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AKAP18:PKA-RII α structure reveals crucial anchor points for recognition of regulatory subunits of PKA

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Abstract

A-kinase anchoring proteins (AKAPs) interact with the dimerization/docking (D/D) domains of regulatory subunits of the ubiquitous protein kinase A (PKA). AKAPs tether PKA to defined cellular compartments establishing distinct pools to increase the specificity of PKA signalling. Here, we elucidated the structure of an extended PKA-binding domain of AKAP18 β bound to the D/D domain of the regulatory RII α subunits of PKA. We identified three hydrophilic anchor points in AKAP18 β outside the core PKA-binding domain, which mediate contacts with the D/D domain. Such anchor points are conserved within AKAPs that bind regulatory RII subunits of PKA. We derived a different set of anchor points in AKAPs binding regulatory RI subunits of PKA. *In vitro* and cell-based experiments confirm the relevance of these sites for the interaction of RII subunits with AKAP18 and of RI subunits with the RI-specific smAKAP. Thus we report a novel mechanism governing interactions of AKAPs with PKA. The sequence specificity of each AKAP around the anchor points and the requirement of these points for the tight binding of PKA allow the development of selective inhibitors to unequivocally ascribe cellular functions to the AKAP18-PKA and other AKAP-PKA interactions.

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AUTHOR CONTRIBUTION

Frank Götz generated recombinant proteins, carried out fine screens for crystallography, ITC, SLS and analytical ultracentrifugation; Yvette Roske collected data at Bessy II, determined the structure and contributed to writing the manuscript; Maike Svenja Schulz generated Flag-tagged AKAP constructs and cAMP-agarose and immunoprecipitations; Katja Faelber participated in the design of experiments; Kerstin Zühlke synthesized and analysed peptide spots; Annika Kreuchwig and Gerd Krause estimated *in silico* binding energies; Karolin Autenrieth and Friedrich Herberg provided PKA constructs, and performed Biacore experiments; Oliver Daumke and Udo Heinemann advised on protein expression and crystallization trials; Yvette Roske, Frank Götz and Enno Klussmann designed experiments and wrote the manuscript. Frank Götz and Yvette Roske contributed equally to the study.

Keywords

A-kinase anchoring protein; compartmentalized cAMP signalling; D/D domain; PKA-binding domain; protein kinase A; protein–protein interaction

INTRODUCTION

A-kinase-anchoring proteins (AKAPs) are a diverse family of around 50 scaffolding proteins with molecular masses ranging from 18 kDa to >500 kDa. They share the capacity to directly interact with protein kinase A (PKA) and thereby to tether the kinase to defined cellular sites. This controls PKA signalling spatially and temporally, and confers specificity to the action of this ubiquitous kinase. However, AKAPs' functions are not restricted to controlling PKA activity. They integrate cellular signalling processes by forming multi-protein complexes through direct protein–protein interactions with PKA substrates and other signalling proteins, such as further kinases, phosphodiesterases (PDEs) or phosphatases; their unique anchoring domains tether AKAP-based protein complexes to defined cellular compartments [1–6].

PKA consists of a dimer of regulatory RI (RI α or RI β) or RII (RII α or RII β) subunits and two catalytic subunits (C α , - β or - γ), each bound to one R subunit [7]. The regulatory subunits of PKA dimerize through interaction of their N-terminal dimerization/docking (D/D) domains (amino acids 1–44). The dimerized D/D domains form a hydrophobic cavity into which the A-kinase-binding domains (AKBs) of AKAPs dock. AKAPs can interact only with dimers of regulatory subunits. A few AKAPs can bind both RI and RII subunits (dual specific AKAPs) [8,9], most AKAPs preferentially bind RII [10,11], and some preferentially or specifically bind RI, such as SKIP [12,13] and smAKAP [14].

NMR analysis has revealed the 3D structure of the D/D domain of RII α in complex with the AKBs of AKAP-Lbc [peptide Ht31(493–515), 23 amino acids] [15] and of AKAP79 [peptide AKAP79(392–408), 16 amino acids] [15,16]. X-ray crystallography resolved 3D structures of the AKBs of D-AKAP2 (amino acid residues 631–651, 20 amino acids) [17] and the *in silico*-designed AKAP-*IS* (amino acid residues 4–20, 16 amino acid residues) [18] with the D/D domain of RII α . All structures show that AKBs are amphipathic helices. The hydrophobic face of the helix docks into the hydrophobic cavity formed by the D/D domains, which have an X-type helix bundle conformation. Thus hydrophobic interactions are considered the backbone of the binding. Comparison with additional AKAPs indicates that the amphipathic helix is a conserved motif within the AKAP family mediating the interaction with D/D domains. The conserved positioning of hydrophobic amino acid residues allowed definition of an AKAP signature motif – *HxxHHxxHHxxHHxxHH* – in which where *H* denotes hydrophobic amino acid residues in conserved positions of AKBs and *x* any amino acid residue [19]. Database screening for this motif, combined with the range of isoelectric points of known AKBs (3.43–6.23), or with the available structural information, has identified novel AKAPs [19,20]. There are hints that allosteric sites such as the RI specifier region in D-AKAPs [21] and a region C-terminal to the D/D domain of RII α

