated in vivo by elegant genetic studies of the two *Drosophila* 14-3-3 genes and their effects on Ras/Raf signaling in the development of the compound eye of the insect.

Cells in the developing *Drosophila* eye differentiate into photoreceptor neurons, or nonneuronal cells upon signaling via the Ras/Raf cascade (Fig. 4). The pathway is activated by ligand binding to the sevenless-receptor tyrosine kinase and transduced via Ras/Raf/MAPK to the nucleus to mediate changes in gene expression requisite for the switch in developmental fate. The sevenless-induced pathway does not affect survival of adult animals. However, perturbation of the signaling pathway either by removal or hyperactivation of one of its components, or reduction of the signaling efficiency results in morphological defects in the eye. These are manifested as misshapen, or noncanonical arrangements of the normally highly ordered array of 800 unit eyes (ommatidia) which constitute the insect compound eye (Wassarman et al., 1995). This sensitive Ras/Raf-signaling detection system can be further sensitized by genetic manipulation of the activity of its components. This "sensitized" system allows detection of a 50% change in the activity of other components of the pathway and genome screens with this system yielded a number of novel members of the Ras/Raf signaling cascade (Dickson et al., 1996; Karim et al., 1996).

The D14-3-3ζ Leonardo, is coexpressed temporally and spatially with other components of the Ras/Raf cascade at the time of the developmental decisions in the compound eye. Mutations engineered to be confined to the eye that lack more than 50% of Leonardo protein result in ommatidia that fail to develop the normal array of photoreceptor neurons. Furthermore, complete lack of the protein results in ommatidial cell death (Kockel et al., 1997). This is consistent with the known function of the Ras/Raf/MAPK cascade in regulation of cell proliferation (Daum et al., 1994), and the embryonic lethality of Leonardo mutations when they are not genetically confined to the nonvital eye tissue (Skoulakis and Davis, 1996; Broadie et al., 1997; and *see* below). Local coexpression in the eye of constitutively active Ras fails to reverse this lethality. In contrast, similar co-expression of constitutively active Raf results in near-normal ommatidia containing photoreceptor neurons. Therefore, artificial activation of Raf rescues the phenotypes caused by lack of Leonardo in the ommatidia and indicates that the D14-3-3ζ acts between Ras and Raf in this signal transduction cascade (Fig. 4).

Mutations in D14-3-3ε were isolated in a genetic screen for novel components of the Ras/Raf/MAPK cascade involved in *Drosophila* eye development. Loss of function mutations in the D14-3-3ε gene suppress the rough eye phenotype caused by the constitutively activated Ras gene indicating that lack of 14-3-3 blocks the cascade between Ras and Raf. Lack of D14-3-3ε suppresses the rough eye phenotype, albeit weakly, caused by activated Raf, but not that of activated transcription factors at the end of the cascade (Chang and

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transducing molecules but act as organizing centers for signaling molecules.

The three conserved domains of Raf1 are illustrated. CR1 and CR2 comprise the regulatory domains, and CR3 the catalytic domain of the kinase. Ras binds on CR1 at the amino-terminal portion and a zinc-finger domain at the carboxy-terminus. CR2 is a serine/threonine-rich region that contains phosphorylation consensus sites for multiple kinases. 14-3-3 dimers are bound on phosphorylated serines within known 14-3-3 binding motifs. 14-3-3 dimers may also bind the zinc-finger domain in CR1. Binding of 14-3-3 to CR2 and CR1 may be requisite for Raf1 activation and binding on the consensus phosphoserine in the catalytic domain (CR3), may follow and promote maintenance of the kinase in the active conformation.

Activated Raf1 phoshorylates and activates the cytoplasmic kinase MEK (D-Sor), which in turn phosphorylates and activates the MAP kinase (Rolled). Activated MAPK translocates to the nucleus where it activates transcription factors required for final neuronal differentiation or embryonic development (tailless, huckebein).

