Association of the type 1 protein phosphatase PP1 with the A-kinase anchoring protein AKAP220

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The cyclic AMP (cAMP)-dependent protein kinase (PKA) and the type 1 protein phosphatase (PP1) are broad-specificity signaling enzymes with opposing actions that catalyze changes in the phosphorylation state of cellular proteins. Subcellular targeting to the vicinity of preferred substrates is a means of restricting the specificity of each enzyme [1,2]. Compartmentalization of the PKA holoenzyme is mediated through association of the regulatory subunits with A-kinase anchoring proteins (AKAPs), whereas a diverse family of phosphatase-targeting subunits directs the location of the PP1 catalytic subunit (PP1c) [3,4]. Here, we demonstrate that the PKA-anchoring protein, AKAP220, binds PP1c with a dissociation constant (K_D) of 12.1 ± 4 nM *in vitro*. Immunoprecipitation of PP1 from cell extracts resulted in a 10.4 ± 3.8-fold enrichment of PKA activity. AKAP220 co-purified with PP1c by affinity chromatography on microcystin sepharose. Immunocytochemical analysis demonstrated that the kinase, the phosphatase and the anchoring protein had distinct but overlapping staining patterns in rat hippocampal neurons. Collectively, these results provide the first evidence that AKAP220 is a multivalent anchoring protein that maintains a signaling scaffold of PP1 and the PKA holoenzyme.

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Received: 29 December 1998
Revised: 25 January 1999
Accepted: 25 January 1999

