Characterization of the Aα and Aβ subunit isoforms of protein phosphatase 2A: differences in expression, subunit interaction, and evolution

Jin ZHOU, Huong T. PHAM, Ralf RUEDIGER and Gernot WALTER[1]

Department of Pathology, University of California at San Diego, La Jolla, CA 92093, U.S.A.

Protein phosphatase 2A (PP2A) is very versatile owing to a large number of regulatory subunits and its ability to interact with numerous other proteins. The regulatory A subunit exists as two closely related isoforms designated Aα and Aβ. Mutations have been found in both isoforms in a variety of human cancers. Although Aα has been intensely studied, little is known about Aβ. We generated Aβ-specific antibodies and determined the cell cycle expression, subcellular distribution, and metabolic stability of Aβ in comparison with Aα. Both forms were expressed at constant levels throughout the cell cycle, but Aα was expressed at a much higher level than Aβ. Both forms were found predominantly in the cytoplasm, and both had a half-life of approx. 10 h. However, Aα and Aβ differed substantially in their expression patterns in normal tissues and in tumour cell lines. Whereas Aα was expressed at similarly high levels in all tissues and cell lines, Aβ expression varied greatly. In addition, *in vivo* studies with epitope-tagged Aα and Aβ subunits demonstrated that Aβ is a markedly weaker binder of regulatory B and catalytic C subunits than Aα. Construction of phylogenetic trees revealed that the conservation of Aα during the evolution of mammals is extraordinarily high in comparison with both Aβ and cytochrome *c*, suggesting that Aα is involved in more protein–protein interactions than Aβ. We also measured the binding of polyoma virus middle tumour antigen and simian virus 40 (SV40) small tumour antigen to Aα and Aβ. Whereas both isoforms bound polyoma virus middle tumour antigen equally well, only Aα bound SV40 small tumour antigen.

Key words: cell cycle, half-life, phylogenetic tree, PP2A, T antigen.

INTRODUCTION

Protein phosphatase 2A (PP2A) exists in cells in two major forms, core enzyme and holoenzyme. The core enzyme is composed of a 36 kDa catalytic C subunit and a 65 kDa regulatory A subunit. The holoenzyme is composed of the core enzyme to which one of several regulatory B subunits is bound (reviewed in [1,2]). The A subunit exists in two forms, Aα and Aβ, which are 86 % identical [3,4]. Both forms were found to be mutated in a variety of human cancers [5–9]. The C subunit also exists in two forms, Cα and Cβ, that are 96 % identical. The B subunits fall into four families designated B, B′ (also called B56), B″ and B‴. The B family has four members, Bα, Bβ, Bγ, and Bδ, each with a molecular mass of approx. 55 kDa. The B′ family consists of numerous isoforms and splice variants, whose molecular masses range from 54 to 68 kDa. The B″ family has four members, which have molecular masses of 48 kDa (PR48), 59 kDa (PR59), 72 kDa (PR72) and 130 kDa (PR130). The latter two are splice variants of the same gene. The B‴ family has two members (reviewed in [2]). Another class of proteins able to associate with the PP2A core enzyme are the tumour (T) antigens encoded by polyoma viruses, which have an important role in neoplastic transformation [10,11]. The combination of all subunits (not including T antigens) could give rise, in theory, to over 70 different forms of holoenzymes, indicating that PP2A is highly regulated and has a regulatory role. However, whether or not all possible forms of PP2A actually exist in cells has not been demonstrated. In fact, the demonstration in the present paper that holoenzymes containing Aβ and Bα do not form reduces the number of potential holoenzymes. The ability of PP2A to associate with 40 other cellular proteins suggests that it regulates many cellular processes (reviewed in [2]).

A model of the PP2A holoenzyme and of complexes of the core enzyme with T antigens is shown in Figure 1. The Aα subunit consists of 15 non-identical repeats. Each repeat is composed of two α-helices connected by an intra-repeat loop. We have shown that the intra-repeat loops have a major role in binding B and C subunits as well as T antigens. Adjacent repeats are connected by inter-repeat loops. Collectively, the repeats form an extended molecule that is stabilized by hydrophobic interactions. The B subunits bind to repeats 1–10, the T antigens within repeats 2–8, and the C subunits to repeats 11–15 [12,13].

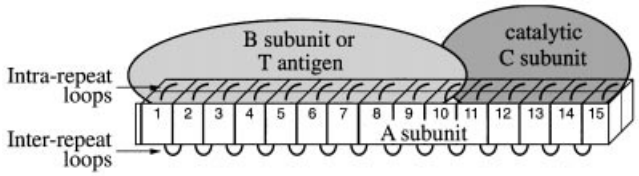

Figure 1 Model of PP2A holoenzymes

The A subunits consist of 15 non-identical repeats. Each repeat is composed of two α-helices connected by an intra-repeat loop which is involved in binding B and C subunits as well as T antigens. Adjacent repeats are connected by inter-repeat loops. The B subunits from three families (B, B′, B″) bind to repeats 1–10, polyoma virus mT and sT antigens bind to repeats 2–8, SV40 sT binds to repeats 3–6, and the C subunits bind to repeats 11–15 of the Aα subunit.

Abbreviations used: DTT, dithiothreitol; E, embryonic day; EE, EEEEYMPME peptide; GM, grey matter; HA, haemagglutinin; KT3, KPPTPPPEPET peptide; MAb, monoclonal antibody; mT, middle T; PP2A, protein phosphatase 2A; sT, small T; SV40, simian virus 40; T antigen, tumour antigen; WM, white matter.

[1] To whom correspondence should be addressed (e-mail GWalter@ucsd.edu).

The genes encoding subunits of human PP2A have been assigned the following names in the GenBank® database: PPP2C encoding the C subunit; PPP2R1 encoding A/R1/PR65; PPP2R2 encoding B/R2/PR55; PPP2R3 encoding B″/R3/PR72; PPP2R5 encoding B′/R5/PR61/B56. For example, PPP2R5γ encodes B′α1/R5γ/PR61γ/B56γ3.

The X-ray structure of the human Aα subunit confirmed our model of the Aα subunit in regard to its α-helical repeat structure and the presence of intra- and inter-repeat loops [14]. It was puzzling that the different B-subunit families seemed unrelated by amino acid sequence, in spite of the fact that all B subunits bind to the same region of the A subunit. However, Li and Virshup [15] recently identified two A-subunit-binding domains in B, B′ and B″ subunits that contained a limited number of conserved residues that are likely to be involved in A-subunit binding.

Although both core enzymes and holoenzymes containing the Aα subunit have been studied in great detail, very little is known about Aβ-containing enzymes, in part because Aβ is expressed at much lower levels than Aα [4]. It appears probable that Aα and Aβ have unique functions, as indicated by the fact that a developmental switch from Aβ to Aα expression takes place during early *Xenopus* development, suggesting that the Aα- and Aβ-containing holoenzymes are involved in different developmental programmes [16]. In addition, the N-terminus of Aβ has an extension of 12 amino acids that is missing in Aα [5]. It seems likely that this extension fulfils a specific function. Furthermore, Aα and Aβ differ in their ability to bind B and C subunits [9]. In order to gather more information about Aβ, we generated Aβ-specific antibodies against the N-terminal extension. Using these antibodies, we determined the cell-cycle expression, subcellular distribution and metabolic stability of Aβ in comparison with Aα and found no qualitative differences between the two isoforms. On the other hand, the patterns of Aβ expression in normal human tissues and in tumour cell lines differed considerably from the patterns of Aα expression. Whereas the level of Aα was high in all tissues examined, the level of Aβ was very low in all tissues except those of the testes. In tumour cell lines, Aα levels were also high and similar between lines, whereas Aβ levels varied greatly. We also developed an *in vivo* binding assay for measuring subunit interactions. Using this assay, we demonstrated that Aα and Aβ have markedly different B- and C-subunit-binding properties. Both isoforms bound polyoma virus middle T (mT) antigen equally well but differed in binding simian virus 40 (SV40) small T (sT) antigen. An analysis of their evolutionary history revealed that Aα and Aβ changed at different rates, consistent with the idea that they have different functions.

