Isoforms of 14-3-3 protein can form homo- and heterodimers in vivo and in vitro: implications for function as adapter proteins

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Abstract 14-3-3 proteins play a role in many cellular functions: they bind to and regulate several proteins which are critical for cell proliferation and differentiation. 14-3-3 proteins exist as dimers, and in this study we have shown that diverse 14-3-3 proteins can form both homo- and heterodimers in vitro (by cross-linking studies) and in vivo (by coimmunoprecipitation and Western blot analysis); this interaction is mediated solely through the N-terminal domain of the proteins. The composition of 14-3-3 dimers within a cell may play a key part in the role of this family of proteins as modulators or adapters which facilitate the interaction of distinct components of signalling pathways.

Key words: 14-3-3; Dimer; Signalling; Raf kinase; MAP kinase

1. Introduction

The 14-3-3 protein family was so named due to its migration position on two-dimensional DEAE chromatography and starch gel electrophoresis [1]. 14-3-3 proteins have a molecular mass of around 30 kDa and are highly conserved in evolution. They have been found in mammals, insects, plants, yeast, amphibians, fish and *Caenorhabditis elegans*, and have been assigned a wide variety of functions (reviewed in [2] and [3]). To date, seven mammalian brain isoforms of 14-3-3 have been described, named α-η after their respective elution positions on HPLC [4]. Five of these have been sequenced (reviewed in [5]), and the α and δ isoforms are phosphorylated forms of the β and ζ isoforms respectively [6]. This phosphorylation is at a consensus motif for proline directed (cyclin-dependent) kinases. Additional isoforms which are absent or present at low levels in brain have been described from other mammalian tissues; these include an isoform found in T-cells [7] and one in epithelial cells [8,9]. These have been named τ and θ respectively [10].

14-3-3 proteins have been ascribed a diverse range of activities including regulation of protein kinase C activity, involvement in exocytosis from adrenal chromaffin cells, transcriptional regulation in plants and regulation of the cell cycle in fission yeast (reviewed in [2] and [3]). Recently the β and ζ isoforms (which are phosphorylated in vivo at a high stoichiometry in brain [6]) have been shown to associate with (and possibly activate) Raf-1 kinase in the cytosol and at the membrane of mammalian cells and in yeast [11–15]. The τ isoform has been shown to bind, and be phosphorylated by, c-Bcr and Bcr-Abl

kinases, and may also regulate these proteins [16]. 14-3-3 isoforms have also been shown to bind polyoma virus middle tumour antigen, a protein which associates with other cellular proteins involved in the regulation of cell proliferation [17].

In this study we have shown that several specimen isoforms can form homo- and heterodimers both in vivo and in vitro. This finding is of importance in the elucidation of the physiological role of this family of proteins.

2. Materials and methods

2.1. DNA manipulations and protein purification

DNA manipulations were performed in *Escherichia coli* TB1 (New England Biolabs). Plasmids pMALc2 (which produces a maltose-binding protein fusion; New England Biolabs) and pKK233-2 (which produces a native protein; Pharmacia) were used as expression vectors. The ζ isoform was cloned from a human T-cell cDNA library constructed in the vector lambda ZAPII (Stratagene, catalogue number 936204). The fragment of DNA containing the open reading frame only, was isolated directly from the library by means of the PCR and ligated into expression vector pKK233-3. Oligonucleotides GGAATTCCATGG-ATAAAAATGAGCTGGTTCAG (which incorporates a *Nco*I site immediately prior to the start codon) and ACGCAAGCTTAATTTTC-CCCTCCTTCTCCTGC (which incorporates a *Hind*III site after the stop codon) were used for this purpose. Optimum recovery of the ζ cDNA was achieved with 1 μl of library (as supplied) in a 50 μl reaction volume. Prior to the addition of buffer, primers and enzyme, the library aliquot was disrupted by incubation at 70°C for 5 minutes, and was then cooled on ice. *Taq* polymerase was added when the reaction mix was at 94°C, and this was subjected to 30 cycles of the PCR of 1 minute at 94°C, 2 minutes at 54°C and 1 minute 45 seconds at 72°C. This was sequenced by the dideoxy chain termination method using the Sequenase kit supplied by USB. Protein purification (both of the fusion proteins, and those expressed as native 14-3-3 proteins) was performed as described in [19]. To generate vectors for expression in eukaryotic cell culture, 14-3-3 isoforms were manipulated into the expression vector pcDNA3 (Invitrogen) using the PCR and suitable oligonucleotides. For expression of isoforms with a C-terminal myc-tag (MEQKLISEE-DLN), the coding DNA was first ligated into a Bluescript vector which already contained the coding sequence for the myc-tag inserted between the *Bam*HI and *Xba*I restriction sites. The 14-3-3 cDNA and tag DNA were then excised together and inserted into pcDNA3.