Published: 15 March 1999

Current Biology 1999, 9:321–324
http://biomednet.com/elecref/0960982200900321

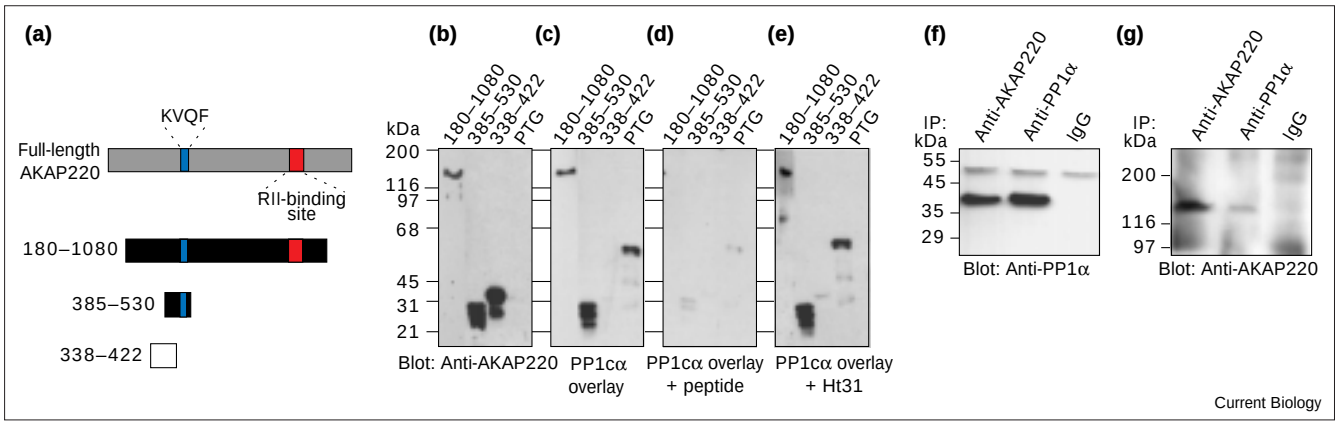
 ISSN 0960-9822

Results and discussion

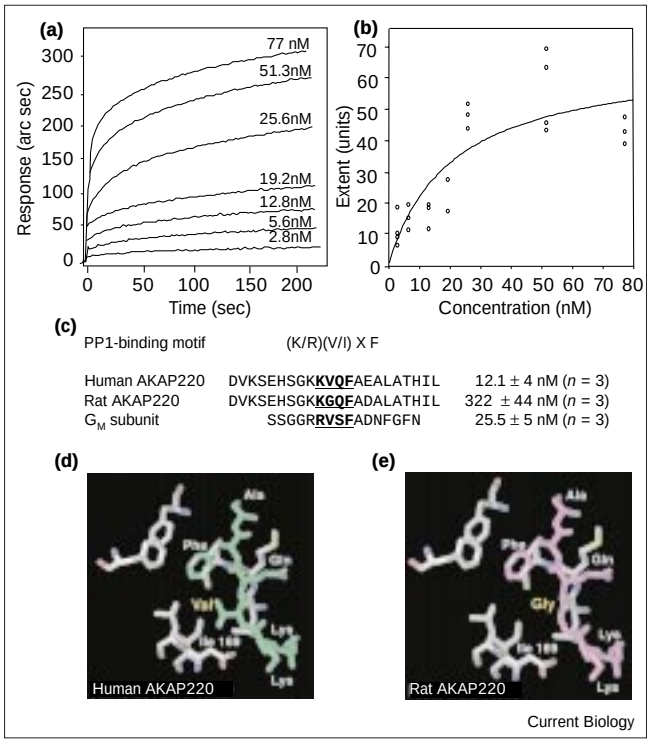
AKAP220 was first identified in a screen for anchoring proteins using radiolabelled regulatory subunit (RII) of PKA as a probe and was shown to interact with the PKA holoenzyme inside cells [5]. Evidence that some AKAPs bind other signaling enzymes [6–8] prompted us to look for other AKAP220-interacting proteins. Residues 501–505 of human AKAP220 contain the amino-acid sequence KVQF (single-letter amino-acid code), which is a hallmark of type I phosphatase targeting subunits (Figure 1a). Others have noted that this loosely defined motif (R/K V/I X F) binds to an allosteric site on the catalytic subunit of the phosphatase [4,9]. We therefore screened a family of AKAP220 fragments for their ability to bind the PP1cα isoform using an overlay assay (Figure 1a,b). In this assay, immoblized proteins are incubated with PP1 in solution and phosphatase binding can be detected on the membrane by western blotting analysis. Fragments encompassing the KVQF consensus motif bound PP1cα, whereas a fragment lacking this sequence did not bind the phosphatase (Figure 1c). Further control experiments demonstrated that solid-phase binding of PP1cα to AKAP220 was blocked by a peptide spanning residues 492–514 of human AKAP220, but was unaffected by preincubation with an RII-anchoring inhibitor peptide derived from the human thyroid anchoring protein Ht31 (Figure 1d,e). Formation of an AKAP220–PP1cα complex in solution was observed in reciprocal co-precipitation experiments. Recombinant PP1cα was isolated upon immunoprecipitation of an AKAP220 fragment (Figure 1f). Similarly, the AKAP220–PP1cα complex was isolated by immunoprecipitation with isoform-specific anti-PP1α antibodies (Figure 1g). Neither protein was immunoprecipitated by immunoglobulin G (IgG) alone (Figure 1f,g). Collectively, these results demonstrate that PP1cα and AKAP220 interact *in vitro*.

Recombinant PP1cα bound the phosphatase-binding-site peptide of human AKAP220 with a dissociation constant of 12.1 ± 4 nM (n = 3), as measured by surface plasmon resonance (Figure 2a,b). Likewise, a peptide containing the PP1-binding site of the muscle glycogen regulatory subunit (G_M) bound PP1cα with an affinity of 25 ± 5 nM (n = 3). However, a peptide derived from the rat homologue of AKAP220 bound the phosphatase with a 20-fold lower binding affinity (322 ± 44 nM, n = 3). Alignment of the human and rat sequences revealed a glycine for valine substitution at position 502 in rat AKAP220 (Figure 2c). Computer modeling of the human and rat PP1-binding peptides using coordinates of the G_M peptide bound to PP1c highlights the importance of this residue [4]. The valine side chain of human AKAP220 participates in the formation of hydrophobic interactions with multiple residues in the phosphatase catalytic subunit including Ile169 (Figure 2d). Although rat AKAP220 lacks this side chain, other critical residues in the PP1-binding site remain and are sufficient to confer a nanomolar binding affinity (Figure 2e). Furthermore, recent studies suggest that sites other than the consensus binding motif contribute to the high-affinity interaction between AKAP220 and the phosphatase [10–12].