MATERIALS AND METHODS

Antibodies

Polyclonal anti-Aβ serum was raised in rabbits against a peptide corresponding to the first 12 amino acids of human Aβ (acetyl-MAGASELGTGPGK). The lysine was added for conjugation to keyhole limpet haemocyanin using glutaraldehyde (Sigma GenoSys). The commercially available antibody against the Aβ subunit from Santa Cruz Biotechnology (PP2A-Aβ, N-20, catalogue number sc-8705) did not recognize Aβ in our experiments. Rat monoclonal antibody (MAb) 6F9 anti-Aα, rat MAb 6G3 anti-Aα/Aβ, mouse MAb anti-EE (where EE is the peptide EEEEYMPME), and mouse MAb anti-KT3 (where KT3 is the peptide KPPTPPPEPET) were described in [9,17]. The IgG fraction of each of these antibodies was isolated by standard procedures using protein G–Sepharose CL-6B beads (GammaBind Plus from Amersham Biosciences), or protein A–Sepharose 4B beads in the case of anti-Aβ [18]. In the case of anti-Aβ, peptide-specific antibodies were affinity-purified using peptide coupled with glutaraldehyde to aminohexane–Sepharose 4B beads (Amersham Biosciences). Mouse anti-(C subunit) [anti-(Cα/Cβ)] MAb was obtained from Marc Mumby. Mouse MAb 419 anti-(SV40 small and large T) (anti-sT) was obtained from Kathy Rundell. Mouse MAb anti-haemagglutinin (anti-HA) was purchased from Roche.

Human cell lines

The human glioma cell lines LN428, U138MG, U373MG, U251MG, LNZ308, A1207, U87MG, T98G, LN319, and LN229 were generously donated by Webster Cavanee at the Ludwig Institute for Cancer Research, University of California at San Diego. Cell lines LN428, LNZ308, LN319, and LN229 were originally obtained from Nicholas de Tribolet [19]. The human lung, breast, cervical and colon cancer cell lines, as well as the adenovirus-transformed primary HEK-293 cells, were obtained from the American Type Culture Collection. The above cell lines were grown in Dulbecco's modified Eagle's medium with 10 % (v/v) foetal bovine serum. Primary normal human bronchial epithelial (NHBE) cells (with retinoic acid) and primary normal human mammary epithelial cells (HMEC) were purchased from Clonetics (BioWhittaker) and grown as described by the manufacturer.

Normal human organ lysates

White matter (WM) and grey matter (GM) of normal (non-cancerous) human brain from an epileptic patient were generously provided by Otmar Wiestler and lysed as described previously (samples 3501, WM, and 3500, GM) [20]. A colon protein extract (ReadyLysate) was purchased from DNA Technologies. Heart, liver, lung, kidney, ovary and testis protein extracts (ChemiLysates) were purchased from Chemicon International.

Western blotting

To measure Aα- and Aβ-subunit levels in whole cell lysates, cells were grown on 10 cm diameter dishes to approx. 70 % confluence, washed with cold PBS and lysed with 500 μl of SDS/PAGE sample buffer [2 % SDS, 100 mM dithiothreitol (DTT), 60 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol and 0.01 % Bromophenol Blue]. Protein concentrations of the cell lysates were determined after precipitation with trichloroacetic acid using the Micro BCA (bicinchoninic acid) protein assay from Pierce. For each lane of 10 % SDS/PAGE gels, 10 μg of total cell lysate was loaded, transferred on to PVDF Immobilon-P membranes (Millipore), and immunoblotted with primary antibodies as indicated on the figures, followed by appropriate secondary antibodies labelled with horseradish peroxidase (Jackson ImmunoResearch), and application of an enhanced chemiluminescence detection system (Western Lightning from PerkinElmer).

Plasmid construction

Bα and B′α1 cDNAs were gifts from Marc Mumby, while B″/PR72 cDNA was generously provided by Brian Hemmings. The entire coding regions of these cDNAs were amplified by PCR using primers designed so that (i) the start methionine was directly preceded by a *Kpn*I site (Bα) or an *Eco*RV site (B′α1 and B″/PR72), and (ii) the stop codon was preceded by a sequence encoding the KT3 tag derived from the C-terminus of SV40 large T (KPPTPPPEPET) and followed by an *Eco*RI site (Bα) or an *Xho*I site (B′α1 and B″/PR72). The DNA fragments were then cloned into pcDNA3 to generate pcDNA3-$B\alpha^{KT3}$, pcDNA3-$B'\alpha1^{KT3}$ and pcDNA3-$B''/PR72^{KT3}$.

Cα cDNA was generously provided by Marc Mumby. Cβ cDNA was generated by site-directed mutagenesis of Cα cDNA using Promega's Gene Editor kit. The entire coding regions of the Cα and Cβ cDNAs were then amplified by PCR using primers designed so that (i) the start methionine was preceded by

a *Kpn*I site and followed by sequence encoding the HA tag (YPYDVPDYA), and (ii) the stop codon was followed by an *Eco*RI site. Finally, the DNA fragments were cloned into pcDNA3 to generate pcDNA3-$^{HA}C\alpha$ and pcDNA3-$^{HA}C\beta$. All constructs were checked by sequencing.

C-terminally EE-tagged wild-type Aα and Aβ as well as Aα mutants were described in [8,9].

An expression vector for SV40 sT antigen, pCEP-sT, was generously provided by Kathy Rundell, and an expression vector for polyoma virus mT antigen, pRSV-mT, was generously donated by Walter Eckhart.

Transfection of HEK-293 cells

Per 10 cm diameter dish, 10^6 HEK-293 cells were plated, grown for 24 h and transfected with a total of 3.9 μg of plasmid DNA using 30 μl of Lipofectamine and 20 μl of PLUS reagent following Invitrogen's instructions. Transfection conditions were optimized for (i) high and similar expression levels of EE-tagged Aα and Aβ, as determined by Western blotting with anti-EE antibodies, (ii) high expression of co-transfected tagged B or C subunits or T antigens, and (iii) high transfection efficiency as determined by staining with X-gal of fixed cells that were co-transfected with a β-galactosidase vector. The cells were usually harvested 48 h after transfection either with SDS/PAGE sample buffer for Western blotting or with TX-100 buffer (0.5 % Triton X-100, 50 mM Tris/HCl, pH 7.5, 150 mM NaCl) for immunoprecipitation.

Immunoprecipitation

Transfected HEK-293 cells on 10 cm diameter dishes were washed twice with cold PBS, placed on ice-cold water, and extracted for 10 min with 450 μl of cold TX-100 buffer containing 3 mM $MgCl_2$, 1 mM DTT and 50 μM leupeptin. The extracts were centrifuged at 14000 ***g*** and 4 °C for 5 min. The cleared supernatants were split into two aliquots and were incubated in the presence or absence of competing peptide with 10 μl of protein A or G beads (see above). The protein A beads contained anti-Aβ and the protein G beads contained anti-EE or 6F9 anti-Aα antibody covalently bound by dimethylpimelimidate. After 1 h of rotation along the tube axes at room temperature (25 °C), the immune complexes were washed three times with TX-100 buffer and boiled in 40 μl of SDS/PAGE sample buffer, of which 20 μl was resolved by SDS/PAGE (10 % gels) followed by Western blotting. In the case of mT immunoprecipitations, a mixture of buffers was used, instead of TX-100 buffer, consisting of two parts of TX-100 buffer and one part of radioimmunoprecipitation (RIPA) buffer (1 % Triton X-100, 0.5 % deoxycholate, 0.1 % SDS, 50 mM Tris/HCl, pH 8.0 and 150 mM NaCl). mT was immunoprecipitated with hamster anti-tumour serum that was not covalently bound to beads.

Immunodepletion

Protein A beads were incubated with anti-Aβ antibodies, and protein G beads were incubated with 6F9 anti-Aα IgG. The amounts of antibodies exceeded the binding capacity of the beads to achieve a high density of antibodies on the beads. Covalent coupling was performed as described above. NCI-H460 cells at 50 % confluence on 10 cm diameter dishes were lysed with 400 μl of TX-100 buffer. The cleared lysate was split into four aliquots and incubated with 30 μl of anti-Aβ or 6F9 anti-Aα beads, or plain protein A or G beads (mock depletions). To deplete highly abundant Aα, two rounds of depletion were performed with 6F9 beads. After centrifugation, the Aα- or Aβ-depleted supernatants were recovered and subjected to SDS/PAGE and Western blotting.

