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Antibodies against the major brain isoforms of 14-3-3 protein

An antibody specific for the N-acetylated amino-terminus of a protein

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14-3-3 proteins are apparently ubiquitous eukaryotic proteins that comprise a large number of isoforms. They have been implicated in the regulation of a wide range of biological processes [reviewed in Aitken et al. (1992) Trends Biochem. Sci. 17, 498–501]. We have raised specific antibodies against each mammalian brain isoform of 14-3-3 employing peptides synthesised from the amino-terminal regions. The peptides were, like the proteins from mammalian brain, N-acetylated. The antiserum specific for the epsilon isoform did not recognise the recombinant form of this protein (lacking the N-acetyl co-translational modification) expressed in *E. coli* until it was chemically acetylated.

14-3-3 protein; N-acetylation; Mass spectrometry; Isoform specific antibody

1. INTRODUCTION

The family of proteins known as 14-3-3 was so named due to their migration position on two dimensional gel electrophoresis [1]. 14-3-3 proteins all have a pI of around 4.5 and M_r of 30,000 Da on SDS-PAGE but 60,000 on size exclusion chromatography [2]. There are seven to eight mammalian brain isoforms of 14-3-3, named α–η after their respective elution positions on HPLC [3]. Five of these have been sequenced (reviewed in [4]). Clones for other HPLC separated brain 14-3-3 proteins have not so far been identified and we present evidence to suggest that in two cases this may be due to identity of the protein primary structures with β and ζ (zeta) isoforms. 14-3-3 has been studied as an activator of tyrosine and tryptophan hydroxylases [5], the rate limiting step in catecholamine and serotonin neurotransmitter synthesis in neurons. However, purified sheep brain 14-3-3 isoforms have been shown not to activate recombinant tyrosine hydroxylase (Sutherland, C. et al. (1993) Eur. J. Biochem., in press). The studies of our own group have focussed on 14-3-3 as a Kinase C Inhibitor Protein [2,6] that we named KCIP-1. A 14-3-3 protein member(s) called EXO1 stimulates calcium dependent exocytosis in permeabilized adrenal medulla cells [7]. The epsilon (ε) isoform of 14-3-3 copurifies with rat pineal N-acetyl transferase, the first enzyme in the pathway for conversion of serotonin to melatonin [8], thereby suggesting a function for 14-3-3 in regulating diurnal rhythm. The eukaryotic host factor that activates exoenzyme S from *Pseudomona aeruginosa* has been shown to be a member of the 14-3-3 family (the ζ isoform, [9]). Exoenzyme S ADP-ribosylates Ras and other GTP-binding proteins. A platelet phospholipase A_2 was identified as 14-3-3 ζ isoform [10] but recent studies [11]; Robinson and Aitken (in preparation) have found no PLA_2 activity in ζ or other isoforms of 14-3-3. Examples of 14-3-3 proteins have also been found in plants [12,13], insects [14] amphibians [15] and yeast [16]. In these non-mammalian species, a variety of roles for regulation of gene expression have been suggested, (reviewed in [4]). More recently, additional evidence for a role in regulation of transcription has come from the group of Ferl [17] who have shown that a plant 14-3-3 homologue binds to the G-box promoter element of inducible genes. In some mammalian tissues, novel isoforms may be expressed at high level. T-cells contain an isoform [18] that is not a major brain isoform. Epithelial cells contain a specific isoform called HME1 [19] or stratifin [20]. In keeping with the established nomenclature for mammalian 14-3-3 isoforms, employing Greek letters, we have named the T-cell isoform, tau (τ) and HME1 or stratifin isoform, sigma (σ). In the present study, antisera were raised against synthetic peptides based on the amino terminal regions of mammalian 14-3-3 isoforms. The peptides were synthesised with the correct processing of the initiator methionine including N-acetylation of the next residue if appropriate. The results indicate that the antisera will prove to be excellent tools for the study of cellular localisation and distribution of 14-3-3 isoforms since the antisera were distinct for each isoform. An interesting and important observation was made with the antiserum raised against the amino terminus of the ε 14-3-3

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isoform – this was specific for the wild type N-acetylated protein and did not recognise the identical (in linear amino acid sequence) recombinant protein. However, the latter protein *was* recognised by the antiserum when chemically acetylated on the initiator methionine. This could be an important point to note if expression libraries are screened using antibodies that may be specific for N-acetylated or other co- or post-translationally modified proteins.