Rubin, 1997). The results indicate that D14-3-3ε acts positively in the Ras/Raf/MAPK cascade in eye development. It acts upstream of the nuclear portion of the cascade, downstream of Ras and either downstream of, or in parallel with Raf. In addition, the weak rough-eye phenotypes of D14-3-3ε mutations are exaggerated in animals that carry mutations in both D14-3-3ε and Leonardo genes. This result is consistent with some functional redundancy between the two isoforms in the eye. An alternative explanation is that the two isoforms are utilized at distinct steps in the signaling pathway. Since Leonardo mutations suppress activated Ras, but not activated Raf, the protein is clearly necessary for transduction of the signal between them. Moreover, the apparent ability of all 14-3-3ζ proteins to bind Raf (Muslin et al., 1996), strongly suggests that this isoform is necessary for activity of the kinase. However, since D14-3-3ε appears to act positively in this signaling cascade, but mutations suppress activated Raf, this isoform could act to maintain activity of the kinase, thus modulating the efficiency of signaling. A role for 14-3-3 proteins as modulators of Raf signaling has been proposed previously (Morrison, 1994), based on the ability of the proteins to bind Raf, but lack of consistent kinase activation in vitro. It is possible then, that different 14-3-3 isoforms may function in two distinct steps in the Ras/Raf signal transduction cascade. Based on results from overexpression studies, it has been proposed that 14-3-3 proteins may have a dual role in regulating Raf activity (Morrison and Cutler, 1997). Binding of 14-3-3 on the regulatory domain of Raf (zinc-finger domain in CR1 and Ser 259 in CR2) may be required for activation of the kinase, whereas binding on the catalytic domain (Ser 621 in CR3) may be necessary for maintenance of the active state (Fig. 4). Although this hypothesis needs to be tested, the results from this genetic analysis demonstrate that 14-3-3 proteins are operant in Ras/Raf signaling.

Is this requirement for 14-3-3 proteins in Ras/Raf signaling specific to that involved in eye development? This cascade is essential for *Drosophila* embryonic development as well. The torso-receptor tyrosine kinase is activated during embryonic development and through Ras/Raf signaling transduces a signal required to define the cell fates at the embryonic anterior and posterior terminals (Fig. 4). Torso activity is graded and plays an instructive role in cell fate determination manifested by the differential activation domains of the tailless (tll) and huckebein (hkb) genes (Perkins and Perrimon, 1991; Perrimon, 1994). Overexpression of a Leonardo transgene enhances signaling through the Ras/Raf cascade manifested by expansion of the tll expression domain. This effect is abolished in the presence of loss of function alleles of either Ras or Raf suggesting that Leonardo acts downstream of Ras and through Raf to transduce the torso signal and activate transcription of tll. In agreement with this result, mutations in Leonardo reduce the tll expression domain (Li et al., 1997). Thus, Leonardo is essential for embryonic development in the torso mediated signaling pathway and these results may in part explain the lethal phenotype of strong mutations in the gene (Skoulakis and Davis, 1996; Broadie et al., 1997). These results, in congruence with the results from eye development, demonstrate that 14-3-3 proteins are bona fide in vivo components of the Ras/Raf/MAPK signaling cascade. Collectively, these recent studies in *Drosophila* provide the first physiological proof that indeed, 14-3-3 proteins are essential for Raf activation, and are integral components of the Ras/Raf-signaling pathway.

14-3-3 Proteins in Neuronal Function

The elevated expression of 14-3-3 proteins in the mammalian brain suggests that these proteins may have a role in brain function. This is underscored by the preferential expression in the hippocampus (Watanabe et al., 1991; Watanabe et al., 1993; Watanabe et al., 1994), a region that is essential for higher brain functions such as learning. The apparent overlap in expression of 14-3-3 isoforms in the mam-

malian brain suggests that a gene knock out approach to investigate their role in mammalian brain function will be difficult. However, a physiological role for 14-3-3 in neuronal function has emerged from the study of mutant animals in *Drosophila*.