[22] are involved in AKAP–PKA interactions but there is no support from structural analyses.

Interactions between AKAPs and PKA are involved in an array of cellular processes including vasopressin-mediated water reabsorption in renal principal cells [23,24], cardiac myocyte contractility [25] and lipolysis in adipocytes [26,27]. AKAP18 (AKAP7) comprises a family of four isoforms, AKAP18 α , - β , - γ and - δ (see Supplementary Figure S1), of which the α , β and γ isoforms are expressed in humans whereas the δ isoform is found in rats [10,28–31]. AKAP18 α (also termed AKAP15 or AKAP7 α) is one of three AKAPs interacting with L-type calcium channels in cardiac myocytes. AKAP18 α is believed to facilitate the phosphorylation of the channel by PKA and thereby to increase Ca²⁺ entry [31]. Compared with AKAP18 α , AKAP18 β carries an insert of 23 amino acid residues in its N-terminus, which leads to apical plasma membrane localization of the AKAP–PKA complex [30]. AKAP18 γ forms a complex with PKA, sarcoplasmic reticulum calcium ATPase 2 (SERCA2) and PDE3A1 in human myocardium. These interactions participate in the control of Ca²⁺ reuptake into the sarcoplasmic reticulum during diastole [32]. The interaction of AKAP18 δ with PKA and phospholamban controls Ca²⁺ reuptake into the sarcoplasmic reticulum of rat cardiac myocytes [33], and apparently plays a role in the control of vasopressin-mediated water reabsorption in renal principal cells [10].

The various biological functions of the AKAP–PKA interactions have mainly been revealed by inhibition with synthetic membrane-permeant peptides. Peptides derived from AKBs of AKAPs such as AKAP-Lbc (peptide Ht31) or AKAP18 δ (peptide AKAP18 δ L314E) bind regulatory subunits of PKA with nanomolar affinity, and thereby globally uncouple PKA from AKAPs [4,34,35]. More recent studies led to peptides that preferentially disrupt AKAP–RI or AKAP–RII interactions [36–41]. However, no agent for selective disruption of any defined AKAP–PKA interaction is to date available; the closest are peptides derived from regulatory subunits of PKA that preferentially interfere with AKAP18–PKA or AKAP79–PKA interactions [42]. The lack of specific inhibitors of defined AKAP–PKA interactions impairs the unequivocal ascription of particular functions. One prerequisite for understanding the molecular mechanisms underlying specific interactions and the design of selective inhibitors is the elucidation of 3D structures of individual AKAP–PKA complexes.

In the present study, we report the 3D structure of a complex of an extended AKB of AKAP18 β with the D/D domain of RII α , which reveals a novel binding frame for RII α , mediated by conserved anchor points in the AKAP sequence. Such anchor points are present in other AKAPs. These sites provide unique opportunities for specific pharmacological interference with a defined AKAP–PKA interaction.

EXPERIMENTAL

Cloning and generation of recombinant AKAP18 β and the D/D domain of RII α

An AKAP18 β cDNA fragment encoding amino acid residues 43–83 (NGGEP DDAEL VRLSK RLVEN AVLKA VQQY LEETQ NKNKP GE) was cloned by ligation-independent cloning (LIC) into vector pET30 Ek/LIC (Merck Millipore). PCR was used for introduction of an additional PreScission recognition site for His-tag removal. The AKAP18 β (43–83)-

encoding cDNA fragment was amplified from a pCMV6-XL5 clone (Origene, NM_138633) and purified, and 0.2 pmol was treated with T4 DNA polymerase and annealed with the vector pET30 Ek/LIC, which was transformed into *Escherichia coli* strain NovaBlue GigaSingles-competent cells (Novagen). The untagged RII α -D/D domain (amino acids 1–44) was cloned into pET46 by removing the N-terminal His-tag from His-D/D-RII α in pET46 Ek/LIC [43] by PCR with the primer pair DD forward: 5'-GGTCA GCCAT GGCAA GCCAC ATCCA GAT-3', reverse: 5'-CGTCT TCTCG AGTTA GCGGG CCTCG CG-3', and restriction with NcoI/XhoI.