2. MATERIALS AND METHODS

2.1. *Materials*

Reagents were analytical reagent grade from Sigma or BDH. Trypsin was obtained from Boehringer Mannheim, and phosphatidylserine was supplied by Sigma. BNPS skatole was from Pierce. Acetonitrile and TFA (both HPLC grade) were from Romil and Pierce respectively. Nitrocellulose (Schleicher and Schuel) and immobilon PVDF membrane (Millipore) were used for electroblotting except for the acetylation experiment when Problott (Applied Biosystems) was used. Recombinant epsilon (ε) and T cell (τ) isoforms of 14-3-3 were expressed in *E. coli* (in preparation).

2.2. *14-3-3 Purification*

14-3-3 was isolated from sheep brain by a combination of anion-exchange and hydrophobic-interaction chromatography steps by a method previously described [6]. Homogeneity was confirmed by the absence of other silver staining proteins on analysis of a heavy loading of protein (30 μg).

2.3. *Reverse-phase separation of 14-3-3*

Purified preparations of 14-3-3 (500 μg) were applied to a reverse-phase HPLC column (Aquapore RP300, 100 mm × 2.1 mm, 7 μ, Brownlee), [6]. Proteins were eluted by the application of a three-step gradient of 0.1% aqueous trifluoroacetic acid to acetonitrile (containing 0.082% by vol. trifluoroacetic acid), with a flow rate of 0.25 ml·min^{-1}. The acetonitrile concentration was increased as follows: 0–45% over 10 mins, 45–60% over 85 mins, 60–90% over 10 mins. A 100 × 4.6 mm column with a flow rate of 0.5 ml·min^{-1} was also used for larger scale (500 μg) separation of isoforms for amino acid sequencing. Peaks (A_{215}) were collected manually. Samples were concentrated and analysed by SDS PAGE and visualized by Coomassie blue or Western blotted.

2.4. *Immunological procedures*

Antibodies against the acetylated N-terminal sequences of sheep 14-3-3 isoforms were raised in rabbits. Acetylated peptides were produced by Peter Fletcher in the peptide synthesis service at NIMR using an Applied Biosystems 430A peptide synthesiser and FMOC chemistry. The peptides synthesised were:

beta (β) isoform	Ac.MDKSELVQKAC
gamma (γ) isoform	Ac.VDREQLVQKAC
zeta isoform (ζ)	Ac.MDKNELVQKAC
epsilon isoform (ε)	Ac.MDDREDLVYQAK
eta isoform (η)	Ac.GDREQLLQRARZ
human T cell (τ) isoform	Ac.MEKTELIQKAC

Peptides were purified by reverse phase HPLC and the structure verified by amino acid analysis and FAB mass spectrometry.

The γ, β, ζ and τ cell peptides were synthesised with a C-terminal cysteine residue, not present in the original sequences, to allow coupling to maleimide-KLH using the inject activated immunogen conjugation kit (Pierce, Rockford, IL, USA). The ε and η peptides were coupled to KLH with 21 mM glutaraldehyde (1 mg peptide: 5 mg KLH). In addition, an antiserum to whole 14-3-3 protein was raised using KLH as a carrier. All antigens were injected subcutaneously with Freund's adjuvant.

2.5. *SDS polyacrylamide gel electrophoresis*

Proteins were run on 12.5% SDS polyacrylamide gels containing SDS [21] and visualised by Coomassie blue and/or silver staining [22].

2.6. *Western blotting*

The antisera were tested by immunoblotting 14-3-3 proteins from SDS-PAGE onto nitrocellulose [23] using a semi-dry blotting apparatus (LKB 2117–250 Novablot). The SDS polyacrylamide gel was soaked for 40 mins in 3-cyclohexyl amino propane-sulfonic acid (CAPS, pH 11), 10% methanol transfer buffer. Transfer was carried out at room temperature at 2 W per 100 cm^2 of gel for 2 h. The gel was stained with Coomassie blue to confirm that transfer had occurred. The nitrocellulose was blocked using 2.5% non-fat-1% milk protein in Tris-buffered saline (20 mM, pH 7.6, TBS) for 1 h. Primary and secondary antibodies were used at dilutions of $^1/_{3,000}$ with the exception of gamma, at a dilution of $^1/_{6,000}$. Non-specifically bound primary antibody was washed off with 5 × 200 ml changes of Tris-buffered saline, 0.1% NP40 (TBS-NP40) every 10 min. Peroxidase-coupled goat anti-rabbit IgG (Biorad) in TBS, NP40 was used as detecting antibody. Non-specifically bound secondary antibody was removed, as for the primary antibody wash and antigens were visualised with the enhanced chemiluminescence (ECL) detection system (Amersham, UK) and exposed to X-OMAT-AR film. Isoforms of 14-3-3 separated by reverse phase HPLC, were blotted onto immobilon PVDF membrane after SDS PAGE and immunodetected in a similar manner.

