REVIEWS

14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins

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A family of proteins known as 14-3-3 is currently receiving increased attention by investigators studying a broad range of biological systems, including plants and invertebrates. The outstanding feature of this family is the extraordinarily high sequence conservation observed. Current thinking indicates that these proteins may function as regulators in signal transduction/phosphorylation mechanisms.

THE 14-3-3 FAMILY OF PROTEINS were originally isolated by Moore and Perez in 1967[1]. The name originated from the nomenclature of their systematic analysis of brain proteins, which were identified as a series of acidic proteins that had molecular masses of around 30 000 kDa and an isoelectric point of around 5 in the two-dimensional polyacrylamide gel electrophoresis (PAGE) system. Subsequent studies have indicated that the proteins exist in dimeric form.

High levels of the proteins were shown to exist in brain, particularly neuronal tissue, and it was initially thought that they were neuronal tissue specific. They have now been shown to be very widely distributed and low levels are expressed in most mammalian tissues. Proteins that show a high degree of similarity have been cloned and sequenced from a wide range of other eukaryotic organisms including plants, insects, amphibians and yeast.

Following the initial discovery period, the 14-3-3 field was relatively quiet for about ten years, until two groups unintentionally ran into these proteins. Isobe and his co-workers discovered that the major components of a brain extract that modifies tryptophan and tyrosine hydroxylase were several proteins which corresponded in size and charge to 14-3-3 proteins. The group then went on to purify seven 14-3-3 isoforms and also cloned the first 14-3-3 isoform. Independently, Aitken and coworkers found that brain protein(s) that inhibits phospholipid/Ca^{2+}-dependent protein kinase C (PKC) were members of the 14-3-3 family. This effort led to the cloning of the second 14-3-3 isoform. Comparison of the sequences of these isoforms and fragments of others provided the first indication that isoforms of this family were remarkably similar. Other investigators have subsequently sequenced unknown proteins of special interest to them, only to discover another 14-3-3 isoform. All animal and plant tissues examined to date contain several isoforms.

Each mammalian 14-3-3 isoform has been assigned a Greek letter (α–η) according to its sequential elution position after reverse-phase high-performance liquid chromatography (HPLC)[2], which is very highly conserved across a range of mammalian species from rat, sheep and cow to humans. On the other hand, each isoform shows distinct differences in discrete regions (Fig. 1). Since the non-mammalian sequences also preserve the highly conserved 'motifs' it would appear that the protein family evolved and diverged before the separation of insects, plants, amphibians and mammals. Indeed, an evolutionary tree of the available sequences shows that the ε sequence is closer to the plant and yeast proteins than to the other mammalian isoforms (Fig. 2).

Distribution of isoforms

The pattern of tissue distribution of 14-3-3 isoforms varies. Preliminary analysis indicates that many isoforms are expressed in most tissues. There are very high levels of many isoforms in brain tissue, particularly in Purkinje cells in the cerebellum (η isoform)[15]. High levels of β and γ isoforms are also found, the latter of which may be brain specific[5]. There are also high levels of some isoforms in adrenal medulla and intestine, platelets and testis[7,16]. Novel isoforms are expressed in spleen. Another isoform is specific to skin, ear and tongue[17]. A subset of the family is expressed in fibroblasts; one of these is down-regulated in proliferating cells compared to SV40 transformed fibroblasts[18], and two isoforms have been identified in the Golgi[17]. Members of the 14-3-3 family of proteins are thus found in an extremely broad range of organisms and tissues.

Mammalian 14-3-3

The 14-3-3 family of proteins clearly has important role(s) in mammalian brain, where levels as high as 13.3 $\mu g\ ml^{-1}$ soluble protein (approximately 1%) have been measured[19]. However, no physiological function had been attributed to 14-3-3 until Ichimura and coworkers[20] showed that it was identical to an activator protein of tyrosine and tryptophan hydroxylases, the rate-limiting enzymes involved in catecholamine and serotonin neurotransmitter biosynthesis, respectively.

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Although tryosine hydroxylase is activated by PKC and cyclic-AMP (cAMP)-dependent protein kinase phosphorylation, the 14-3-3 protein was necessary for activation after phosphorylation by Ca^{2+}/calmodulin-dependent (CAM) kinase II. All three kinases phosphorylate an identical site on tyrosine hydroxylase[21], but CAM kinase II phosphorylates an additional unique site. The proposed mechanism for activation involves the acidic carboxyl terminus of 14-3-3 binding to the regulatory domain of the phospho-form of the hydroxylase to induce an active conformation. This is a distinct second step in hydroxylase activation that is not required for the phosphorylation of the enzyme by CAM kinase II. In addition to this function, there are clearly other roles for 14-3-3, since it is found in tissues and cells that do not contain these hydroxylases.