Cell synchronization and FACS

NCI-H460 cells were plated at 6×10^5 cells per 10 cm diameter dish and grown for 24 h. To obtain S phase cells, a double thymidine block was performed: the cells were arrested in G1/S with 2 mM thymidine for 24 h, released from the block with fresh medium for 6 h, again arrested with 2 mM thymidine for 27 h and released again with fresh medium for harvest 3 h later when they reached S phase. Mitotic cells were obtained by blocking with 40 ng/ml nocodazole for 16 h; rounded-up cells were harvested by shaking them off the dish. To synchronize in G1, cells were first blocked with 2 mM thymidine for 28 h, released with fresh medium for 3 h and then blocked with 40 ng/ml nocodazole for 16 h. Rounded-up cells were collected by shaking them off the dish. They were released from their mitotic block with fresh medium for 9 h to progress to G1. For harvesting, the cells were washed twice with PBS, either on the dish for S and G1 cells or by resuspending them in PBS for M phase cells. To prepare whole cell lysates, 500 μl of SDS/PAGE sample buffer were added per dish. To prepare cytoplasmic extracts, 500 μl of cold TX-100 buffer containing 3 mM $MgCl_2$, 1 mM DTT and 50 μM leupeptin were added for 10 min, then removed from the plate and centrifuged at 14000 ***g*** and 4 °C for 5 min. To the cleared supernatant, 100 μl of 6× concentrated SDS/PAGE sample buffer was added. The nuclei and cytoskeletons that remained on the dish after cytoplasmic extraction were lysed with 300 μl of SDS/PAGE sample buffer. Cell-cycle synchronization was verified by FACS. Approx. 10^6 cells were trypsinized, washed with PBS, resuspended in 2 ml of PBS and fixed by dropwise addition of 2 ml of 100 % ethanol during gentle mixing. The fixed cells were analysed on an Epics Elite cell sorter (Coulter) using Multi Cycle software (Phoenix Flow Systems) by Judy Nordberg of the Flow Cytometry laboratory at the Veterans Affairs Hospital, La Jolla, CA, U.S.A.

Pulse–chase experiments

NCI-H460 cells were grown on 6 cm diameter dishes to 70 % confluence, incubated for 1 h in methionine-free medium containing 10 % dialysed bovine serum (Gibco), and pulsed for 1 h in 1 ml of fresh methionine-free medium containing 10 % dialysed foetal bovine serum and 250 μCi/ml of [^{35}S]methionine at a specific radioactivity of >1000 Ci/mmol (Amersham Biosciences). The cells were then chased in fresh complete medium for various time intervals and harvested with 300 μl of TX-100 buffer. The extracts were split into two aliquots and used to immunoprecipitate the ^{35}S-labelled Aα or Aβ subunits with 6F9 anti-Aα or anti-Aβ antibodies in the presence and absence of 200 μg (6F9) or 20 μg (anti-Aβ) of competing peptide. Following SDS/PAGE, the proteins were visualized and quantified by phosphorimaging. Values for each band were obtained by subtracting the counts of the corresponding peptide control background bands.

Phylogenetic analysis

Phylogenetic trees were constructed by progressive alignment of amino acid sequences using the program ProPack as described by Feng and Doolittle [21]. Sequences deposited under the following GenBank® accession numbers were used: NP_055040 (human Aα), AAC69624 (human Aβ), NP_058587 (mouse Aα), P54612 (pig Aα), P54613 (pig Aβ), S65953 (*Xenopus laevis* Aα), S65952 (*Xenopus laevis* Aβ), P36179 (*Drosophila melanogaster*

PR65), NP_498162 (*Caenorhabditis elegans* PP2A), AAB60713 (*Arabidopsis RCN1*), T44416 (*Schizosaccharomyces pombe* PP2A A chain), AAC04941 (*Saccharomyces cerevisiae* PP2A regulatory subunit A). We assembled the sequence for mouse Aβ using the following 14 expressed sequence tags: BG088452, BF164408, AA512085, AI596686, D21768, AI789475, AA461879, AI117417, AA726823, AI385987, BF181861, AA033221, BI149803 and BI663256. For tree construction, the A subunits were cropped corresponding to amino acids 4–589 of human Aα.

RESULTS

Cell-cycle expression, nuclear versus cytoplasmic distribution, and metabolic stability of Aβ

At the present time, the only information about Aβ-subunit expression is based on Northern blots [4] and direct measurements of Aβ-protein levels are lacking. In order to measure Aβ levels, antibodies against the unique N-terminus of Aβ were generated. Initially, we demonstrated that these antibodies recognize *in vitro* synthesized Aβ, whereas pre-immune serum did not (results not shown). The antibodies were then tested on cell lysates. As shown in Figure 2, anti-Aβ immunoprecipitated Aβ from HEK-293 cell extracts and recognized it on Western blots (panel a, lane 1). The immunoprecipitation of Aβ was inhibited by an excess of peptide (panel a, lane 2). As expected, the antibodies did not recognize Aα immunoprecipitated with Aα-specific antibodies (panel a, lane 3). The same Western blot was re-probed with

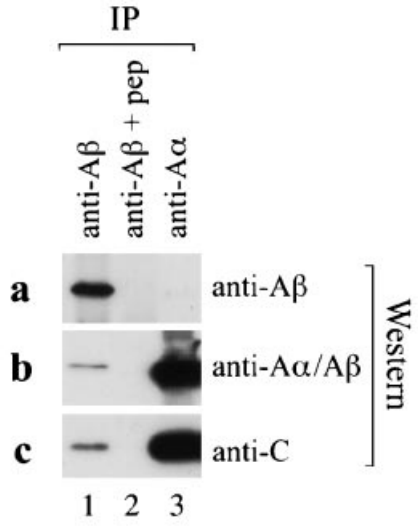

Figure 2 Immunoprecipitation and Western blotting with Aβ-specific anti-peptide antibodies

Using HEK-293 cell extracts, immunoprecipitations were carried out with peptide-purified anti-(Aβ peptide) antibodies in the absence (lane 1) or presence (lane 2) of competing peptide used for immunization, and with MAb 6F9 specific for Aα (lane 3). The immunoprecipitates were analysed by Western blotting with (**a**) anti-Aβ, (**b**) 6G3 MAb recognizing Aα and Aβ [anti-(Aα/Aβ)] and (**c**) anti-(C subunit) MAb.

Table 1 FACS analysis of synchronized NCI-H460 cells

Details of synchronization are described in the Materials and methods section. The bold numbers indicate the enrichment of cells in S, G2/M, and G1.

	% of cells in		
Sychronization for phase	S	G_2/M	G_1
S	**87**	0	13
M	14	**81**	5
G_1	23	18	**59**
Random	45	5	50

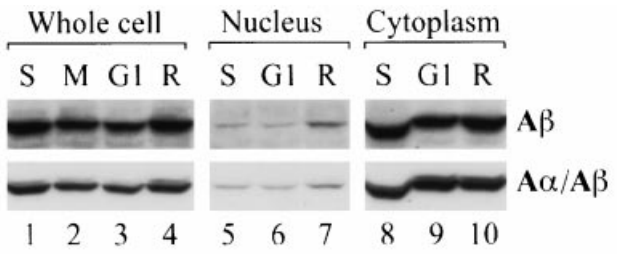

Figure 3 Constant expression of the Aα and Aβ subunits during the cell cycle; higher abundance of Aα and Aβ in the cytoplasm compared with the nucleus

NCI-H460 cells were synchronized in the S, M and G1 phases of the cell cycle, and whole cell lysates or cytoplasmic and nuclear extracts were prepared as described in the Materials and methods section (R, random, unsynchronized cells). In each lane, 6 μg of total protein was analysed by Western blotting with anti-Aβ antibodies (upper panel) or 6G3 anti-(Aα/Aβ) MAb (lower panel). Aβ is separated from Aα in the lower panel, as detected by a weak shadow above Aα.

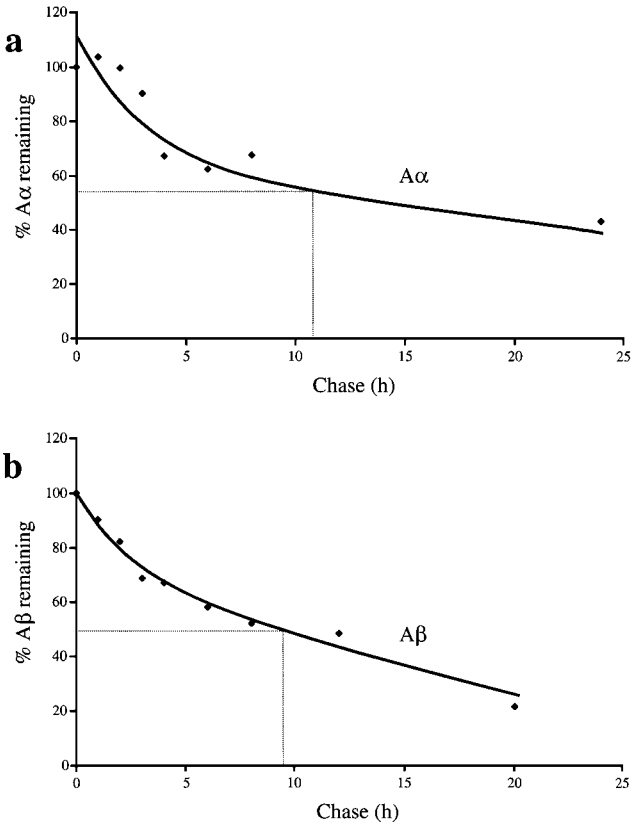

Figure 4 Aα and Aβ subunits have similar half-lives of about 10 h

Pulse–chase experiments were carried out with NCI-H460 cells as described in the Materials and methods section. Aα and Aβ were immunoprecipitated and quantified by phosphorimaging. The percentage of labelled Aα and Aβ remaining at each time point was calculated relative to the time zero chase. The data shown are representative of two experiments.