For crystallization, the two proteins were co-expressed in *E. coli* strain Rosetta 2 DE3 in the presence of the antibiotics kanamycin, ampicillin and chloramphenicol. Pre-cultures from cryo stocks were grown in 50 ml of lysogeny broth (LB) medium at 37 °C overnight, centrifuged and resuspended to inoculate up to four 500-ml Overnight Express Instant TB Medium specimens (autoinduction medium, Novagen). Cultures were grown for at least 24 h at 37 °C under constant agitation (110 rev./min) in 2-l Erlenmeyer flasks. Cells were harvested by centrifugation, washed with PBS and stored at – 80 °C.

Lysates were prepared in 40 mM phosphate buffer, pH 7.5, 300 mM NaCl, 5 mM imidazole, 2 mM MgCl₂, 5 mM 2-mercaptoethanol, 0.5 mM PMSF, a protease inhibitor tablet (Roche) and benzonase (5 μ l/100 ml) using a fluidizer. Cell debris was removed by centrifugation at 21000 rev./min at 4 °C for 30 min. The supernatant was cleared by filtration through a 0.45- μ m filter and applied to a TALON cobalt affinity column (Clontech) in an ÄKTA system pre-equilibrated with 40 mM phosphate buffer, pH 7.5, 300 mM NaCl and 5 mM imidazole. The TALON column was washed with equilibration buffer with 10 mM imidazole and eluted with 40 mM phosphate buffer, pH 7.5, 300 mM NaCl and an imidazole gradient up to 300 mM. His-AKAP18 β (43–83)–RII α -D/D complex-containing fractions were pooled. After dialysis against 20 mM phosphate, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, the His-tag of AKAP18 β (43–83) was removed overnight with His-PreScission protease. His-tag and His-PreScission were separated from the tag-free AKAP18 β (43–83)–RII α -D/D complex using a TALON cobalt affinity column chromatography under identical buffer conditions. The final polishing step was gel filtration with a HiLoad 16/600 Superdex 75 (GE Healthcare) in 20 mM Hepes, pH 7.5, 150 mM NaCl, 0.2 mM MgCl₂. The purest fractions were pooled and stored at – 80 °C.

For interaction studies with ITC (Biacore), a double mutant of AKAP18 β (43–83), AKAP18 β (43–83)D49A/E74A, and a triple mutant, AKAP18 β (43–83)D49A/Q70A/E74A, in pET30 Ek/LIC-AKAP18 β (43–83) were generated. The corresponding point mutations were introduced into pET30 Ek/LIC AKAP18 β (43–83) in three consecutive rounds of PCR by using Pfu Ultra II polymerase (primer pair AK18 β -D49A forward: 5'-GGAGG GGAGC CCGAT GCCGC TGAAC TAGTA AGG-3', reverse: 5'-CCTTA CTAGT TCAGC GGCAT CGGGC TCCCC TCC-3'; and primer pair AK18 β -E74A forward: 5'-GTCCA GCAGT ATCTG GAGGC AACA CAGAA TAAAA AC-3', reverse: 5'-GTTTT TATTC TGTGT TGCCT CCAGA TACTG CTGGA C-3' to generate the double mutant AKAP18 β (43–83)D49A/E74A; primer pair AK18 β -Q70A forward: 5'-GCTCA AGGCT GTCCA GGCGT ATCTG GAGGC AACA-3', reverse: 5'-TGTTG CCTCC AGATA CGCCT GGACA GCCTT GAGC-3' to obtain the triple mutant AKAP18 β (43–83)D49A/Q70A/E74A). After

DpnI digestion, the reactions were transformed in Strata Clone Solo Pack-competent cells (Agilent). Mutations were verified by sequencing. AKAP18 β (43–83), AKAP18 β (43–83)D49A/E74A and AKAP18 β (43–83)D49A/Q70A/E74A were expressed in *E. coli* (strain Rosetta 2 DE3) in the presence of the antibiotics kanamycin and chloramphenicol. Purification of the proteins was carried out as described above for the AKAP18 β (43–83)–RII α -D/D domain complex. The D/D domains of RII α or full-length RII α were obtained as previously described [43].