Figure 1



PP1α binds AKAP220 *in vitro*. (a) Schematic diagram of AKAP220 fragments used in overlay analysis: the amino-acid regions spanned by each fragment and the location of consensus PP1-binding sites (KVQF, blue boxes) and RII-binding sites (red boxes) are indicated. The fragments shaded black (180–1080 and 385–530) bind PP1, the unshaded fragment (338–422) does not bind PP1, and the full-length AKAP220 protein has not been assayed for PP1 binding *in vitro*. (b) Western blot detection of human AKAP220 fragments. Histidine-tagged AKAP220 fragments were purified by FPLC, separated on SDS–polyacrylamide (4–15%) gradient gels, and transferred to Immobilon membrane; the phosphatase-targeting protein PTG [22] was used as a control. Protein fragments were detected by western blotting using a polyclonal antibody to human AKAP220. (c) Solid-phase detection of PP1cα-binding proteins by overlay analysis. Immobilon membranes containing AKAP220 protein fragments and PTG were incubated with 0.5 μg recombinant PP1cα in TTBS (0.03% Tween, Tris-buffered saline) for 2 h at room temperature. PP1cα was detected by western blot analysis using a polyclonal anti-PP1cα antibody and chemiluminescence (Pierce). (d) PP1 overlay in the presence of 2 μM peptide corresponding to the PP1-binding site of AKAP220 (DVKESEHSGKKVQFAEALATHIL). (e) PP1cα overlay in the presence of 2 μM RII-anchoring inhibitor peptide Ht31 (DLIEEAASRIVDAVIEQVKAAGA) [23]. (f,g) Co-precipitation of PP1cα and AKAP220. Recombinant PP1cα and AKAP220 fragment containing amino acids 180–1080 were incubated for 30 min on ice in 50 mM Tris buffer pH 7.4, 0.1 mM EDTA, 1 mM DTT, 0.1% bovine serum albumin, 1 mM $MnCl_2$. Monoclonal antibodies to PP1cα or AKAP220 (Transduction Labs), or mouse IgG (10 μg antibody in each case) were added for 2 h at 4°C. Immune complexes were isolated by incubation with protein A–Sepharose beads for 1 h at 4°C. After four washes with TBS, 0.5 M NaCl, 0.5% Triton X-100, the beads were boiled in sample buffer, separated by SDS–PAGE, and transferred to Immobilon membrane for western blot analysis to detect (f) PP1cα and (g) AKAP220.