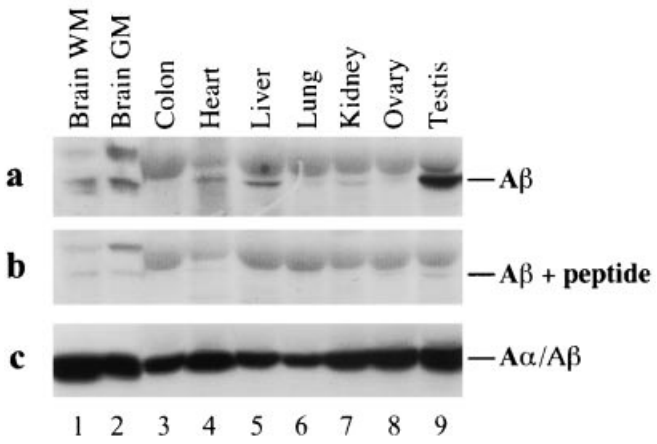

Figure 5 Differences between Aα and Aβ expression in normal organs from adult humans

Organ lysates (10 μg of total protein each), were analysed by Western blotting with anti-Aβ antibodies in the (**a**) absence or (**b**) presence of competing peptide, and with (**c**) 6G3 anti-(Aα/Aβ) MAb.

MAb 6G3, recognizing both Aα (panel b, lane 3) and Aβ (panel b, lane 1). Probing this blot with anti-(C subunit) antibodies showed that the C subunit was co-immunoprecipitated with both anti-Aα and anti-Aβ antibodies (panel c).

We showed previously that the expression of Aα is constant throughout the cell cycle [22]. To measure the cell-cycle expression of Aβ, we used the lung cancer cell line H460 because it expresses a relatively large amount of Aβ (see Figure 6, panel a, lane 3). Cells in S phase were obtained by release from a double-thymidine block, mitotic cells were generated by nocodazole treatment and G1 cells by release from a nocodazole block. The degree of synchrony as determined by FACS analysis is shown in Table 1. Whole cells, as well as nuclear and cytoplasmic fractions, were analysed by Western blotting with anti-Aβ antibodies. As shown in Figure 3, the same levels of Aβ were observed in synchronized (S, M, G1) and random (R) cells, whether whole cells (lanes 1–4) or nuclear (lanes 5–7) and cytoplasmic fractions (lanes 8–10) were analysed (upper panel). Very similar results were obtained with MAb 6G3, recognizing Aα and Aβ (lower panel). Since Aβ represents only about 10% of the total A-subunit amounts (see below), the signal obtained with 6G3 represents predominantly Aα. The nuclear fractions contained approx. 10-fold less Aβ and Aα than the cytoplasmic fractions.

Pulse–chase experiments were carried out to measure the half-life of Aα and Aβ in H460 cells. The cells were labelled with [^{35}S]methionine and chased for various lengths of time. Cytoplasmic extracts were prepared from which Aα and Aβ were immunoprecipitated. The precipitates were analysed by SDS/PAGE and quantified with a phosphorimager as described in the Materials and methods section. As shown in Figure 4, the half-lives of Aα and Aβ were approx. 10 h.

Levels of Aα and Aβ subunits in normal human tissues, human tumour cell lines and normal human primary epithelial cells

To measure Aβ-subunit levels in primary human tissues, the tissues were solubilized in SDS/PAGE sample buffer or lysates

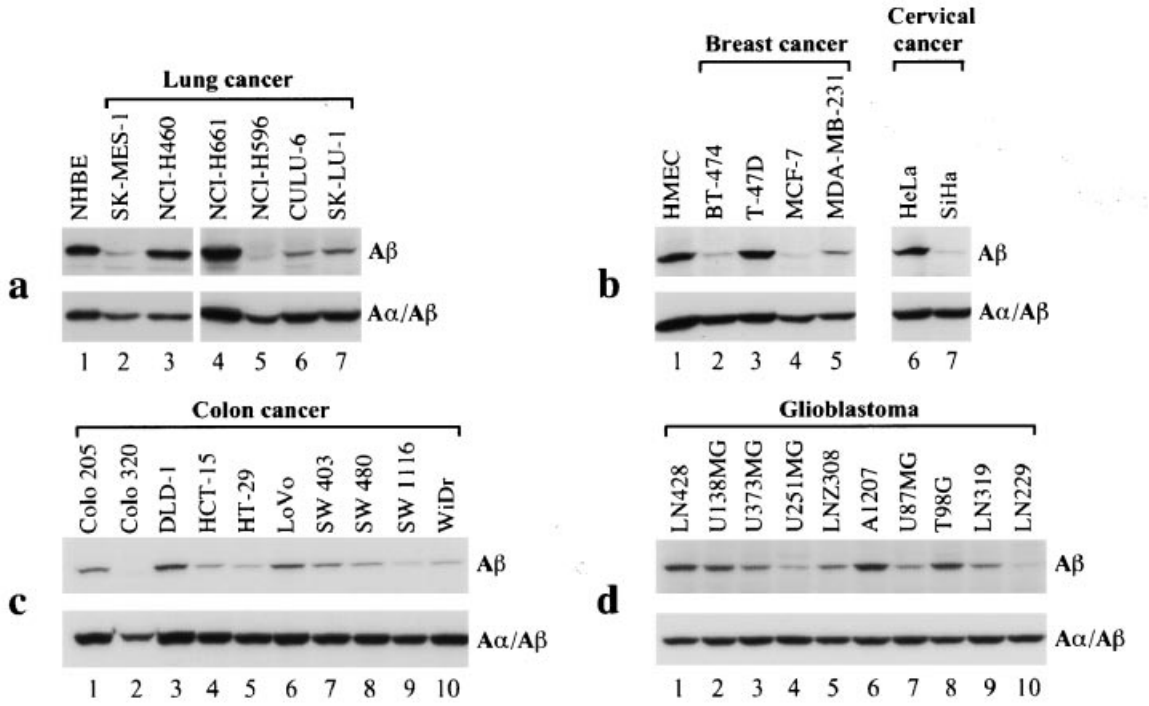

Figure 6 Reduced expression of Aβ in approx. 50% of cancer cell lines compared with primary normal human epithelial cells

Equal amounts of protein from each cell line (10 μg) were analysed by Western blotting using anti-Aβ antibodies or 6G3 anti-(Aα/Aβ) MAb. Primary normal human bronchial epithelial cells (NHBE) (panel a, lane 1) and primary normal human mammary epithelial cells (HMEC) (panel b, lane 1) were used as standards for normal Aβ expression.