Crystallization and data collection

The purified complex AKAP18 β (43–83)–RII α -D/D(1–44) was concentrated to 49 mg/ml and mixed with an equal volume (200 nl) of reservoir solution containing 21 %PEG3350, 10 mM cadmium chloride hydrate, 0.1 M sodium acetate, pH 4.5. The crystallization experiments were performed using the sitting-drop, vapour-diffusion method at 20 °C with a Gryphon pipetting robot (Matrix Technologies Corp.) and a Rock Imager 1000 storage system (Formulatrix).

Crystals of the complex appeared after 2–3 weeks and were flash frozen in liquid nitrogen in a cryo-solution containing an additional 20 % glycerol relative to the reservoir solution. Diffraction data were collected on BL14.1 operated by Helmholtz-Zentrum Berlin (HZB) at the BESSY II electron storage ring. The data were processed and scaled using the XDSAPP suite [44].

Structure determination

Phases for the AKAP18 β –RII α -D/D complex were obtained by molecular replacement with PHASER [45] using the RII α -D/D domain of the AKAP-*IS*–RII α -D/D domain complex (Protein Data Bank or PDB: 2IZX) as a search model [18]. The AKAP18 β –RII α -D/D complex structure was manually built using COOT [46] and iteratively refined using REFMAC. Two prominent electron density maxima at the centre of the AKAP18 β –RII α -D/D pseudo-trimer (see Results) were assigned as Cd²⁺ positions based on the presence of cadmium chloride in the crystallization buffer. The presence of Cd²⁺ was inferred from the short distances of the Cd–O bonds (typically 2–2.5 Å, 1 Å = 0.1 nm) and the coordination geometry [47]. Data collection and refinement statistics are reported in Table 1. Analysis with MolProbity (<http://molprobity.biochem.duke.edu/>) shows that 98.5 % of the modelled residues are in favoured regions of the Ramachandran map with no outliers. Figures were prepared using PyMOL Molecular Graphics System (version 1.3, Schrödinger, LLC). Domain superimpositions were also performed with PyMOL, which aligns the sequences and computes a structural alignment with several cycles of refinement in order to reject structural outliers found during the fit. The angle calculation of the AKAP helices bound to the D/D dimers was performed with the Python module ‘anglebetweenhelices’ implemented in PyMOL.

Static light scattering

Static light scattering (SLS) experiments were carried out using a Superdex 75 10/300 SEC analytical column attached to an ÄKTA FPLC system (both GE Healthcare), coupled to VE3580 RI and 270 Dual detectors (Viscotek). Evaluation of the data was performed using

the OmniSec software (Viscotek) and the RI signal. The protein concentration of the complex AKAP18 β (43–83)–RII α -D/D(1–44) was adjusted to 200 μ M in 20 mM Hepes, 150 mM NaCl, pH 7.5, complemented with 2 mM CdCl₂ or 2 mM EDTA. All samples were injected in 200- μ l aliquots. The Superdex 75 10/300 column was equilibrated with the same buffer, and run at a flow rate of 0.5 ml/min.

Analytical ultracentrifugation

Sedimentation velocity experiments of the complex AKAP18 β (43–83)–RII α -D/D(1–44) were carried out at 20 °C with an Optima XL-i analytical ultracentrifuge (Beckman) at 35000 rev./min with a 60-Ti rotor (Beckman Instruments). The analysed samples were prepared in 20 mM Hepes, 150 mM NaCl, pH 7.5 with either 2 mM CdCl₂ or 2 mM EDTA at a protein concentration of 0.6 mg/ml. Two-channel Epon centrepieces with an optical path of 12 mm were used, and all experiments were performed using sapphire windows. Scans were recorded at 280 nm with radial spacing of 0.005 cm. The program Sednterp was used to estimate the partial specific volume from amino acid composition as well as the density, ρ , and viscosity, η . Data were then analysed using the program SEDFIT [48] with a continuous $\alpha(s)$ distribution model. Theoretical sedimentation coefficients for monomeric and higher oligomeric protein species were calculated using the following equation:

$$s = \frac{M_r(1-\rho)}{N_A(f/f_0)6\pi\eta\sqrt[3]{3N/\pi N_A}}$$

with N_A is Avogadro's number, and M_r and f/f_0 the protein's molecular mass and frictional ratio, respectively. Assuming a frictional ratio of 1.25, the theoretical sedimentation coefficients of 1.7 and 2.2 were calculated for a monomeric or dimeric complex, respectively (one copy of AKAP18 β (43–83) and two copies of RII α -D/D(1–44). The monomeric complex has a molecular mass of 15.0 kDa.