The acidic proteins of 29–33 kDa isolated from sheep brain that are potent inhibitors of PKC (Ref. 22) were called KCIP-1 (kinase C inhibitor protein). A search of the EMBL database revealed that peptides sequenced from the KCIP-1 isoforms had a high degree of sequence identity with bovine brain 14-3-3 protein[23] and subsequent studies showed that they are members of the 14-3-3 family[14]. The inhibitory mechanism of KCIP-1 does not appear to involve competition with PKC, its substrates, ATP or co-factors [Ca^{2+} or diacylglycerol (DAG)]. There was also no effect on [^{3}H]phorbol dibutyrate binding, although the presence of the phorbol ester, TPA, could overcome this inhibition[22].

Examination of the sequences in Fig. 1 suggests a possible mechanism for PKC inhibition. Residues 60–63 are reminiscent of part of the 'pseudosubstrate' domain of protein kinase C[3]. The pseudosubstrate hypothesis has been proposed to account for the inhibitory sequences in the regulatory domains of a wide range of second messenger-dependent protein kinases. These sequences contain all the elements necessary for a recognition site for the particular kinase, but the phosphorylatable Ser or Thr is replaced by another residue, usually Ala. Competitive inhibition by this domain is relieved by the conformational change induced by second messenger binding. It should be noted that Ser residues at positions 64 and 65 are potential substrate sites for PKC, while cAMP-dependent kinase and Ca^{2+}/calmodulin kinase could phosphorylate Ser65 and Ser70, respectively. A subset of the KCIP/14-3-3 family is in