The N-terminal 17 kDa portion of 14-3-3 proteins forms a domain which is stable and soluble when expressed in *E. coli* [19]. This domain of ε 14-3-3 was also inserted into pcDNA3 as a myc-tag, and expressed well. N-terminally deleted ε (which can be expressed in *E. coli* [19]) did not produce any detectable protein expession however. Full details of plasmids generated can be obtained from the authors.

2.2. Protein cross-linking

20 μg of ε or τ maltose-binding protein fusions or MBP alone, and 10 μg of non-fusion ζ or τ protein were mixed as required (this gives near equal molar ratios of the proteins) and made up to 100 μl in 20 mM Tris pH 7.5 + 1 mM DTT. A solution of 8M guanidine HCl was then added to each tube to a final concentration of 6 M and these were incubated at room temperature for 2 hours. Samples were then dialysed against several changes of PBS + 1 mM DTT. Titration of BS^3 (bis[sulphosuccinimidyl] suberate) cross-linking reagent (Pierce)

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against ζ isoform indicated that a molar ratio of 140:1 BS[3]: ζ gave approximately 50% cross-linking after one hour at room temperature, which gave sharp protein bands on SDS-PAGE (data not shown). Excess cross-linker resulted in blurred cross-linked protein bands. Freshly prepared BS[3] was therefore added to the reaction tubes to the required concentration, and incubated at room temperature for 1 hour. The reaction was stopped by the addition of Tris pH 7.5 to 100 mM. The sample volume was reduced by freeze-drying and the mixes were then analysed by 8% SDS-PAGE. One gel was stained with Coomassie blue and one gel was analysed by Western blotting.

2.3. Electroporation of COS cells and immunoprecipitation

Rapidly growing cells were harvested and 2×10^6 cells were electro-porated (using a Bio-Rad Gene Pulser at 0.27 V, 125 μFd) with 3 μg plasmid DNA + 20 μg sonicated herring sperm carrier DNA. Cells were grown for a further 48–60 hours, with one change of medium. Cell monolayers were harvested by scraping cells from the bottom of the dish in the presence of ice-cold detergent lysis buffer (20 mM Tris pH7.5, 150 mM NaCl, 1% NP40, 5 mM iodoacetamide, 5 mg/ml each aprotinin and leupeptin, 0.1 mM vanadate). This was rotated at 4°C for 1 hour, then debris was removed from the supernatant by microcentrifugation at 4°C for 15 minutes.

Monoclonal antibody 9E10 was bound to protein A Sepharose beads (Pharmacia) by the high salt method [20]. Beads were mixed with the cell lysate for 2 hours at 4°C, then washed with lysis buffer. Samples were analysed by SDS-PAGE analysis [21].

2.4. Western blot analysis

This is described in [10]. Antibodies specific to each 14-3-3 isoform are described in [10], and antibody specific to the C-terminus of ε is described in [19].