In silico binding energy estimation

The free energies of binding G_{bind} (kJ/mol) were estimated using the equation:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}})$$

where G_{complex} is the optimized free energy for the complex, and G_{protein} and G_{ligand} are the optimized free energies for the free D/D domain and free peptide, respectively. Each energy term was calculated by a combination of molecular mechanics energy, implicit solvation energy and surface area energy.

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed in 20 mM Hepes, pH 7.5, 150 mM NaCl using a VP-ITC titration microcalorimeter (GE Healthcare). The D/D domain of RII α (20 μ M) was loaded into the calorimeter cell. The titration syringe was filled with either AKAP18 β (43–83), AKAP18 β (43–83)D49A/E74A or AKAP18 β (43–83)D49A/Q70A/E74A at 100 μ M. Titrations were carried out using 25–30 injections of 10–12 μ l each

injected at 5- to 20-min intervals at a constant temperature of 25 °C. Raw data in the form of the incremental heat per mole of added protein were fitted with the ORIGIN7 software by non-linear least squares using a one-site binding model for the D/D domain of RII α -AKAP18 β (43–83) titration. The protein concentration was adjusted by this fit. In the case of the mutants AKAP18 β (43–83)D49A/E74A and AKAP18 β (43–83)D49A/Q70A/E74A, fitting of the data to a one-site binding model was not possible.

Surface plasmon resonance measurements

Interaction studies were performed using a Biacore T200 instrument (GE Healthcare). RII α subunits were captured as previously described [49]. Measurements were performed in running buffer containing 20 mM Mops, 150 mM NaCl, 3 mM EDTA, 0.05 % (v/v) surfactant P20, pH 7. In brief, 8-AHA-cAMP (BIOLOG Life Science Institute) was covalently coupled to CM5 sensor chips (GE Healthcare) using standard *N*-hydroxysuccinimide (NHS)/*N*-ethyl-*N*-(3-(dimethylamino)propyl)carbodiimide (EDC) chemistry [50]. Each flow cell was activated, coupled and deactivated individually with a flow rate of 5 μ l/min at 25 °C. Purified human PKA RII α was injected in running buffer containing 2 mg/ml BSA for reversible capture on the 8-AHA-cAMP surface (surface density 290 resonance units). An 8-AHA-cAMP surface without captured R subunit served as reference in order to test for unspecific binding. After each cycle, regeneration of the surface was performed by two short injections (15 s each) of 0.1 % (w/v) SDS, followed by a single injection of 3 M guanidinium hydrochloride (30 s) and an injection of running buffer with 1 M NaCl (30 s). Kinetic analyses were performed by injection of increasing concentrations (0.2–146 nM) of the AKAP18 β variants at a flow rate of 100 μ l/min at 37 °C. Association signals were recorded for 60 s and the dissociation of the highest AKAP18 β concentration (146 nM) was monitored for 2 h. After subtracting the reference cell signal, the resulting binding signals were evaluated using BIAevaluation software version 4.1.1 (Biacore Life Science, GE Healthcare, Freiburg, Germany). The dissociation phase was fitted separately using the same software.

Generation of Flag-AKAP18 γ and Flag-smAKAP

These plasmids were generated by standard PCR using Pfu Ultra II polymerase. Full-length human AKAP18 γ (D293A/Q314A/E318A) was generated by sequential mutagenesis of plasmid AKAP18 γ (wild-type AKAP18 γ purchased as GeneArt Strings DNA fragment, Life Technologies, and inserted into pCMV6-Entry, Origene, PS100001). The primer pairs for PCR were: AK18 γ -E318A forward: 5'-GTCCA GCAGT ATCTG GAGGC AACAC AGAAT AAAAAC-3', reverse: 5'-GTTTT TATTC TGTGT TGCCT CCAGA TACTG CTGGAC-3'; primer pair AK18 γ -Q314A forward: 5'-GCTCA AGGCT GTCCA GCGT ATCTG GAGGC AACAC-3', reverse: 5'-TGTTG CCTCC AGATA CGCCT GGACA GCCTT GAGC-3'; and AK18 γ -D293A forward: 5'-GAGCC CGATG CCGCT GAACT AGTAA GGC-3', reverse: 5'-GCCTT ACTAG TTCAG CGGCA TCGGG CTC-3'. After DpnI digestion Strata Clone Solo Pack-competent cells were transformed with the DNAs. Mutations were confirmed by sequencing.

For the generation of wild-type smAKAP and smAKAP (D72A/Q76A) synthetic DNA sequences (Eurofins Genomics; sequences are shown in the supplementary information) were inserted into plasmid pCMV6-Entry (Origene, PS100001).