2.7. *Stripping of immobilon membranes*

The membrane was submerged in 100 mM 2-mercaptoethanol 2% SDS, 62.5 mM Tris-HCl, pH 6.7 and incubated at 50°C for 8 h with occasional agitation. The membrane was washed 4 × 10 min in TBS/0.1% NP40 at room temperature using 400 ml of buffer. The membrane was reprobed with secondary antibody and incubated with ECL detection reagents to ensure complete removal of primary antibody had occurred before reprobing with the next antiserum.

2.8. *Sequence analysis*

2.8.1. Protein Cleavage

Sequence analysis was performed on peptides from both cyanogen bromide, CNBr [24] and BNPS-skatole [25] digests of purified 14-3-3 isoforms. Samples for cleavage with CNBr were reduced with 0.2 M 2-mercaptoethanol (2 h at 30°C), lyophilised and redissolved in 50% v/v formic acid containing CNBr (approximately 100-fold excess over methionine residues). The samples were incubated under nitrogen for 24 h at 4°C in the dark. The solution was diluted to 5% formic acid and lyophilised. BNPS-skatole digestion was carried out in the presence of a 100-fold excess of reagent in glacial acetic acid for 4 h at 47°C. The reagent was removed by ether extraction.

2.8.2. Peptide purification and sequencing

Peptides were fractionated on HPLC using a water/ acetonitrile gradient (0–50% in 0.1% v/v TFA) on a Vydac reverse-phase C_{18} column (25 × 0.46 cm). Peaks (A_{215}) were collected, concentrated and the peptides sequenced on Applied Biosystems 470A gas-phase and 477A pulsed liquid-phase protein sequencers with Applied Biosystems 120A on-line phenylthiohydantoin (PTH)-amino acid analysers [26]. Data collection and analysis were performed with an Applied Biosystems 900A module calibrated with 25 pmol PTH-amino acid standards.

2.9. *Fast atom bombardment mass spectrometry*

Structural analysis of KCIP 1 peptides was performed by fast atom bombardment mass spectrometry on a VG-70 250SE mass spectrometer [27]. Peptides were air dried to 1–2 μl on the stainless steel probe and mixed with 1 μl of thioglycerol matrix containing 1% v/v TFA. Spectra were recorded using multi channel analysis in the positive ion

mode on a VG 70–250 SE mass spectrometer at an operating voltage of 8 kV using a 30 kV caesium ion gun. The results are expressed in average mass units (amu).

Mass spectrometry of intact 14-3-3 proteins was carried out on a VG electrospray mass spectrometer.

2.10. *Acetylation with acetic anhydride*

Recombinant 14-3-3 ε isoform was electroblotted from multiple lanes of an SDS PAGE gel onto Problott (PVDF membrane), as described above and the blot was divided into two sections. Both were placed in 400 ml beakers, wetted with methanol and 5 ml of 50 mM Na_2HPO_4 (pH 8) added to each.

25 μl acetic anhydride (Sigma) were added to one section and the beaker shaken for 5 mins. After washing with high purity water the electroblot was rewetted with methanol and the procedure repeated three times. The conditions used permit a lower degree of acetylation of the ε-amino group of lysine residues although in the present study lysine was fortuitously present only at the C-terminus of the ε14-3-3