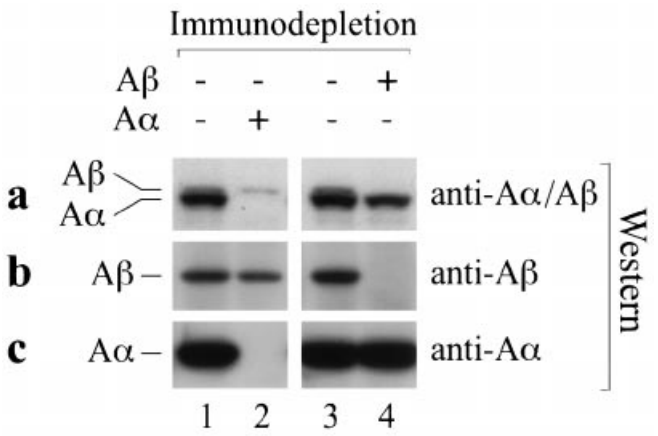

Figure 7 Epithelial cells express approx. 10% as much Aβ as Aα

Extracts of NCI-H460 cells, which contain Aβ levels similar to primary normal human epithelial cells (Figure 6), was depleted of Aα with 6F9 (anti-Aα) (lane 2) or of Aβ with anti-Aβ antibodies (lane 4). The antibodies used for immunodepletion were coupled to protein A or protein G beads, as described in the Materials and methods section. Mock depletions were carried out with plain beads without antibodies (lanes 1 and 3). Western blotting was carried out to demonstrate high depletion efficiencies for Aβ (panel b, lane 4 cf. lane 3) and Aα (panel c, lane 2 cf. lane 1). Western blotting the depleted samples with 6G3, a MAb that recognizes Aα and Aβ equally well (results not shown), allows direct comparison of Aα and Aβ levels (panel a, lane 2 cf. lane 4).

were obtained from commercial sources and analysed by SDS/PAGE and Western blotting with Aβ-specific antibodies. An equal amount of total protein was analysed from each sample. As shown in Figure 5, testis was the only tissue with a high level of Aβ, whereas low levels were detected in kidney, lung, liver, heart and brain (panel a). Peptide inhibition verified that the bands that were recognized by the antibodies, including the weak ones from the heart, liver, lung and kidney samples, represented Aβ (panel b). In contrast with Aβ, Aα was expressed at high levels in all tissues (panel c). The striking difference between the Aα and Aβ expression patterns indicates that these proteins fulfil different functions.

In previous studies, we observed that the Aα subunit was highly expressed in all cells analysed, irrespective of tissue origin and animal species, and whether transformed or untransformed ([17,22] and R. Ruediger and G. Walter, unpublished work). As shown in Figure 6, this observation was confirmed when a large number of human tumour cell lines derived from lung, colon, breast, cervix and brain was analysed, all of which show similarly high expression of Aα. In contrast, the levels of Aβ varied greatly, raising questions about the level of Aβ in normal human epithelial cells from which carcinomas are derived. Either the high level of Aβ that is found in some tumour cell lines corresponds to that in normal cells, and the low level in other lines results from down-regulation, or normal epithelial cells might express a low level of Aβ, and the high level in some tumour cell lines is caused by up-regulation. To distinguish between these two possibilities, primary normal human lung and breast epithelial cells were analysed in parallel with lung and breast tumour cell lines. As shown in Figure 6, normal lung (panel a, lane 1) and breast (panel b, lane 1) epithelial cells expressed high levels of Aβ, implying that Aβ is suppressed to various degrees in many tumour cell lines. On the other hand, the level of Aα was roughly the same in normal epithelial cells and all tumour cell lines. These findings suggest that the expression of Aα is regulated differently from that of Aβ.

Although the levels of Aβ were high in testis, primary epithelial cells and some tumour cell lines, they were still low when compared with Aα. This was demonstrated by immunodepletion of either Aα or Aβ from H460 cell extracts and subsequent analysis of the depleted extracts on SDS/PAGE (7% gel) Tris-acetate gels (Invitrogen), which separate Aα from Aβ. Aβ migrates slightly slower than Aα on account of its N-terminal extension. This was shown first for *in vitro* synthesized Aα and Aβ (results not shown). As shown in Figure 7 (panel a, lanes 1 and 2), immunodepletion of Aα from an H460 cell extract with MAb 6F9 caused complete removal of the strong lower band (Aα) but did not affect the weak upper band (Aβ). For this Western blot, MAb 6G3 was used, which recognizes both Aα and Aβ equally well (results not shown). That Aα was completely removed by immunodepletion was also shown by Western blotting with MAb 6F9, which recognizes Aα but not Aβ (panel c, compare lanes 1 and 2). The difference in intensity between the Aα and Aβ bands (panel a, lanes 2 and 4) is approx. 10-fold. As expected, depletion of Aα (lane 2) had no effect on the level of Aβ (panel b, compare lanes 1 and 2), while depletion of Aβ (lane 4) had no effect on Aα (panel c, compare lanes 3 and 4). The depletion of Aβ is also apparent, although weakly, from the disappearance of the upper band in panel a, lane 4. We estimated that in tumour cell lines with low levels of Aβ, such as SK-MES-1 (Figure 6, panel a, lane 2), the Aβ levels are 5- to 10-fold lower than in H460 or primary cells, implying that they range between 1 and 2% of Aα levels. We have previously shown that Aα represents approx. 0.1% of total cellular proteins in many cell lines [22]. The present results show that the high expression of Aβ in testis, primary epithelial cells and certain tumour cell lines corresponds to approx. 0.01% of total cell protein, whereas the low expression in most tumour cell lines is equivalent to approx. 0.001–0.002% of total cell protein.

The Aα and Aβ subunits differ in their binding to B and C subunits *in vivo*

Using an *in vitro* binding assay, we showed previously that the Aα and Aβ subunits differ markedly in binding B and C subunits [9]. In contrast with the Aα subunit, the Aβ subunit did not bind Bα and B′ subunits. In addition, binding of B″/PR72 to Aβ was reduced 3-fold in comparison with Aα, and C-subunit binding was reduced 7-fold. No distinction was made between Cα and Cβ. It seemed possible that the *in vivo* binding properties of the A, B, and C subunits differ from the *in vitro* binding properties due to modification by phosphorylation or methylation that might occur in intact cells, but not in the reticulocyte lysate used for *in vitro* subunit synthesis (reviewed in [2]). Therefore we developed an *in vivo* binding assay. In this assay, HEK-293 cells were transfected with plasmids encoding EE-tagged Aα or Aβ subunits, KT3-tagged Bα, B′α1 or B″/PR72 subunits, and HA-tagged Cα or Cβ subunits. The EE-tagged A subunits were immunoprecipitated with anti-EE antibodies, and the precipitates were analysed by SDS/PAGE and Western blotting using anti-KT3 and anti-HA antibodies to identify co-immunoprecipitated B and C subunits.

As shown in Figure 8, the binding of all C and B subunits to Aβ was significantly reduced (lane 5) as compared with Aα (lane 3). Importantly, no binding of Bα to Aβ was detectable. Control immunoprecipitations were carried out using extracts from cells that were transfected with control vector (lanes 1 and 2) and by the addition of competing EE-peptide (lanes 2, 4, and 6). Lanes 8 and 9 show that the amounts of extract used for immunoprecipitation contained equal amounts of the tagged A, B, and C

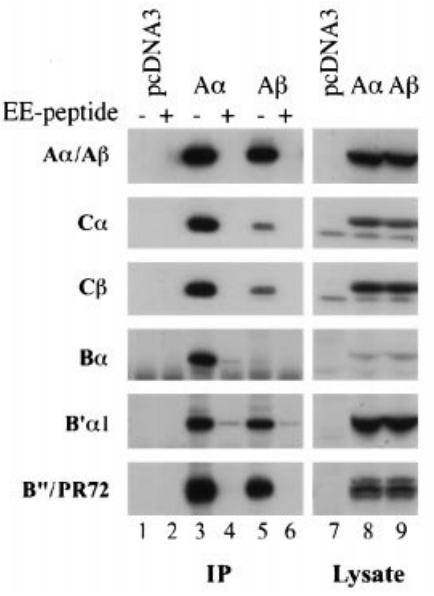

Figure 8 Aβ is a weak binder of B and C subunits compared with Aα

HEK-293 cells were co-transfected with EE-tagged Aα- or Aβ-subunit constructs and with vectors for KT3-tagged B subunits or HA-tagged C subunits. Complexes were immunoprecipitated with anti-EE antibodies, and B or C subunits that bound to the A subunits were identified with anti-KT3 or anti-HA antibodies by Western blotting. Transfections with empty vector (pcDNA3) (lanes 1, 2 and 7) and inhibition of immunoprecipitation with EE-peptide (+) were used as negative controls. Western blotting of cell lysate aliquots shows that similar amounts of A, B or C subunits were used for the immunoprecipitations (lanes 8 and 9). Using serial dilutions of lysates from Aα co-transfected cells (results not shown), we determined that, in comparison with Aα, Aβ binds Cα and Cβ 8-fold weaker, Bα undetectably, and B′α1 and B″/PR72 3-fold weaker (lane 5 cf. lane 3).