Cell culture, AKAP expression, immunoprecipitation and Western blotting

HEK293 cells were grown in Dulbecco's Modified Eagle Medium or DMEM (GlutaMAX, 10 % FBS, Thermo Fisher Scientific) and transfected with the above-mentioned plasmids. The cells were lysed in lysis buffer (140 mM NaCl, 3 mM KCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 0.2 % Triton X-100, 2 mM EDTA, 2 mM EGTA) containing protease inhibitors (cOmplete, Roche) and phosphatase inhibitors (Phosphostop, Roche). The lysates were cleared by centrifugation (20 000 g, 4 °C, 10 min). Proteins were precipitated using anti-FLAG M2 magnetic beads (Sigma-Aldrich) [51] and eluted from the beads using 0.1 M glycine buffer, pH 2.5. The eluate was neutralized with 1 M Tris, pH 10.6, and incubated with Laemmli sample buffer for 8 min at 95 °C. Flag-tagged AKAPs, RI α , RII α and RII β were detected by Western blotting with specific primary and horseradish peroxidase-coupled secondary antibodies (anti-Flag antibody: Sigma Aldrich #Flag M2; anti-RI α : BD Biosciences no. 610610; anti-II α : BD Biosciences no. 612243; anti-II β : BD Biosciences no. 610626; anti-DDK (Flag): Origene TA50011, POD-anti-mouse IgG: Immuno Research no. 715-035-151) [10,19].

Peptide spot array assay

Peptides derived from the AKBs of AKAP18 β were generated by automatic SPOT synthesis on Whatman 50 cellulose membranes using 9-fluorenylmethyloxycarbonyl chemistry and the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments). The interaction of membrane-bound peptides with RII α was investigated by RII overlay. The membrane-bound peptides were overlaid with recombinant, radioactively labelled RII α subunits (³²P) as described previously [19,35,52]. Interaction was detected by autoradiography.

ACCESSION NUMBERS

Coordinates and structure factors for the human AKAP18 β -RII α -D/D have been deposited in the PDB under accession code 4ZP3.

RESULTS AND DISCUSSION

Overall structure and crystal packing of the AKAP18 β -RII α -D/D complex

AKAP18 β (amino acid residues 43–83) and the D/D domain of RII α (amino acid residues 1–44) were co-expressed in *E. coli* and the AKAP18 β -RII α -D/D complex purified. Its structure was determined by molecular replacement from a crystal diffracting to 2.63 Å resolution, and refined to a R_{work} and R_{free} of 22.2 % and 26.8 %, respectively (see Table 1).

The asymmetrical unit in the crystals contained six AKAP18 β -RII α -D/D complexes. Each complex is composed of one AKAP18 β molecule bound to an RII α -D/D dimer. The six AKAP18 β -RII α -D/D complexes are arranged as two identical pseudo-trimers in a propeller-like fashion (see Supplementary Figure S2A). The central pseudo-3-fold propeller axis is

defined by two cadmium ions bound to Glu⁴¹ of the helices B and B' of all RII α -D/D dimers (see Supplementary Figure S2B). The pseudo-trimeric arrangement is further stabilized by two symmetrical salt bridges between Arg³⁸ and Glu³⁴ of two adjacent D/D dimers (see Supplementary Figure S2C). In each pseudo-trimer, the 3-fold symmetry is broken by one complex that is rotated 180° with respect to the two others, so that its bound AKAP18 β extends in the opposite direction. However, the binding mode of AKAP18 β to the RII α -D/D dimer in the rotated complex is identical to the other two complexes. Overall, the superimposition of all six complex structures, found in the asymmetrical unit of the crystal, revealed no major structural differences except for some termini in the molecules (see below). The six complexes align with averaged RMSDs between equivalent C α atoms ranging from 0.330 Å to 0.318 Å.

SLC coupled to size exclusion chromatography and analytical ultracentrifugation was performed to investigate a putative physiological relevance of the bound cadmium ion in the formation of higher oligomeric AKAP18 β -RII α -D/D complex structures. Both datasets show that the AKAP18 β -RII α -D/D complex has an absolute molecular mass between 13 and 17 kDa in the presence or absence of 2 mM CdCl₂ or 2 mM EDTA, which is in agreement with the calculated molecular mass of 15 kDa of the AKAP18 β -RII α -D/D (1:2 molar ratio) complex (see Supplementary Figure S3). No formation of higher oligomeric AKAP18 β -RII α -D/D complexes was observed, which indicates that cadmium mediates only the packing of AKAP18 β -RII α -D/D complexes in the crystal and does not have a biological relevance for this complex. Only in the presence of cadmium were crystals of the AKAP18 β -RII α -D/D complex obtained that diffracted to a suitable resolution for structure determination.