3. Results and discussion

Mammalian 14-3-3 proteins have a molecular weight of circa 30 kDa (with the exception of ε which has an apparent M_r of 33 kDa) and isoelectric points of circa 4.6. As a result most cannot be separated clearly on SDS-PAGE. Cross-linking of individual isoforms gives no useful data as all 60 kDa cross-linked species have a virtually identical mobility. By crosslinking a 14-3-3 fusion protein with a native protein this problem is overcome. It is important to note that the fusion used in this work is the monomeric maltose binding protein (MBP). In contrast, glutathione S-transferase is dimeric in its native state. Fig. 1 shows the Western blot of τ and ζ non-fusion proteins

and τ-MBP in all combinations, with or without cross-linker. τ and ζ native proteins cross-link efficiently to give a 60 kDa product. τ-MBP, which has a monomeric molecular mass of 72 kDa, cross-links less efficiently to give a product of approximately 200 kDa; while this appears high, it should be remembered that the protein now has some covalent structure and is not a totally denatured linear polypeptide. The N-terminal amino acids of 14-3-3 are important for dimer formation [19], and as the MBP is attached to the N-terminus, the less efficient cross-linking may be due to steric effects.

When τ-MBP is cross-linked to ζ or τ 14-3-3, the 60 kDa and 200 kDa protein species are again evident. In addition, and with equal intensity for both isoforms, a doublet protein species of intermediate size (approximately 140 kDa) is present; this is τ-MPB cross-linked to τ and ζ. Why this should be a doublet is unclear, though it could be an artefact depending on where the cross-linker binds in the protein sequence.

To determine whether 14-3-3 proteins can form homo- and heterodimers in vivo, epitope tagged ε 14-3-3 was transfected into COS cells and subsequently immunoprecipitated by means of the myc-tag. Any non-tagged 14-3-3 precipitated must be dimerised or complexed with the myc-tagged form. Tagged 14-3-3 proteins have an apparent molecular weight approximately 3 kDa higher than untagged proteins, thus any 14-3-3 protein bound to the tagged isoform will be clearly visible on SDS-PAGE. However, transfection with tagged 14-3-3 alone resulted in very little co-immunoprecipitation of other 14-3-3 proteins (data not shown). It has been observed previously (from in vivo phosphorylation studies) that 14-3-3 proteins have a relatively long half-life (Aitken and Hernandez, pers. obs.). Thus the transfected, tagged 14-3-3 proteins, which should be translated at a high rate, may associate predominantly with themselves, and not with endogeneous proteins which are being produced at a much slower rate. To overcome this, double transfections of both tagged and non-tagged isoforms were performed. Upon immunoprecipitation it was clear that non-tagged proteins were dimerising efficiently with the myc-tagged versions. Fig. 2 shows that when cells are transfected with ε-myc and either native ε or ζ 14-3-3, the non-tagged proteins are precipitated with ε-myc (lanes 5 and 9). In
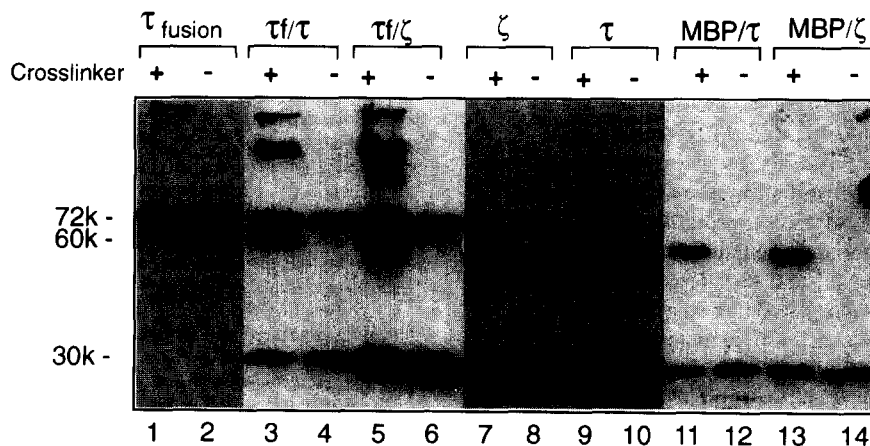