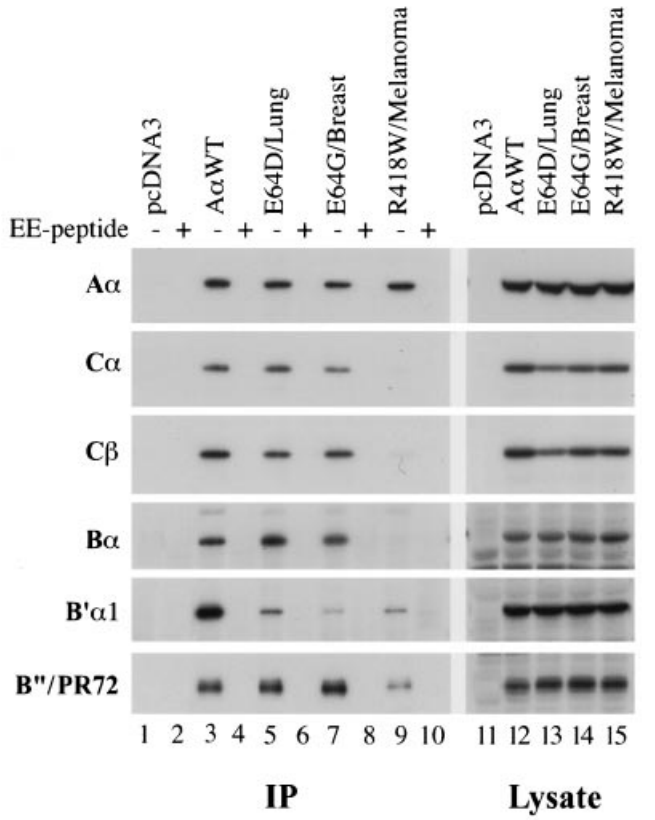

Figure 9 Binding deficiencies of Aα-subunit mutants detected in lung, breast and skin cancers

HEK-293 cells were co-transfected with constructs expressing EE-tagged wild-type or mutant Aα subunits, and with vectors for KT3-tagged B subunits or HA-tagged C subunits. Immunoprecipitation and Western blotting were performed as described in the legend to Figure 8, which also describes the controls. Using serial dilutions of lysates from Aα co-transfected cells (results not shown), we determined that, in comparison with wild-type Aα, C-terminal mutant R418W is completely defective in Cα, Cβ and Bα binding, while it binds B′α1 10-fold weaker and B″/PR72 4-fold weaker (lane 9 cf. lane 3). N-terminal mutants E64D and E64G were defective only in B′α1 binding by factors of 10 and 20 respectively, whereas their binding to Cα, Cβ, Bα and B′/PR72 was unaffected (lanes 5 and 7 cf. lane 3).

subunits. The *in vivo* binding assay shown here yielded similar results to the *in vitro* binding assay reported previously, suggesting that protein modification, if it took place *in vivo*, but not *in vitro*, did not significantly alter subunit interactions.

We reported previously that B′α1 does not bind to Aβ when assayed *in vitro* [9], whereas now we consistently find binding. The previous failure to detect B′α1 binding was caused by a high background in the control reaction.

Although Cα and Cβ are 96% identical, we were still surprised to find no difference between Cα and Cβ in binding to Aα or Aβ. However, in spite of the strong similarity, Cβ cannot substitute for Cα *in vivo* since Cα knock-out mice die on embryonic day (E) 6.5, suggesting that functional differences between these two isoforms do exist [23]. It has also been reported that before E6.5, Cα is predominantly localized at the plasma membrane whereas Cβ is found in the cytoplasm and nucleus [24]. According to our data, these differences between Cα and Cβ cannot be explained on the basis of their interaction with Aα and Aβ. It remains to be examined whether or not Cα- and Cβ-containing core enzymes differ in their enzymic activity or if they interact differently with B subunits.

Before performing *in vivo* binding assays with tagged B and C subunits, we determined that the tags do not interfere with subunit interactions. Tagged and untagged B and C subunits as well as EE-tagged Aα and Aβ were synthesized *in vitro* in the presence of [^{35}S]methionine, incubated with each other in various combinations and immunoprecipitated with anti-EE. When comparing tagged with untagged constructs, identical amounts of Bα, B′α1, B″/PR72, Cα or Cβ were co-immunoprecipitated with Aα or Aβ (results not shown). The question could be raised whether our binding assays truly reflect normal subunit interactions, since the transfected subunits might have been overexpressed. However, in comparison with the highly abundant endogenous Aα, Bα and Cα subunits, the transfected subunits were expressed at similar levels and therefore were not really overexpressed (results not shown). In the case of Aβ, the level of endogenous Aβ is considerably lower than that of transfected Aβ. However, the co-transfected binding partners of Aβ (C and B subunits) were expressed at similarly high levels. Therefore we expect no distortion of subunit interactions.

Cancer-associated Aα-subunit mutants are defective in binding B, or B and C subunits *in vivo*

Previously, we found that all Aα-subunit mutants that were detected in human cancers are strikingly defective in B-, or B- and C-subunit binding, as determined by *in vitro* binding assays [8]. We proposed that these binding defects may be related to the loss of PP2A tumour-suppressor activity in cancer. Because of

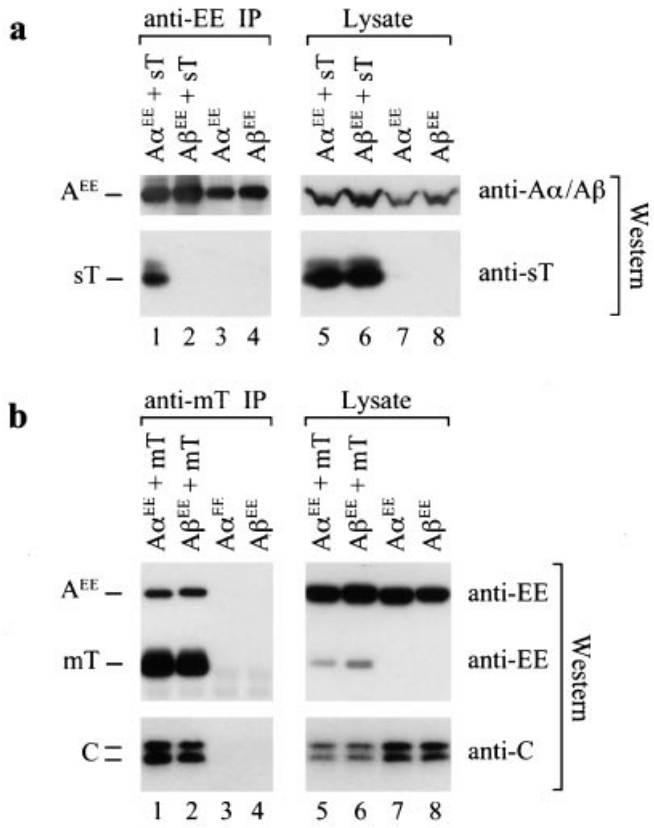

Figure 10 Polyoma virus mT, but not SV40 sT, antigen binds to Aβ

(**a**) HEK-293 cells were co-transfected with EE-tagged Aα or Aβ-subunit constructs and with a vector for SV40 sT. The A subunits were immunoprecipitated with anti-EE antibodies, and the co-precipitation of sT was analysed by Western blotting with MAb 419 (anti-sT). (**b**) HEK-293 cells were co-transfected with EE-tagged Aα- or Aβ-subunit constructs and with a vector for mT. mT was immunoprecipitated with a specific tumour serum (anti-mT) that had no anti-EE activity (A^{EE} lanes 3 and 4 cf. lanes 1 and 2). Co-immunoprecipitated $A\alpha^{EE}$ or $A\beta^{EE}$ were detected by Western blotting with anti-EE antibodies. Co-immunoprecipitated endogenous C subunit was also detected by Western blotting with an anti-C MAb.

the potential importance of these mutants for elucidating the role of PP2A in tumour suppression, we investigated their binding properties further using the *in vivo* binding assay described above. Three Aα mutants were analysed: Glu^{64}→Asp (E64D) from lung carcinoma, Glu^{64}→Gly (E64G) from breast carcinoma and Arg^{418}→Trp (R418W) from melanoma. E64D and E64G are located close to the intra-repeat loop of repeat 2, and R418W is located in the intra-repeat loop of repeat 11 (Figure 1). Figure 9 demonstrates that, in comparison with wild-type Aα (lane 3), the mutants E64D (lane 5) and E64G (lane 7) were specifically defective in B′α1 binding, but were normal in binding Bα, B″/PR72, Cα and Cβ. Mutant R418W was defective in binding all B and C subunits (lane 9). Control transfections were carried out with pcDNA3 (lanes 1 and 2). Control immunoprecipitations were carried out in the presence of competing peptide (lanes 2, 4, 6, 8 and 10). Lysates containing equal amounts of A, B and C subunits were used for immunoprecipitation (lanes 12–15). The results of the *in vivo* binding assay confirm what we found *in vitro*. We conclude that modifications of the A, B and C subunits that might occur *in vivo* do not significantly alter the binding properties of Aα.