In two D/D complexes, the two N'-termini are well ordered and positioned along the AKAP18 β helix, as in the AKAP-*IS*-RII α -D/D complex [18]. In the remaining four complexes, the N-termini point ~90° away from the amphipathic helix and are less well ordered (see Supplementary Figure S3). In addition, AKAP18 β in all complexes varies at the N- and C-termini. The first five to eight N-terminal residues and the last three to eight C-terminal residues are disordered in the various molecules (see Supplementary Figure S4B).

The hydrophobic core region is stabilized by flanking salt bridges

For a detailed description of the structure, two AKAP18 β -RII α -D/D complexes were superimposed with an RMSD of 0.305 Å, to derive a model with the maximum length of the AKAP18 β amphipathic helix in N- and C-terminal directions from amino acid positions 48–80 (Figure 1). The RII α -D/D dimer shows the known X-type helix bundle formed by the helices A, B, A' and B' (Figure 1A) [15–18]. The hydrophobic face of the amphipathic AKAP18 β helix is composed of nine turns (I–IX) and docks into the hydrophobic cavity of the RII α -D/D dimer, formed by helices A and A' of the two protomers of the D/D domains (Figures 1B and 2). Helices B and B' are not involved in the interaction (Figure 1A). AKAP18 β shows an asymmetrical binding to the RII α -D/D dimer (Figure 2) as also observed for AKAP-*IS* and D-AKAP2 [17,18]. Our complex structure indicates that the asymmetrical orientation of the AKAP helix is governed by the localization of the conserved hydrophobic core sequence for RII binding, *HHxxHHxxHHxxHHxxHH*, of the amphipathic

helix and is not caused by crystal contacts or by a flexible N'-tail of the RII α -D/D dimer as assumed for the AKAP-*IS* and D-AKAP2 complex structures [17,18].

Eleven residues in the AKAP18 β helix (Leu⁵², Val⁵³, Leu⁵⁵, Leu⁵⁹, Val⁶⁰, Ala⁶³, Val⁶⁴, Ala⁶⁷, Val⁶⁸, Tyr⁷¹ and Leu⁷²) engage in hydrophobic interactions with the RII α -D/D dimer (Figures 1B and 2A). The amphipathic hydrophobic core is localized within turns I–VI of the AKAP18 β helix and constitutes the backbone of the interaction. In addition, Leu⁵², Leu⁵⁵, Leu⁵⁹, Val⁶⁸, Tyr⁷¹ and Leu⁷² project horizontally along the D/D dimer and mediate binding to a hydrophobic surface in the RII α -D/D N-termini. Of note, Asp⁴⁹ and Glu⁷⁴ are engaged in salt bridges with Arg²² and Arg^{22'} located in the RII α -D/D helices A and A', respectively (Figure 2A). The binding of the C-terminus of the AKAP helix to the RII α -D/D dimer is further stabilized by an H-bond formed by Gln⁷⁰ of AKAP18 β with Gln¹⁴ of the RII α -D/D dimer. This polar interaction was also observed in the binding of AKAP-*IS* to the RII α -D/D dimer [18]. A schematic representation of the interactions is shown in Figure 2B.

Anchor points in AKAP helices define specificity for PKA binding

Surprisingly, two additional polar interactions of Arg^{22'} and Arg²² of the RII α -D/D dimer with the AKAP18 β residues Asp⁴⁹ and Glu⁷⁴, respectively, were observed. In addition, Gln¹⁴ of the RII α helix A forms a hydrogen bond with AKAP18 β residue Gln⁷⁰. Thus Asp⁴⁹, Gln⁷⁰ and Glu⁷⁴ appear to serve as hydrophilic anchors in the long AKAP helix to facilitate a stable and specific binding to RII α -D/D dimers (see below). This set of three additional interactions was not found in the AKAP–RII α -D/D dimer complex structures known to date due to the limited lengths of the bound AKAP helices (17–24 amino acids) compared with the long AKAP18 β helix reported here (32 residues).