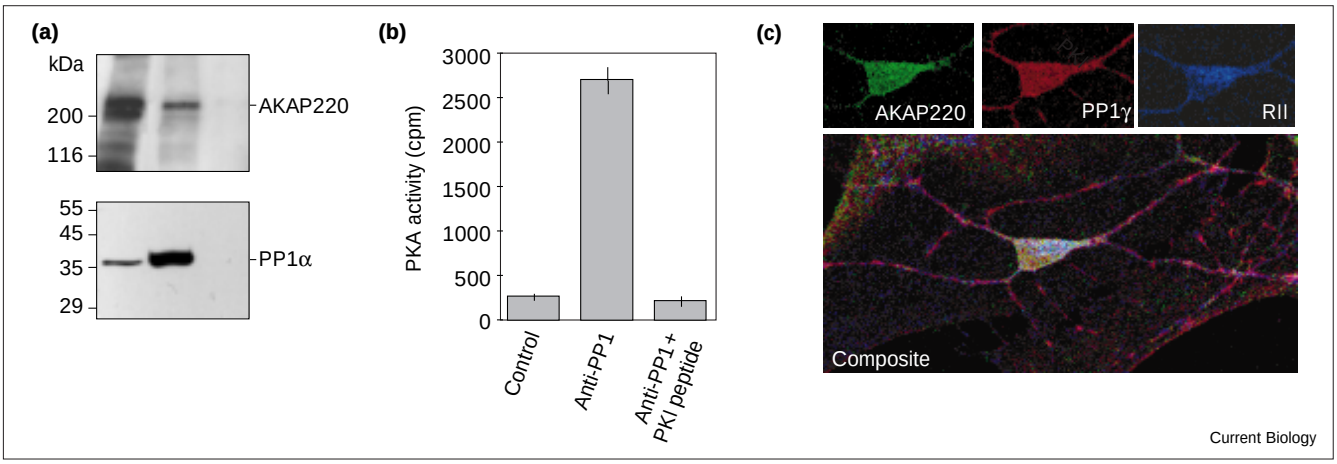


In the light of the evidence that the rat AKAP220 homologue bound to PP1 with a lower binding affinity than the human homologue, it was imperative to determine whether the proteins interacted inside cells. AKAP220 and PP1 were isolated from rat brain extracts by affinity chromatography on microcystin–sepharose (Figure 3a). Control experiments illustrated that isolation of both proteins was

Figure 2

Human and rat AKAP220 interactions with PP1α. (a,b) Affinity measurements of the PP1cα interaction with immobilized human AKAP220 peptide by surface plasmon resonance, as described [24]. (a) Representative data of the real-time binding to a peptide corresponding to amino acids 492–514 of human AKAP220 over a range of PP1cα concentrations (2.8 nM–77 nM). (b) The graph of extent of binding (units) plotted against concentration (nM) that was used to determine the dissociation constant (K_D). (c) Comparison of PP1cα binding to the human AKAP220 peptide, the rat AKAP220 peptide, and the G_M subunit peptide (± standard error). K_D values were calculated using nonlinear regression (Marquardt analysis) of a single binding site equation with Kaleidograph™, and are the average from three independent experiments. (d,e) Computer-generated modeling of (d) human AKAP220 peptide (green) and (e) rat AKAP220 peptide (pink) using coordinates from the G_M peptide–PP1 structure (white). Red, methyl group; blue, carboxyl group; yellow, amine group.

Figure 3



Rat AKAP220 interacts with PP1 and PKA inside cells. (a) PP1α and AKAP220 can be isolated from rat cell extracts. Rat brain was pulverized in liquid nitrogen and dounce-homogenized in Hendit buffer (20 mM HEPES pH 7.4, 20 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 1 mM AEBSF, 2 μg/ml leupeptin, and 1 mM benzamidine), incubated on ice for 30 min and lysates were prepared by centrifugation at 10,000 × *g* for 15 min at 4°C. Phosphatase complexes were purified by affinity chromatography on microcystin–sepharose [25]. Control extracts were preincubated with excess free microcystin (0.5 μM) for 30 min on ice. Samples were eluted in sample buffer, then separated by SDS–PAGE and transferred to Immobilon membrane for western blot analysis with monoclonal anti-rat AKAP220 antibodies (upper panel) or anti-PP1α antibodies (lower panel). (b) Immunoprecipitation of PKA activity from extract with PP1β antibodies. Rat testis extract was prepared as described above. The extract (1 ml) was incubated with either rabbit anti-PP1β antibody sera (anti-PP1) or pre-immune sera (control) for 2 h at 4°C. Immune complexes were isolated by incubation with protein A–Sepharose beads (30 μl) for 1 h at 4°C. After extensive washing with 1 M NaCl Hendit buffer, the catalytic subunit was eluted with 1 mM cAMP and activity was assayed using Kemptide as substrate [26]. PKA activity is defined as those counts (cpm) blocked by the addition of the PKI 5–24 inhibitor peptide (PKI peptide) [27]. A representative of three experiments is presented, with the error bars showing the standard error within that one experiment. (c) AKAP220, PP1γ and PKA occupy the same focal plane in neurons. Confocal analysis of AKAP220 (green), PP1γ (red), and the RII subunit of PKA (blue) in rat primary hippocampal neurons: a composite of the three images is also shown. Neurons were fixed with 3.7% formaldehyde in PBS for 5 min, washed twice with PBS, and permeabilized with ice-cold acetone for 1 min. After washing twice with PBS/0.1% BSA, primary antibodies (monoclonal anti-AKAP220 antibodies, rabbit polyclonal anti-PP1γ1 antibodies, and goat polyclonal anti-RII antibodies) were added for 1 h. Following three washes with PBS/BSA, secondary antibodies (fluorescein-isothiocyanate-conjugated donkey anti-mouse antibodies, Texas-Red-conjugated donkey anti-rabbit antibodies, and Cy-5-conjugated donkey anti-goat antibodies) were added for 1 h. The slides were washed with PBS/BSA and mounted using Prolong mounting reagent (Molecular Probes Inc). Confocal microscopy was performed on a MRC1024 Biorad UV/Vis system.