Polyoma virus mT, but not SV40 sT, binds to Aβ

A remarkable feature of the PP2A core enzyme is its ability to bind SV40 and polyoma virus T antigens [10,11]. All previous studies were carried out under conditions that measured T antigen binding to Aα, whereas binding to Aβ was never assayed. In the present study, we used an *in vivo* assay for measuring the binding of SV40 sT and polyoma virus mT to Aα and Aβ. HEK-293 cells were transfected with plasmids encoding SV40 sT antigen or polyoma virus mT antigen together with EE-tagged Aα or Aβ. To assay for SV40 sT binding, the A subunits were immunoprecipitated with anti-EE antibodies and co-immunoprecipitated sT was detected by Western blotting with a specific antibody. As shown in Figure 10(a), SV40 sT binds to Aα (lane 1) but not to Aβ (lane 2). Figure 10(a) also shows that similar amounts of Aα and Aβ were immunoprecipitated (lanes 1–4) and that the Aα and Aβ lysates contained equal amounts of SV40 sT (lanes 5 and 6). To assay for polyoma virus mT binding, mT was immunoprecipitated with a specific antiserum and the co-immunoprecipitated A subunits were assayed by Western blotting with antibodies against the EE-tag. As shown in Figure 10(b), mT bound equally well to Aα and Aβ (lanes 1 and 2). Furthermore, the mT-Aα and mT-Aβ precipitates contained equal amounts of catalytic C subunit (lanes 1 and 2). Therefore, of all the proteins tested that bind to PP2A core enzymes (Figures 8 and 10), mT is the only protein that shows no preference for Aα (Figure 10b). Figure 10(b) also shows that (i) Aα and Aβ were expressed at similar levels (lanes 5–8), (ii) all four lysates contained similar amounts of C subunit (lanes 5–8), and (iii) mT was expressed at similar levels in the Aα and Aβ sample (lanes 5 and 6). While the lysates contained more A subunit than mT (lanes 5 and 6), the immunoprecipitation enriched for mT, as expected (lanes 1 and 2).

Evolution of Aα and Aβ

In the context of our studies on the structure and function of the A subunits, it is instructive to look at their evolutionary history. It was noted several years ago that the C subunit of PP2A is one of the most highly conserved enzymes [25]. Since then, more sequence information has become available demonstrating that the human, mouse, pig and frog genomes encode both Aα and Aβ subunits whereas fruit fly, nematode and yeast genomes encode only one subunit. Alfalfa encodes at least two and *Arabidopsis* encodes three A subunits, all of which are closely related to each other. The existence of three A-subunit genes in *Arabidopsis* could be due to an A-subunit gene duplication as well as a whole-genome duplication followed by gene loss [26]. Therefore, an A-subunit duplication has occurred twice in evolution, once in vertebrates and once in plants. Interestingly, the *Arabidopsis* gene *RCN1*, encoding one of the three A subunits, is a positive regulator of PP2A activity [27] and *RCN1* mutants are defective in root development [28]. As illustrated by the phylogenetic tree of the A subunits (Figure 11a), Aα is more conserved than Aβ. The length of the horizontal lines, as expressed by the numbers, is proportional to the number of evolutionary amino acid replacements. For example, the human Aα subunit is 0.2 and the human Aβ subunit is 1.62 length units removed from their common precursor with the corresponding pig or mouse A subunits, indicating that human Aα had 8 times fewer amino acid replacements than human Aβ during the same time span. The difference between Aα and Aβ is less pronounced when we compare frog Aα and frog Aβ with the corresponding human A subunits.

A striking feature of A-subunit evolution is the dramatic drop in the rate of change over evolutionary time. This is illustrated by

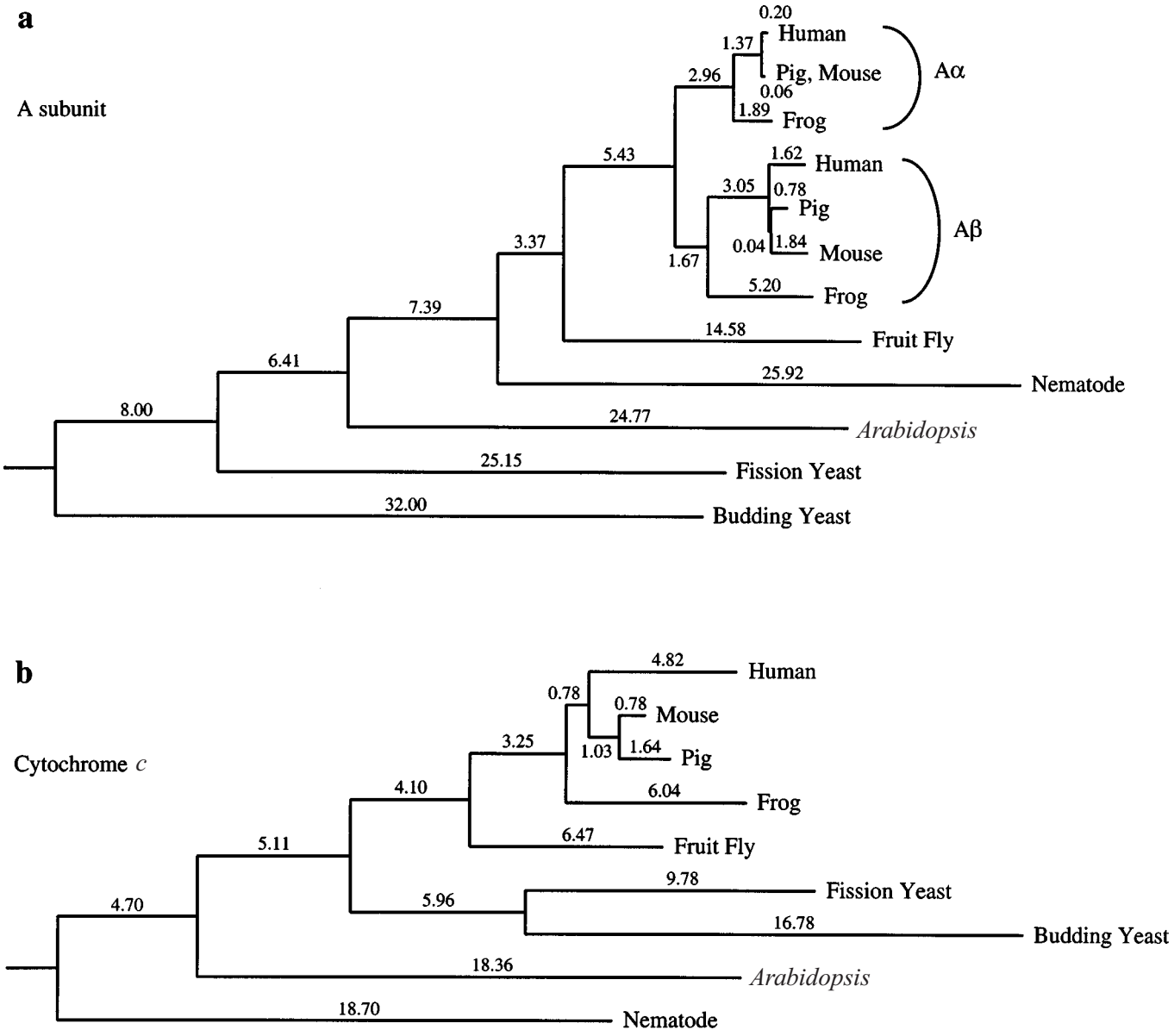

Figure 11 Evolutionary trees of A subunits and cytochrome *c*

See text for details.