Fig. 1. Western blot of 14-3-3 proteins in the presence (+) or absence (−) of cross-linking reagent. The blot was probed with antibodies specific for the N-terminal regions of τ and ζ 14-3-3 isoforms [10]. Lane 1 shows the presence of the 200 kDa cross-linked τ-MBP fusion protein (labelled τ fusion), and lanes 7 and 9 show the 60 kDa cross-linked product of native ζ and τ respectively. There is no effect on this cross-linking when MBP alone is present (lanes 11 and 13). When τ-MBP fusion protein (labelled τf) is cross-linked with either τ or ζ however (lanes 3 and 5), an additional band of intermediate size is apparent; the dimer of τ-MBP/τ (lane 3) and τ-MBP/ζ (lane 5).

Fig. 2. Western blot (probed with antibodies specific for the N-terminal regions of ε and ζ 14-3-3 isoforms) of total soluble cell extract (T) and protein immunoprecipitated with anti-myc 9E10 monoclonal antibody (B). Cells were transfected with vector alone (pcDNA3, lanes 2 and 3) or with vectors expressing ε-myc, $1/2\varepsilon$-myc (a fusion with the N-terminal domain of ε only), ε or ζ (lanes 4 through 11) as shown. Lanes 1 and 12 are brain-purified 14-3-3 protein as a marker: ε is the upper band (33 kDa) and ζ is the lower band (30 kDa). The antibody against ζ shows less avidity than the ε antibody. In all 'total' (T) lanes, background ε and ζ 14-3-3 (at 33 kDa and 30 kDa) are apparent. Lane 4 shows an additional higher molecular weight band at approx. 36 kDa (ε-myc), which when immunoprecipitated (lane 5) also brings down ζ 14-3-3 (and a small amount of background ε). An equivalent pattern is seen with half-length ε-myc fusion (with an M_r of 17 kDa, $1/2\varepsilon$-myc, lane 7). The immunoprecipitate of cells transfected with ε-myc and ε shows that ε is precipitated with the tagged form, and again some background ζ is precipitated (lane 9). This is also seen in cells transfected with half-length ε-myc fusion and ε (lane 11).

each case endogenous ε or ζ are also visible albeit as much fainter bands (for reasons described above). The tagged 17 kDa N-terminal domain of ε 14-3-3 also precipitated intact 14-3-3 ε and ζ efficiently (lanes 7 and 11). This is predicted from the crystal structure of 14-3-3, which verifies that the N-terminal region of 14-3-3 mediates the dimerisation [22].