prevented by preincubation with excess free microcystin (Figure 3a). Complementary experiments demonstrated that immunoprecipitation of the PP1β isoform resulted in a 10.4 ± 3.8-fold (n = 3) enrichment of cAMP-dependent kinase activity over the control (Figure 3b). As PKA activity was blocked by a peptide corresponding to amino acids 5–24 of the specific PKA inhibitor PKI, it is reasonable to assume that the catalytic subunit of the kinase co-purified with the phosphatase. Finally, confocal analysis of endogenous AKAP220, PP1γ and RII in rat hippocampal neurons indicated that the staining patterns for all three proteins were distinct yet overlapping. PP1γ and RII were detected in the cell body and the neurite extensions, whereas the majority of AKAP220 staining was located in the cell body (Figure 3c). Similar results were observed when the distribution of AKAP220 was compared with PP1β (data not shown). The evidence that a subset of all three proteins occupy the same focal plane, when combined with *in vitro* and cell-based data, lead to the proposal that AKAP220 interacts with a proportion of PP1 and PKA inside cells.

Targeting kinases and phosphatases close to their sites of action allows for tight control of the phosphorylation state of certain substrates. In some cases both enzymes directly bind to each other [13–15], but often localization is mediated by interactions with a targeting protein [16]. Although numerous PP1 regulatory subunits have been identified [17–21], AKAP220 is the first example of a type 1 phosphatase targeting subunit that also binds to the PKA holoenzyme. Our data suggest that a subpopulation of each enzyme interacts with the anchoring protein. Our data further illustrate that AKAP220 can interact with multiple PP1 isoforms. While we have demonstrated that PP1α interacts with AKAP220 *in vitro*, our cell-based assays and immunoprecipitation data indicate that the anchoring protein interacts with the PP1β and PP1γ isoforms inside cells. On the basis of these findings it would appear that AKAP220 associates with multiple PP1 isoforms and conceivably could function to position PP1 and PKA where they can reversibly modulate the phosphorylation state of as yet unidentified substrates.

Acknowledgements

The authors thank Adam Kashishian (ICOS Corporation) for providing a partial clone of human AKAP220, Ernest Lee for purified PP1 catalytic subunit, Alan Saltiel for PTG protein, Brain Wadzinski and Alistair Sim for PP1 antibodies, Ann Westphal and Greg Scott for expert technical assistance, Derrick Carter and Richard Brennan for help in the computer modeling, and colleagues at the Vollum Institute for critical reading of the manuscript. Supported in part by NIH grant DK48239 to J.D.S.

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