Table 2 High evolutionary stability in vertebrates of the Aα and Aβ subunits in comparison with cytochrome *c*

	Evolutionary distance* (arbitrary units)			Relative rate of change compared with cytochrome *c*†	
Common precursor	Aα	Aβ	Cytochrome *c*	Aα	Aβ
Pig/mouse	0.2	1.6	4.8	0.04	0.33
Frog	1.57	4.67	5.6	0.28	0.83
Fruit fly	9.96	11.8	8.85	1.12	1.33
Budding yeast	35.13	36.9	12.95	2.71	2.85

* Evolutionary distances of human Aα, Aβ and cytochrome *c* from their respective common precursors with pig/mouse, frog, fruit fly and budding yeast (values taken from Figure 11).
† Ratio of evolutionary distances of Aα and Aβ to cytochrome *c*.

comparing the phylogenetic tree of the A subunits (Figure 11a) with that of cytochrome *c* (Figure 11b), which serves as a standard with a constant rate of change [29]. Whereas the distance of human Aα from its common precursor with pig or mouse is 0.2 length units, the corresponding distance for cytochrome *c* is 4.82 length units. Therefore, during the evolutionary period from mouse or pig to human, cytochrome *c* changed 24 times faster (4.82/0.2) than Aα. From frog to human, cytochrome *c* changed only 3.6 times faster than Aα, and from fruit fly to human, both proteins changed with equal rates. On the other hand, over the entire period from yeast to human, Aα changed 2.7 times faster than cytochrome *c*. These results are summarized in Table 2. Overall, during the evolution from yeast to human, a 67-fold (2.71/0.04) drop in the rate of change of Aα as compared with cytochrome *c* took place, whereby Aα changed faster than cytochrome *c* initially, but much slower at recent time. The rate of change of Aα during mammalian evolution is very similar to that of histone H4, one of the most highly conserved proteins known. Table 2 also shows the evolutionary development of Aβ in comparison with that of cytochrome *c*. Although Aβ's rate of change during mammalian evolution is 8-fold (0.33/0.04) higher than that of Aα, it is still lower than that of cytochrome *c*.

DISCUSSION

The A subunit of PP2A fulfils a dual function. On the one hand, it acts as a structural base on which a regulatory B subunit can interact with a catalytic C subunit [12,13]. The B subunits may modify the substrate specificity or activity of the catalytic subunit [30], or direct the holoenzyme to a particular subcellular location [31,32]. They may also interact with other cellular proteins and thereby direct PP2A to a specific signalling pathway (reviewed in [2]). On the other hand, the A subunit plays a purely regulatory role by altering the substrate specificity of the catalytic subunit in the absence of a B subunit [33,34]. A remarkable property of the A subunit is its ability to bind to many forms of B subunits, which are weakly related or unrelated to each other, and two isoforms of C subunits. This property permits the formation of a large number of different holoenzymes. The versatility of the A subunit is further enhanced by the existence of two isoforms, Aα and Aβ (reviewed in [2]).

Nearly all studies on the A subunit involved the highly abundant Aα isoform. We began to investigate the Aβ isoform after Wang et al. [5] reported that this form is mutated in a variety of human cancers. Importantly, many of the cancer-associated Aβ mutants are defective in binding B and C subunits [9]. These findings suggested that Aβ plays a unique role in growth control. Owing to the fact that human Aβ has an N-terminal extension of 12 amino acids that is not present in Aα, we were able to generate Aβ-specific antibodies that were used to investigate Aβ in comparison with Aα. We found that Aα and Aβ are expressed at constant levels throughout the cell cycle, and that both are enriched in the cytoplasmic fraction. Their half-lives are approx. 10 h. However, Aα and Aβ differ markedly in their levels of expression. Whereas Aα is highly abundant in all cells and normal tissues (0.1 % of the total cell protein), Aβ is 10-fold less abundant even in cells with highest Aβ expression. In low-expressing tumour cell lines and tissues, Aβ is 50- to 100-fold less abundant than Aα. Therefore, even cells that express relatively high levels of Aβ still express a large molar excess of Aα. This suggests that the function of Aβ must be qualitatively different from that of Aα since, quantitatively, Aβ plays a minor role. It should be pointed out that during mention of 'high' levels of Aβ, it is understood that they are 10-fold lower than Aα levels.

A dramatic difference between Aα and Aβ was found when we analysed their expression in various human tissues. Aβ was expressed at very low levels in all tissues with the notable exception of testis, while Aα was highly expressed and similarly abundant in all tissues including testis. Since 90 % of the testis consists of sperm-producing seminiferous cells, Aβ may play a role in spermatogenesis. Furthermore, since the testis is also rich in Bβ [35] and Cβ subunits [36], a testis-specific holoenzyme consisting of Aβ, Bβ and Cβ may be involved in spermatogenesis. Interestingly, other phosphatases including PP1 [37] and PP2B [38] are also enriched in the testis. We suggest that Aα-containing PP2A has housekeeping functions besides specific regulatory roles, whereas Aβ-containing core or holoenzymes fulfil mostly specialized tasks.

Another remarkable difference between Aα and Aβ was found when we analysed their expression in a large number of cancer cell lines. Whereas the levels of Aα were high and similar in all tumour cell lines, the levels of Aβ varied greatly between the lines. We also analysed Aα and Aβ expression in primary lung and breast epithelial cells. Importantly, the high Aβ levels in some tumour cell lines corresponded to the levels in the primary normal lung and breast epithelial cells as well as to the high level in the testis. These results suggest that Aβ has been suppressed in many carcinomas with low Aβ expression. Whether or not this reduced expression of Aβ signifies a loss of the presumed tumour suppressor activity [5] of PP2A remains to be investigated. Previously, we found that Aα levels were strongly reduced in 43 % of primary human gliomas [20], whereas in the present study, we find that levels were high in ten established glioblastoma cell lines. The reason for this apparent paradox is unknown.

The high level of Aβ in primary epithelial cells from normal human breast and lung seems to conflict with the low expression of Aβ in normal lung and breast tissue. However, only a relatively small fraction of breast and lung tissue consists of epithelial cells, presumably expressing high amounts of Aβ, whereas the bulk of the tissue consists of stromal cells in lung and stromal and fat cells in breast, which presumably express low levels of Aβ. In addition, only a subfraction of the epithelial cells in breast and lung, e.g. dividing cells, may express a high amount of Aβ. These questions could be answered by immunostaining with Aβ-specific antibodies. Unfortunately, our Aβ-specific antibody, although good for immunoprecipitation and Western blotting, cannot be used for immunostaining because it cross-reacts with other proteins.

A striking difference between Aα and Aβ was revealed when we studied their ability to bind B and C subunits. The present investigation was carried out *in vivo*, whereas previously an *in vitro* binding assay was used [9]. Both assays demonstrated that the Aα and Aβ subunits differ considerably in their ability to bind B and C subunits. The most remarkable difference was that Aβ was unable to bind the Bα subunit. In addition, its ability to bind B′, B″/PR72, Cα and Cβ was strongly reduced compared with Aα. Since we only measured the binding properties of one member from each B-subunit family (Bα, B′α1 and B″/PR72), the possibility that other family members bind preferentially to Aβ has not been excluded. On the other hand, it seems likely that all the members of one B-subunit family are highly conserved in their A-subunit-binding domains and, therefore, have similar binding properties. It is also possible that Aβ binds to unknown B-like subunits that do not bind to Aα. Our finding that SV40 sT binds to Aα, but not to Aβ, indicates that the transforming activity of SV40 sT is mediated by the Aα–C core enzyme. On the other hand, polyoma mT, which binds to both Aα and Aβ, could function in growth control by binding to Aα- and Aβ-containing core enzymes.

A comparison of the Aα and Aβ amino acid sequences may provide some clue to the question of what makes Aβ unique. Most noticeably, the human Aβ subunit has an N-terminal extension of 12 amino acids that is absent from the Aα subunit. This extension is conserved in mouse and pig Aβ but is absent from *Xenopus* Aβ, suggesting that it was acquired during the evolution of mammals. This unique sequence could either be required for the binding of an unknown, Aβ-specific regulatory subunit, or it may direct Aβ-containing holoenzymes to a specific subcellular location where it fulfils a unique function that cannot be carried out by Aα-containing holoenzymes. This function may be required by mammals but not amphibians. Aside from the N-terminal extension, Aβ differs from Aα at 83 positions located throughout the entire protein. Some of these differences could explain the reduced binding of B and C subunits to Aβ compared with Aα.

Our studies provide the first biological and biochemical data suggesting that the Aα and Aβ subunits have unique functions. Further support for this idea comes from an analysis of the evolutionary history of the A subunits. Most remarkable is the extremely low rate of change of the Aα sequence during vertebrate and particularly mammalian evolution. Since the main role of Aα is to bind B and C subunits, as well as numerous other proteins, its sequence may not be altered without obstructing the interaction with these proteins. Aβ is less conserved, suggesting that it interacts with fewer proteins than Aα. Consistent with this notion is the finding that Aβ does not bind Bα and SV40 sT antigen.

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