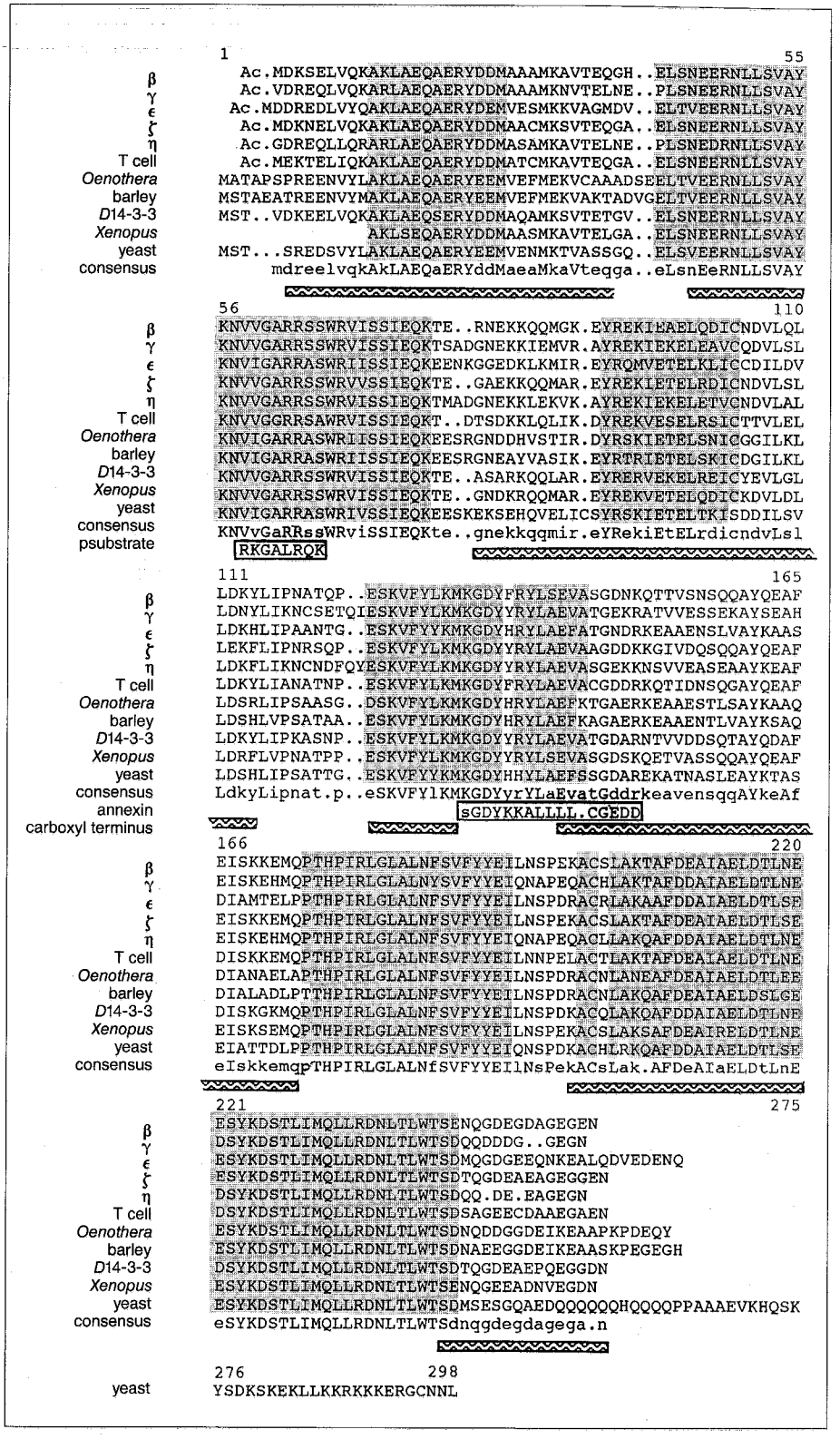

Figure 1

Sequence alignments of 14-3-3 family members. The sequence around the 'pseudosubstrate' Ala residue and the similarity with the carboxyl terminus of the annexin family is shown. Note that the ζ isoform of PKC has the sequence GARR (Ref. 3). The actual sequence of the carboxyl terminus is that of human annexin V, although some members of this family contain a conserved Lys residue at the position of the lower case Ser[4]. The sequences of 14-3-3 are bovine β and γ (Ref. 5); sheep ε (Ref. 6); rat ζ (Ref. 7); bovine η[2]; a human 14-3-3 T-cell sequence[8]; sequence deduced from a *Drosophila* clone (*D*14-3-3)[9]; yeast[10]; plant, *Oenothera hookeri*[11] and barley[12] 14-3-3 homologues and the consensus sequence. The *Xenopus* sequence[13] is not completely full length. It is not known whether the non-mammalian proteins are *N*-acetylated. Circular dichroism has confirmed the high degree 64% of α-helical structure predicted[14]. These regions are shown as a bar underneath the alignment. The invariant (or very highly conserved) regions are boxed.

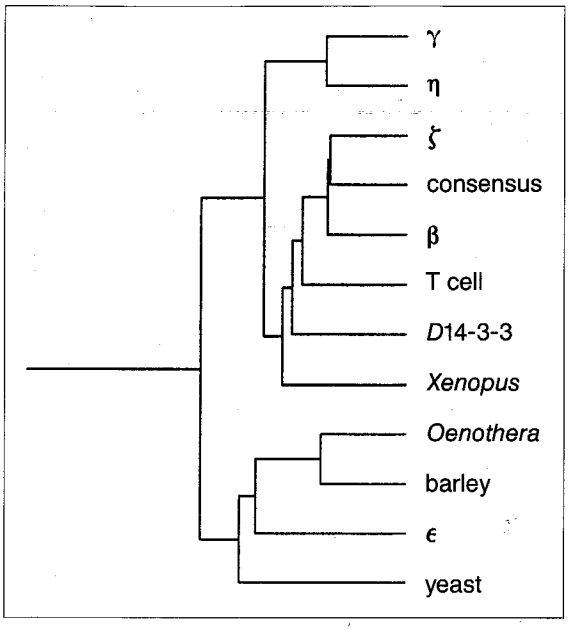

Figure 2
Computer-generated line-up of sequences. Note that the *Drosophila* sequence is closer to the β, ζ and human sequences and that the plant and yeast sequences are closer to the ε isoform. Similarly, the *Drosophila, Xenopus* and three of the mammalian 14-3-3 sequences are closer to each other. This suggests that the divergence of the isoforms was an early event in evolution and that the sequences have subsequently remained highly conserved. The sequence comparison was generated using the program 'pileup' in the GCG sequence analysis package. This generates a multiple sequence alignment to show relations between sequences, but is not a phylogenetic tree.

fact phosphorylated by PKC itself but not by a wide range of other protein kinases[14].