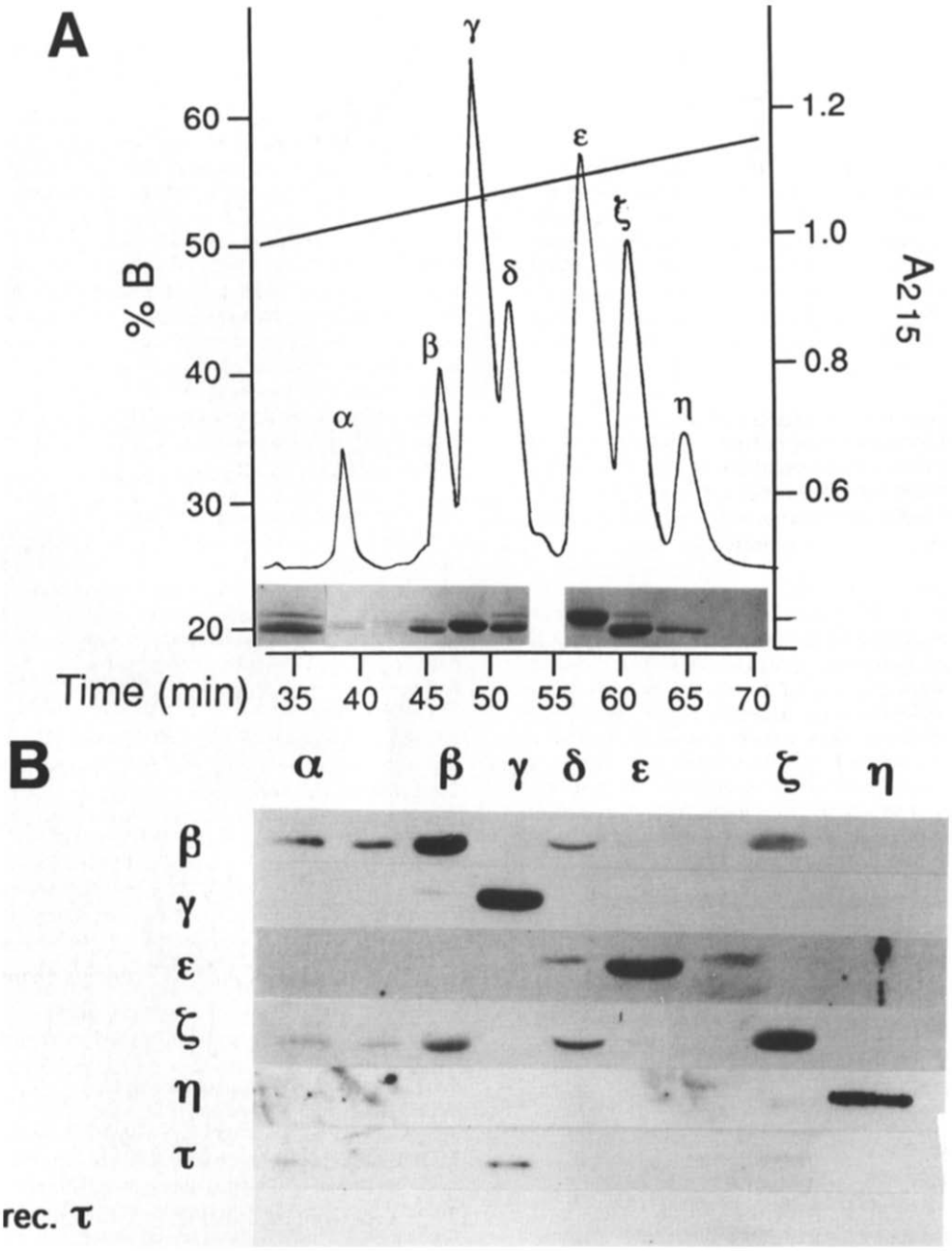

Fig. 1 Specificity of 14-3-3 antisera. (A) Elution profile of a pure preparation of sheep 14-3-3 from the reverse-phase HPLC column as described in Section 2. The diagonal line represents the acetonitrile gradient. The inset underneath the HPLC profile is a 12.5% SDS PAGE analysis of each protein peak. The left lane contains a mixture of brain isoforms. Proteins were visualised by coomassie blue staining. (B) Western blots of the HPLC peaks shown in Fig. 1A. The Greek letters across the top of the strips denote the major 14-3-3 isoform eluted in the peak. The strip was incubated in turn with the antisera raised against the particular amino-terminal peptide sequence α to η in descending order. The T-cell (τ) protein was probed using a separate blot from SDS PAGE of 3 μg of protein from each HPLC peak. This gel included a similar 3 μg loading of recombinant T-cell protein (rec. τ). This control verified that τ isoform elutes between γ and δ 14-3-3 but is not a major isoform in brain. (Depending on the exact timing of the manual collection of the peaks τ isoform is sometimes detected only in the γ peak – as in the example shown here).