Drosophila are amenable to behavioral manipulation being capable of both associative and nonassociative learning utilizing olfactory, visual and tactile stimuli. Mutants have been used extensively to dissect and characterize various behaviors exhibited by the flies (Davis, 1996). The preferential expression of D14-3-3ζ Leonardo in the mushroom bodies suggested that this protein may have a role in learning and memory processes. Mutations in other genes with preferential mushroom-body expression have been shown to compromise behavioral plasticity of the animals (Skoulakis et al., 1993; Davis, 1996). Mutations in Leonardo that do not affect the vital developmental functions of the gene, but impair preferential expression in the mushroom bodies were generated. The gross brain neuroanatomy of these animals is indistinguishable from that of normal animals, indicating that elevated expression is not required for development of the mushroom bodies or other brain areas (Skoulakis and Davis, 1996). Reduction in the amount of Leonardo in the mushroom bodies does not deprive the sensory abilities of the animals, but compromises integration of the stimuli manifested as learning and memory deficits (Skoulakis and Davis, 1996). These results provided the first physiological evidence of an essential role for 14-3-3 proteins in neuronal functions such as learning and memory.

The specific role for Leonardo in mushroom-body neurophysiology is unknown. It is tempting to speculate that the Ras/Raf/MAPK cascade is operant in these brain centers, as in the eye and in embryonic development. In support of this hypothesis, components of this signaling pathway are expressed in the adult mammalian brain (Finkbeiner and Greenberg, 1996). It is not currently known whether members of the *Drosophila* Ras/Raf/MAPK cascade are expressed in the mushroom bodies or other adult brain neuronal centers. An alternative hypothesis is that the behavioral deficits of Leonardo mutants are caused by disruption of signaling via PKC, the other major neuronal kinase known to bind 14-3-3. Two of the three *Drosophila* PKC genes are expressed in the adult brain (Rosenthal et al., 1987; Schaeffer et al., 1989), but mutations are not yet available. It would be interesting to examine the learning and memory ability mutants in PKC when available, in combination with Leonardo-deficient animals to address this hypothesis.

The impaired behavioral neuroplasticity in many of the *Drosophila* learning mutants is also reflected in deficits in synaptic plasticity at the larval neuromuscular junction (NMJ) (Davis, 1996). Since investigation of synaptic physiology is not currently possible in the *Drosophila* brain, the NMJ has served as an important model in elucidating the role of many molecules in synaptic physiology and plasticity (Broadie, 1994; Davis, 1996). Leonardo is enriched at the presynaptic side of the embryonic NMJ (Broadie et al., 1997). Since severe Leonardo mutations are lethal, electrophysiological investigation was performed in the mature embryonic NMJ. The morphology and basal excitation-secretion functions appear normal at the NMJs, suggesting that synaptogenesis is normal in loss of function Leonardo-mutant embryos. However, synaptic transmission, amplitude and fidelity are impaired, especially when the demand for neuronal output is high (high stimulation frequencies, or low external Ca^{2+}). Furthermore, plastic properties of the NMJ, such as long-term facilitation and posttetanic potentiation, are disrupted. These effects on synaptic physiology and plasticity do not appear to be the result of decreased numbers of synaptic vesicles, but rather a failure to mobilize them properly (Broadie et al., 1997). These defects in synaptic function and neuroplasticity may underlie all or part of the behavioral defects of Leonardo mutants.

Interestingly, a role in regulated exocytosis in permeabilized mammalian adrenal chro-

maffin cells has been ascribed to 14-3-3 proteins (Morgan and Burgoyne, 1992; Burgoyne and Morgan, 1993; Roth et al., 1993). Addition of 14-3-3 proteins enhances exocytosis in these cells by modulating the availability of secretory vesicles (Chamberlain et al., 1995). This, in turn, appears to result from 14-3-3-mediated reorganization of the cortical actin network to allow access of the vesicles to the membrane (Roth and Burgoyne, 1995). The number of available secretory vesicles appears to be regulated by activated PKC also (Morgan and Burgoyne, 1992; Gillis et al., 1996), suggesting an interaction between the kinase and 14-3-3 at the synapse (Fig. 5). The effect of 14-3-3 then, could be to either activate or keep PKC active. Alternatively, 14-3-3 dimers could bind the positively charged actin and PKC simultaneously and mediate reorganization of the actin network by PKC phosphorylation. Consistent with this role in exocytosis, most neuronal 14-3-3 isoforms are present in purified synaptic and synaptosomal membranes from mammalian brains (Martin et al., 1994).