The observation that several specimen isoforms of 14-3-3 can form homo- and heterodimers has important ramifications. ζ has been shown to bind to and activate c-Raf kinase and τ binds to and is phosphorylated by Bcr and Bcr-Abl kinases. Whether each 14-3-3 isoform has a specificity for a single cellular protein, is as yet unclear. However it has been shown that
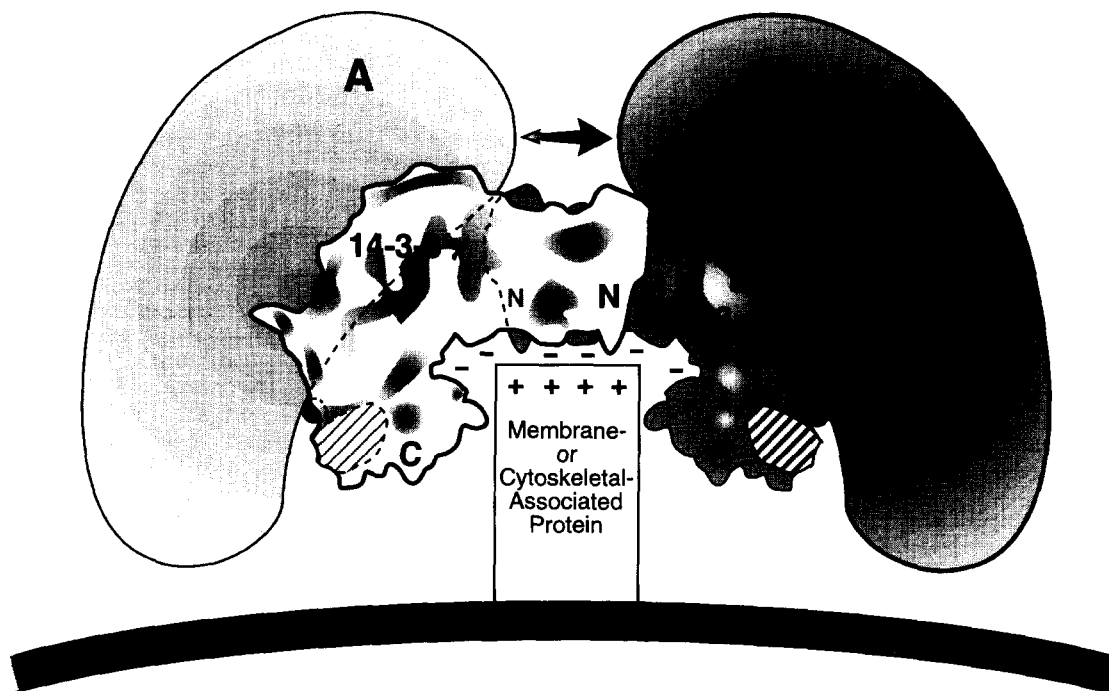

Fig. 3. Putative function of 14-3-3 heterodimers (labelled 14-3-3 X and Y), as adaptors to bring together distinct regulatory proteins (labelled A and B) of signalling pathways. The interaction between A and B may be directed to a membrane, cytoskeletal or organellar location as depicted, if 14-3-3 interacts through its conserved, negatively charged pocket with a positively-charged protein. The shape of the 14-3-3 dimer is modelled on the surface electrostatic potential [22]. N and C denote the amino- and carboxy-termini of each subunit. The filled circle indicates the position of the in vivo phosphorylated Ser185 [6] present in the phospho forms of β and ζ 14-3-3. The adjacent shaded portion is the annexin-similarity domain which is also on the surface of 14-3-3 and which has also been proposed to be involved in protein–protein interaction [18]. The double-headed arrow indicates interaction between the hypothetical signalling molecules A and B. It is also possible that interaction with adjacent organelle- or membrane-associated protein(s) could be mediated by formation of the complex.

a number of isoforms can regulate the activity of protein kinase C, with different potencies [18]. ε 14-3-3 is more similar in sequence to the plant and yeast homologues than it is to other mammalian isoforms [5] (indeed this is the reason it was chosen for detailed study), so if this isoform can form a heterodimer with ζ, it suggests all 14-3-3s can similarly dimerise. The ability of diverse members of this family to form heterodimers may play an important role in properties and functions: ζ 14-3-3, for instance, is phosphorylated to approximately 40% in the brain (when it is called the δ isoform), and this site is not present in τ. Other isoforms may have different potential regulatory sites which could modulate activity.

The crystal structure of a 14-3-3 protein has recently been elucidated [22]. This shows that residues within the region Gln8-Asp21 in one monomer form contacts with residues Ile65-Glu89 in the opposing monomer. The data reported here confirm that the N-terminal 17 kDa domain alone is sufficient for dimer formation. The dimeric protein forms a large negatively-charged channel, the interior of which contains amino acids which are almost invariant in 24 members of the family (from plants, mammals, yeast and amphibians). This is potentially involved in locating the 14-3-3 dimer at the surface of a cell membrane via a conserved cytoskeletal or membrane-associated protein (see Fig. 3). This could mediate the targetting to a specific location of the signal transduction proteins with which 14-3-3 interacts and lends further support for an 'adapter' function for these proteins.

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