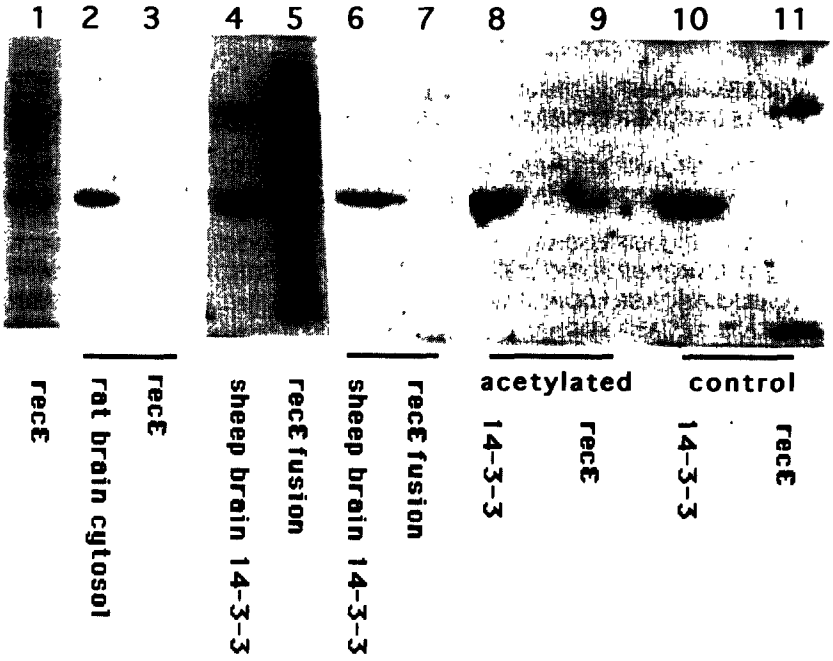

Fig. 2. Antisera raised against the N-terminus of 14-3-3 ε only recognises the acetylated protein. Lane 1 is a Coomassie blue-stained gel of partially purified recombinant 14-3-3 ε (rec ε) while lane 3 shows a Western blot of an identical sample. Lane 2 is a Western blot of crude rat brain cytosol as a control. Lanes 4 and 6 are SDS PAGE and Western blot of identical samples of purified sheep brain 14-3-3 while lanes 5 and 7 are identical samples of SDS PAGE and Western blots of recombinant ε 14-3-3 expressed as a fusion with maltose binding protein. This is indicated by the arrow. Lanes 8 and 10 are Western blots of a mixture of purified sheep brain 14-3-3. Lanes 9 and 11 are recombinant ε 14-3-3 protein blots. The portion of the blot in lanes 8 and 9 was treated with acetic anhydride as described in the text while lanes 10 and 11 were treated with a similar amount of acetic acid, as a control.

peptide against which the antisera were raised. This lysine would also have been involved in coupling with glutaraldehlyde. Acetylation of this lysine did not prevent recognition of the wild type protein by the antisera. As a control, to the other section of the blot, 100 μl of glacial acetic acid were added and this part treated as above (but without acetic anhydride).

2.11. *Amino acid analysis and protein concentration*

The concentration of the purified proteins was estimated by amino acid analysis (on a Beckman 121MB or a Beckman 6300 Analyser with post column ninhydrin detection), or by the Biorad protein concentration assay (according to manufacturers instructions).

3. RESULTS AND DISCUSSION

Fig. 1 shows western blots of HPLC fractions of sheep brain 14-3-3 proteins separated by reverse phase HPLC. These were probed with antisera raised against peptides corresponding to the β, γ, ζ, ε, η and T-cell (τ) isoforms of mammalian 14-3-3 proteins.

These antisera were quite specific for each isoform (with the exception of the α and δ HPLC peaks – see below). There was a low level of cross reaction between β and ζ antisera. The N-termini of these isoforms differ only in a Ser→Asn substitution. Nevertheless the amino terminal region of 14-3-3 isoforms proved to be a useful part of the sequence against which specific antisera could be raised. Previously, high titre antibodies have proved difficult to obtain against either whole 14-3-3 proteins or synthetic peptides based on other specific regions of the primary structure. Antisera raised against a peptide synthesised according to the amino terminal region of the σ isoform [19,20] did not cross-react with any brain isoform, while there was a strong response in Western blots to a ca. 30,000 Da protein in cytosolic extracts from the epithelial HEp-2 human laryngeal carcinoma cell line at an antibody dilution of $^1/_{3,000}$ (data not shown).