Although the kinase inhibitor and 14-3-3 are apparently not Ca^{2+}-binding proteins[19] and there is no evidence of similarity with any putative Ca^{2+}-binding domains, one region (residues 134 to 150) shows close similarity with the conserved carboxyl terminus of the family of Ca^{2+}- and lipid-binding proteins, the annexins[4] (Fig. 1). This could be the binding site for the regulatory domain of PKC (the proteins do not inhibit PKM, the catalytic domain of PKC[14]). Annexin V has been shown to inhibit PKC[24]. Mochly-Rosen and colleagues[25] have shown that PKC binds to cytoskeletal or cytoskeletal-associated proteins when the cytosolic enzyme is activated by the second messenger DAG, in the presence of elevated levels of Ca^{2+}. She has named these RACKs (receptors for activated C kinase). Following her observation that members of the annexin family could act as RACKs, combined with the report that a particular region on 14-3-3/KCIP-1 had close similarity with the carboxyl terminus of lipocortin/annexins[23], she has tested the ability of the carboxy-terminal region of annexins to prevent PKC association with the plasma membrane. A synthetic peptide based on this region in 14-3-3/KCIP-1 that is similar to the annexins has also been shown to prevent PKC binding to RACKs. These observations point to a potential physiological role for an isoform(s) of 14-3-3 as mediators of PKC translocation. Thus, members of this protein family may regulate the subclass of PKC isoforms (α, β and γ) that are Ca^{2+} dependent and translocate to the plasma membrane as part of their activation mechanisms.

The ε and ζ isoforms of the 14-3-3 family co-purify with the ovine pineal serotonin *N*-acetyltransferase and may be involved in the regulation of the enzyme, the rate-controlling step in the conversion of serotonin to melatonin[6]. Recently, two proteins named Exo1 and Exo2 have been isolated from brain cytosol by Burgoyne *et al.*[26] These proteins stimulate Ca^{2+}-dependent exocytosis in permeabilized adrenal chromaffin cells. Exo1 protein(s) migrate on SDS-PAGE as a group of polypeptides of approximately 30 kDa, and peptide sequencing has revealed these to be members of the 14-3-3 family. The ability of Exo1 to reactivate exocytosis is potentiated by PKC, which suggests a role for Exo1 in the PKC-mediated control of Ca^{2+}-dependent exocytosis. A protein with phospholipase A_2 (PLA_2) activity, distinct from extracellular PLA_2, has recently been shown to be a member of the 14-3-3 family[27]. This is presumably the human ζ isoform since there are only two conserved amino acid changes from rat ζ 14-3-3. This PLA_2 has been purified as at least three chromatographically distinct isoforms from sheep platelets. The activity(ies) was highly selective for the release of arachidonic acid from choline and ethanolamine glycerophospholipids. A stable acyl-enzyme intermediate was formed and a role in the cellular trafficking of the arachidonyl moiety was proposed. Arachidonic acid stimulates secretion from many cell types, which may be relevant to the role of Exo1.

14-3-3 in other organisms

The partial clone from *Drosophila* was initially thought to be a domain of an alternatively spliced form of EGF receptor but was later shown to be a cloning artefact[28]. The function of this protein(s) in insects remains unknown. However, the recent cloning of the intact gene from *Drosophila*[9] has enabled the study of the expression of the gene during development. The level of one of three mRNA species peaked between 12 and 15 h of embryogenesis. The levels of all three mRNA species dropped to almost basal level in larvae and pupae, then rose again in the adult stage. There are high levels in embryo ventral nerve cord and in neural tissues.

The yeast homologue of 14-3-3 (Ref. 10) may have a function in growth regulation. Yeast strains with the gene on multicopy plasmids and disruption mutants showed no loss of cell viability but showed a 30% reduction in growth rate using glucose as the carbon source. However, by contrast to the wild-type and disruption mutants, the strains with the gene on a multicopy plasmid hardly grew with acetate or glycerol as carbon source.

A plant 14-3-3 homologue has been isolated from pea and has been shown to be inhibitory in the PKC assay[11]. Subsequently, cDNA coding for plant 14-3-3 was cloned and sequenced from spinach and *Oenothera*. A role for 14-3-3 in the regulation of protein kinases in plants has also been proposed, although a plant protein kinase that is Ca^{2+}, phospholipid and DAG dependent, with similarity to mammalian PKC has not yet been described[11]. Protein kinases are involved in the regulation of light harvesting in the chloroplast thylakoid membrane and it is interesting that a protein encoded by a gene upstream of a gene that responds to changes in light intensity, cloned from a cyanobacterium[29], has sequence similarity with another PKC inhibitor protein, called PKCI-1, isolated by Walsh and colleagues[30]. Cyanobacteria have the oxygen-evolving plant chloroplast type of photosynthetic apparatus. 14-3-3 protein-like cDNAs from barley leaves have been identified on the basis of sequence similarity and serological similarity to the mammalian sequences[12]. The level of mRNA corresponding to at least two of the three different se-