The neurophysiological results on impairment of synaptic transmission amplitude and fidelity in Leonardo mutants are congruent with the role of 14-3-3 proteins in exocytosis. However, the plastic properties of the synapse—long-term facilitation and posttetanic potentiation—appear to be regulated by distinct signal-transduction cascades (Kullmann and Siegelbaum, 1995) including those mediated by protein kinase A, Ca^{2+}/calmodulin-dependent kinase II and PKC. In addition, the Ras/Raf/MAPK appears to play a role in synaptic function at the *Drosophila* NMJ (Zhong, 1995; Zhong and Pena, 1995). It is possible then, that Leonardo has a dual role at the NMJ synapse. One role is to regulate vesicle dynamics by interactions with the actin network and PKC, and another is in synaptic plasticity by modulating the activities of Raf, PKC, and other unknown kinases (Fig. 5). Impairment of all or some of these processes may underlie the mushroom-body-mediated behavioral deficits of Leonardo mutants. Interestingly, Leonardo appears enriched in mushroom-body synapses, but it is present at high levels throughout these cells (Skoulakis and Davis, 1996), a fact that may reflect involvement of the protein in multiple processes in these neurons.

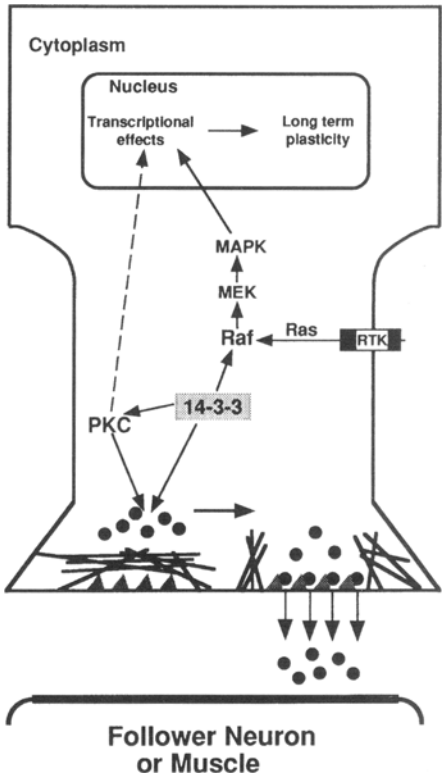

Fig. 5. A model for the neuronal functions of 14-3-3. The diagram depicts a mushroom body neuron or a motor neuron. PKC, in concert with 14-3-3 causes a reorganization of the actin cytoskeleton (dark network of lines) at the synapse, enabling synaptic vesicles (dark circles) to occupy docking sites (gray triangles) for release. In addition, interaction of 14-3-3 with PKC is required for activation of the kinase and subsequent signaling to the nucleus (dashed arrow) where changes in transcription of target genes may mediate long term changes in neuroplasticity. Moreover, activation of a putative RTK in the neuron may activate the Ras/Raf cascade, which independently or in concert with PKC activation, may mediate changes in neuroplasticity. The 14-3-3 proteins are depicted inside boxes to suggest participation in the pathways but not transduction of signals.

Concluding Remarks

Thirty years after their discovery and following a long history as biological oddities the 14-3-3 family of proteins has come of age. Their involvement in fundamental biological processes suggested by their high evolutionary conservation has been established firmly with the discoveries of in vivo physiological roles ranging from neuronal development to function at the level of one synapse or an entire organism. Their seemingly gratuitous involvement in diverse processes can easily be explained if the entire 14-3-3 dimer is viewed as a module, akin to SH2 or SH3 domains, involved in coordination and modulation of the activity of its ligands. Specificity for such interactions is not inherent only in binding specific motifs on their target proteins. Specificity could be further refined by differential affinities of homo- and heterodimers among the isoforms for their ligands. This must be especially important in cells like neurons that require temporal and spatial coordination of multiple signal-transduction pathways and processes. This requirement may be reflected in the high expression level of 14-3-3 genes in neuronal tissues in species ranging from *Drosophila* to humans.

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