The antiserum raised against the amino terminus of the τ isoform recognised a minor component eluting between the γ and δ peaks. This was a small part of the total antigenicity of mixed isoforms from brain while the antisera strongly recognised a similar level of recombinant τ protein (Fig. 1B). In this case at least some of the population of polyclonal antibodies did not require the N-acetyl-group, which (like recombinant ε 14-3-3) was absent. In contrast the ε antisera only recognise the protein when it is acetylated (Fig. 2) and indicates the importance of including the correct structure of a peptide if the amino-terminal region is used as an epitope to raise antibodies. Even the fusion protein is not recognised although it contains a glycine residue (structurally very similar) at the position of the N-acetylation on the wild type protein. Recombinant ζ 14-3-3 (not-*N*-acetylated) is also not recognised by its antiserum (data not shown). In the literature there is widespread and incorrect translation of c-DNA sequences into protein sequences without assessing the possible co- or post-trans-

A

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     1                                                                  65
α    AcMDKSELVQKAKLAEQAERYDDMAAAMKAVTEQGHELSNEE                    WRVISS
β   AcTMDKSELVQKAKLAEQAERYDDMAAAMKAVTEQGHELSNEERNLLSVAYKNVVGARRSSWRVISS

δ    AcMDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNLLSVAYKNVVGArrSSWRVVSS
ζ    AcMDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNLLSVAYKNVVGARRSSWRVVSS

γ    AcVDREQLVQKARLAEQAERYDDMAAAMKNVTELNEPLSNEERNLLSVAYKNVVGARRSSWRVISS
ε   AcMDDREDLVYQAKLAEQAERYDEMVESMKKVAGMDVELTVEERNLLSVAYKNVIGARRASWRIISS
η    AcGDREQLLQRARLAEQAERYDDMASAMKAVTELNEPLSNEDRNLLSVAYKNVVGARRSSWRVISS
σ    AcMERASLIQKAKLAEQAERYEDMAAFMKGAVEKGEELSCEERNLLSVAYKNVVGGQRAAWRVLSS
τ    AcMEKTELIQKAKLAEQAERYDDMATCMKAVTEQGAELSNEERNLLSVAYKNVVGGRRSAWRVISS

     66                                                                130
α     IEQKTE..RNEKK QMGKEYREKIEAELQDI                              MKGDY
β     IEQKTE..RNEKKQQMGKEYREKIEAELQDICNDVLQLLDKYLIPNATQP..ESKVFYLKMKGDY

δ     IEQKTE..GAEK                                                 MKGDY
ζ     IEQKTE..GAEKKQQMAREYREKIETELRDICNDVLSLLEKFLIPNASQA..ESKVFYLKMKGDY

γ     IEQKTSADGNEKKIEMVRAYREKIEKELEAVCQDVLSLLDNYLIKNCSETQIESKVFYLKMKGDY
ε     IEQKEENKGGEDKLKMIREYRQMVETELKLICCDILDVLDKHLIPAANTG..ESKVFYYKMKGDY
η     IEQKTMADGNEKKLEKVKAYREKIEKELETVCNDVLALLDKFLIKNCNDFQYESKVFYLKMKGDY
σ     IEQKSNEEGSEEKGPEVREYREKVETELQGVCDTVLGLLDSHLIKEAGDA..ESRVFYLKMKGDY
τ     IEQKT..DTSDKKLQLIKDYREKVESELRSICTTVLELLDKYLIANATNP..ESKVFYLKMKGDY

    131                                                               195
α     FRYLSEVASGDNKQTTVSNSQQAYQEAFEIS   MQPTHPIRLGLALNFS
β     FRYLSEVASGDNKQTTVSNSQQAYQEAFEISKKEMQPTHPIRLGLALNFSVFYYEILNSPEKACS

δ     YRYLAEVAAGDDKKGIVDQSQQAYQEAFEISKKEMQPTHPIRLGLALNFSVFYYEILNSPEkA
ζ     YRYLAEVAAGDDKKGIVDQSQQAYQEAFEISKKEMQPTHPIRLGLALNFSVFYYEILNSPEKACS

γ     YRYLAEVATGEKRATVVESSEKAYSEAHEISKEHMQPTHPIRLGLALNYSVFYYEIQNAPEQACH
ε     HRYLAEFATGNDRKEAAENSLVAYKAASDIAMTELPPTHPIRLGLALNFSVFYYEILNSPDRACR
η     YRYLAEVASGEKKNSVVEASEAAYKEAFEISKEHMQPTHPIRLGLALNFSVFYYEIQNAPEQACL
σ     YRYLAEVATGDDKKRIIDSARSAYQEAMDISKKEMPPTNPIRLGLALNFSVFHYEIANSPEEAIS
τ     FRYLAEVACGDDRKQTIDNSQGAYQEAFDISKKEMQPTHPIRLGLALNFSVFYYEILNNPELACT

    196                                                               257
α                                          WTsENQGDegda
β     LAKTAFDEAIAELDTLNEESYKDSTLIMQLLRDNLTLWTSENQGDEGDAGEGEN