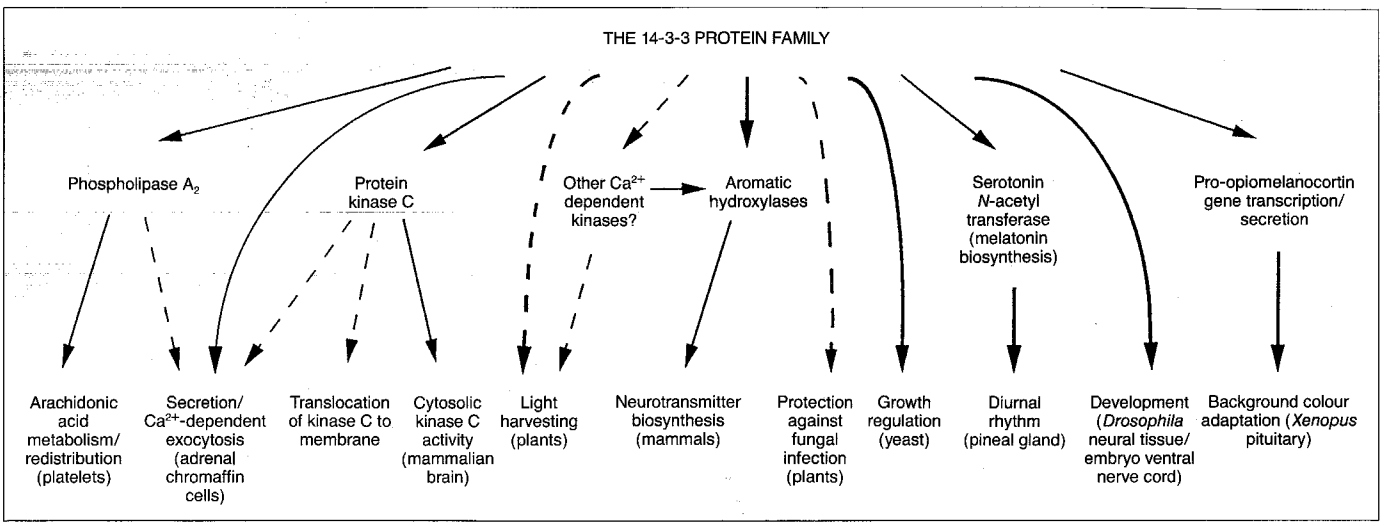

Figure 3

This figure summarizes the current state of knowledge of the biological roles of the 14-3-3 family of proteins. Full lines indicate that a link has been established between a 14-3-3 protein, an enzyme activity or regulatory function and the physiological process. The dashed lines indicate that the connection is tentative.

quences identified increased following inoculation with fungal pathogen *Erysiphe graminis*, the powdery mildew fungus; this rise was concomitant with increased levels of other mRNAs believed to encode products involved in defence. The role of 14-3-3 protein in defence, if any, is unknown; however, various possibilities are raised from its functions in other systems. For example, signal transduction is central to the activation of defences since elicitors, produced by pathogens, induce defence responses in the host following recognition. It has been shown that the protein phosphorylation state can be affected by elicitor action. 14-3-3 protein might act as a regulator of these processes. In the interaction between barley and *Erysiphe graminis* the enhanced transcription of defence-related genes, including the 14-3-3 homologue, is correlated with the development of papillae (extracellular local reinforcement to the inner side of the cell wall comprising callose, phenolic compounds and proteins). Cytoplasmic activity increases adjacent to the nascent papilla, presumably indicating enhanced exocytosis. Thus the possibility that 14-3-3 protein has a role in Ca^{2+}-mediated exocytosis might indicate that this is the major role during defence. In analogy with the role of 14-3-3 in regulating tryptophan and tyrosine hydroxylases, it is also conceivable that this protein acts to regulate the biosynthesis of phenolic compounds produced in the papillae.

A 14-3-3 homologue has also been cloned and sequenced from *Xenopus*[13]. It was identified as a gene coordinately expressed with the prohormone pro-opiomelanocortin (POMC) in the melanotrope cells of *Xenopus* pituitary gland. Transcription of the POMC gene increased 20–30 times when the toad was placed on a black background. This 14-3-3 homologue may therefore be involved in secretion from the pituitary gland during background adaptation[13].

The known functions of this novel class of protein are illustrated in Fig. 3. These include a wide range of cell-signalling processes as well as development and growth regulation. However, much work remains to elucidate the exact physiological role(s) of each mammalian isoform (there may be 10–12 distinct gene products) and their counterparts in other organisms.

Acknowledgements

The authors wish to thank the many colleagues and collaborators who contributed to the work described in this article. These include D. Klein (NIH), H. Thordal-Christensen, J. Brandt and K. Vad (Copenhagen).

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