δ                                    MQLLRDNLTLWTSDTQGDEA
ζ     LAKTAFDEAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDTQGDEAEAGEGGEN

γ     LAKTAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDQQDDDG..GEGN
ε     LAKAAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTLWTSDMQGDGEEQNKEALQDVEDENQ
η     LAKQAFDDAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDQQ.DE.EAGEGN
σ     LAKTTFDEAMADLHTLSEDSYKDSTLIMQLLRDNLTLWTADNAGEEG..GEAPQEPQS
τ     LAKTAFDEAIAELDTLNEDSYKDSTLIMQLLRDNLTLWTSDSAGEECDAAEGAEN
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Fig. 3. (A) Sequence alignment of α and δ 14-3-3 with the other mammalian 14-3-3 isoform sequences. Residues identical in all known mammalian 14-3-3 proteins are underlined. Bold characters in the single letter code indicate variable residues within an isoform (see text for details). Lower case letters in the α and δ sequences indicate tentative assignments. The sequences are from references [2,3,4,8,10,18,19,20,29,30]. Ac denotes N-acetyl group and the threonine suggested as the first residue in the β sequence is shown in italics. (B) Matrix of identities between the sequences shown in Fig. 2A. This compares only the identities between the isoforms in the regions that were sequenced for the α and/or δ isoforms. This also excludes residues that are completely conserved across the range of known 14-3-3 proteins. The figure shows a percentage comparison of identical residues for part of the sequence alignment shown in Fig. 2A. 81 residues from the α sequence and 80 residues from the δ sequence were compared to the other isoforms. *71 residues from the δ sequence were compared to α (see Fig. 2A).

B

	α	β	γ	ε	δ	ζ	η	σ	τ
α	100	100	46	26	66*	65	48	33	58
β		100	47	29	63	70	49	35	59
γ			100	26	43	48	74	31	35
ε				100	30	34	24	22	25
δ					100	100	52	42	63
ζ						100	49	43	59
η							100	28	40
σ								100	42
τ									100

Fig. 3. *(continued)*

lational processing that may occur. This depends on the nature of the residue adjacent to the indicator methionine. This methionine is generally acetylated, the N-acetyl-Met may then be removed and the next residue may or may not be acetylated [28]. Alternatively an exposed glutamine may cyclise to pyroglutamic acid or a glycine may be myristoylated (or undergo other fatty acyl modification).

Two of the antisera cross-reacted with two additional isoforms that elute at distinct positions on reverse phase HPLC. These were α, which is probably identical to β isoform and δ which is probably identical to ζ isoform. Purified preparations of α and δ isoforms separated by reverse-phase HPLC (Fig. 1A) were cleaved with CNBr and BNPS-skatole. Peptides fractionated by HPLC were analysed by direct protein sequencing and primary structures aligned according to 14-3-3 protein [4]. 138 residues of α 14-3-3 were sequenced and 57 of these are absolutely conserved between known mammalian sequences (Fig. 3). Comparison was therefore made with the other 81 residues. The matrix (Fig. 3B) was plotted from the programme 'distances' in the GCG sequence analysis programme using modified parameters which allows only identical residues to be scored. The figure shows the α isoform to be identical to β in 81 out 81 positions that were compared while the identities between other isoforms of 14-3-3 vary from 26 to 66%. Similarly, 162 amino acids from δ 14-3-3 were sequenced and 80 residues were compared to the other isoforms. There was 100% identity with the ζ sequence but 30 to 66% identity with the other isoforms. The ζ isoform has been sequenced from sheep brain protein in addition to having been cloned and sequenced from rat, bovine and human species. These are all identical in primary structure – which reflects the general high level of sequence conservation of each isoform. The only sequence variations are Arg^{112} and Pro^{115} in rat ζ while the mammalian ζ sequences from the other species have Ala^{112} and Ala^{115}. In other mammalian 14-3-3 isoforms that have been sequenced from more than one species, bovine and rat η contain Ala^{102}, Asn^{144} and His^{164} while these are Ser^{102} and Gln^{164} in human η. Residue 144 is variable in human 14-3-3 η. It is asparagine according to the cDNA sequenced by Ichimura-Ohshima et al. [29] or threonine according to Leffers et al. [20]. Human stratifin σ 14-3-3 isoform [20] has been sequenced in another laboratory and has been named HME1 [19]. A variation of Val^{247} in the mammary gland protein (HME1) which is Ala^{247} in stratifin has been reported. A possible explanation for the apparent identity of α and β isoforms is that an alternative initiator methionine, two codons upstream gives rise to a slightly more hydrophobic form. The group of Celis and coworkers have cloned and sequenced a 14-3-3 isoform (called IEF1054, [20]) which is undoubtedly the human equivalent of mammalian 14-3-3 β. It is identical in sequence to bovine β 14-3-3 [30] with the exception of Glu^{102} in human, Gln^{102} in bovine) but these authors suggest that the upstream methionine codon is the initiator. Neither of these codons is surrounded by a perfect consensus sequence for initiation [31]. Therefore the protein starting Ac.MDKSEL.....may be the α isoform while the β isoform commences Ac.TMDKSEL..... In these two cases the consensus processing of the initiator

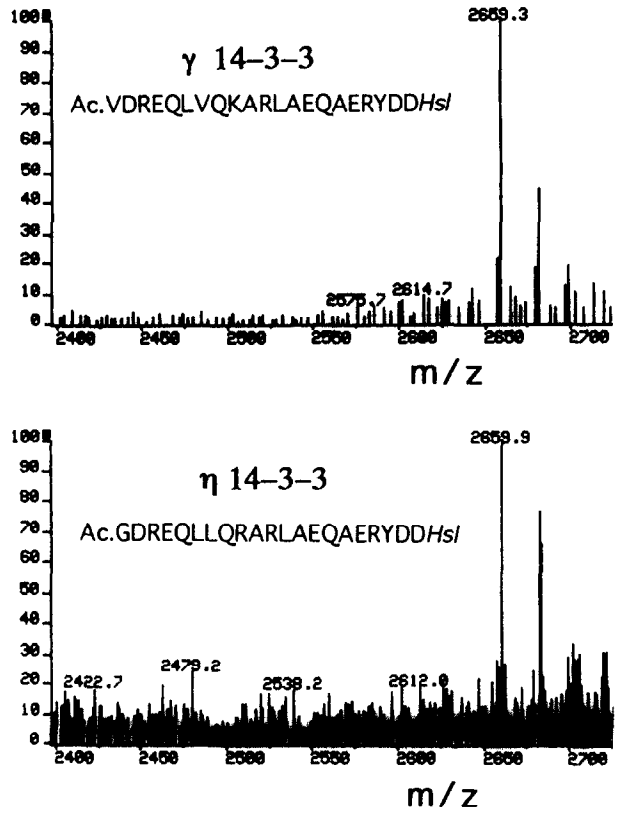

Fig. 4. FAB MS of the amino terminal cyanogen bromide peptides obtained from γ and η isoforms of 14-3-3 protein. By coincidence, both have a pseudomolecular ion mass (M+H)+ at 2659 for the homoserine lactone (Hsl) form. The amino acid analyses (which are quite distinct, not shown) clearly prevents confusion as to their identity. The peaks at higher mass are sodium and potassium adducts.

methionine is assumed to be occuring [28,32]. In both isoforms on digestion with CNBr a peptide commencing KDSEL..... will be formed. In the γ and η isoforms, with Val and Gly as the second residues, respectively, the acetylated initiator methionine undergoes further co-translational processing. It is removed and the Val and Gly undergo a further round of acetylation. This is verified by fast atom bombardment mass spectrometry of the N-terminal CNBr peptides (Fig 4). Since the complete amino acid sequence of sheep β 14-3-3 has not yet been determined, the exact molecular weight is unknown. However, it is likely to be as highly conserved as the counterparts of other isoforms from a range of mammalian species. We have determined the sequence of peptides corresponding to more than 70% of sheep β 14-3-3 (unpublished results) and can detect no sequence variations from *bovine* β 14-3-3 [30]. Electrospray mass spectrometry suggests that the molecular weight of sheep β 14-3-3 is approximately 96 Da higher than that of α 14-3-3 (data not shown, the residue weight of Thr is 101.1). Future work will focus on the exact structural differences between α/β and δ/ζ isoforms which may in fact be due to a sub-stoichiometric post-translational modification. This could explain the ca. 8 min differences in elution position on the shallow HPLC gradient between each pair of proteins.

The role for N-acetylation of proteins may be protection against proteolysis and when proteins have been expressed in acetylated or non-acetylated forms the cellular half life of the former is longer [32]. Whatever the biological role for co-translational acetylation, the importance of taking into account the correct biochemical moiety at the amino-terminus of a protein has been emphasised by the present findings especially if one were to use these antibodies to screen expression libraries. The antibodies described in the present study will be used to probe the location of specific isoforms in organelles or sub-cellular distribution of specific 14-3-3 isoforms.

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