Besides the five conserved hydrophobic core patches (Figure 2B), the three additional anchor points support the orientation of the AKAP helix relative to the RII α -D/D dimer. The long straight AKAP18 β helix is bound with an angle of 44° to the RII α N-terminal helix A (Figure 3A). Sequence alignment of RI-, RII- and dual-specific AKAPs revealed a conservation of the three anchor points for RII-binding AKAPs (Figure 3E). Analyses of the structurally known complexes of RII α -D/D bound to AKAP79 (AKAP5) and D-AKAP2 (AKAP10) revealed a similar binding angle for the AKAP helix of 46° and 47°, respectively (Figures 3B and 3D). We assume that AKAPs with either an aspartate or a glutamate, in anchor point positions I and III in the helix, bind with an angle between 44° and 47° to the RII α helix A. The distance of the C α atoms of anchor points I and III is 37 Å, spanning 25 residues. This supports a rigid binding mode for RII-specific AKAPs because the RII α -D/D dimer presents two Arg²² residues, which are the counterparts for salt bridge formation with anchor points I and III (Figure 3A). Gln¹⁴ in the RII α -D/D dimer constitutes the binding site for anchor point II and allows for a greater variety of interacting amino acids in the AKAP helix, such as glutamine, asparagine, glutamate, aspartate, lysine and arginine. These residues have the potential to engage in hydrogen bonds with Gln¹⁴ to further stabilize the AKAP–RII α -D/D interaction.

Based on sequence alignments, we expect the RII-specific AKAP18 α , AKAP18 δ , AKAP6 and AKAP14 to bind in a similar fashion to RII (Figure 3E), because they contain the same three anchor points. RII-specific AKAPs that do not have the conserved anchor point

residues may adopt a more flexible binding mode, giving the freedom for bending of the AKAP helix, such as that seen in the AKAP13 (AKAP-Lbc)–RII α -D/D complex (Figure 3C). The bent AKAP-Lbc helix binds with an angle of approximately 29° to the RII α helix A. A similar binding behaviour is expected for the RII-specific AKAP8, AKAP9, AKAP12 and GSKIP because they also lack the three required anchor point residues. This shows that there are two different binding modes to RII α . Even more important, the three identified anchor points not only are involved in orienting the AKAP helix with regard to the RII α -D/D domain, but also further define the binding frame for RII-specific AKAPs (Figures 3A and 3E). The previously known five hydrophobic patches of the amphipathic AKAP helix (Figure 3B) are now flanked by the three anchor points in the fashion

$N_{xx}H_{xx}HH_{xx}HH_{xx}HH_{xx}HH_{xx}P_{xx}N$, where N stands for negatively charged, H for hydrophobic, P for polar and x for any residue. Sequence alignments reveal that the dual-specific AKAP5, AKAP10 (D-AKAP2), Ezrin, AKAP1 and MAP2D may also bind RII α in a similar fashion to that observed for AKAP18 β (Figure 3A) in our structure (Figure 3E). Only AKAP4 (FSC1A) and AKAP11 of the dual-specific AKAPs lack all, and AKAP3 one, of the anchor points. These AKAPs are probably more flexible in binding to RII α , as also seen for AKAP13.

On closer inspection of the structures of the dual specific D-AKAP2 bound to RI α (PDB: 3IM4) and RII α (PDB: 2HWN), together with sequence alignment analyses, it also becomes evident that there is a RI α -specific anchor frame that differs from the RII α anchor frame (Figures 3D and 3E). The AKAP RI α anchor frame follows the sequence pattern $N_{xx}HH_{xx}HH_{xx}HH_{xx}P_{xx}HH_{xx}NH$. The RI α anchor point II is inserted between hydrophobic patches 3 and 4 and anchor III between patches 4 and 5 (Figure 3E). This is in contrast to the RII α anchor points II and III, which are located C-terminally after patch 5. The central hydrophobic core patch in the AKAP sequence makes a helical register shift of one turn in the C-terminal direction, compared with the RII α frame. The shift was already reported in the comparison of the two D-AKAP2 complex structures [53]. We observe that the RI α anchor point III is placed by the helical shift in the same local position of anchor point II in the RII α -bound AKAPs (Figure 3E). However, as the D-AKAP2 helix in the RII α -D/D domain-bound complex is too short, the two different anchor frames for RI α and RII α were not recognized. The two specific frames at the AKAP helix, together with the complementing amino acids in the D/D domains, become visible on superimposition of the D-AKAP2–RI α and D-AKAP2–RII α complex structures (Figure 3D). In the D-AKAP2–RI α complex anchor points I (Glu⁶³²) and III (Gln⁶⁴⁹) bind to the complementary, positively charged Lys³⁰ residues in the RI α -D/D dimer. This is comparable with anchor points I and III in RII α -specific AKAPs that bind the complementary Arg²² residues in the RII α -D/D dimer, but with the difference that the RI α -specific anchor points I and II have a closer C_{α} distance of 25 Å to each other, than seen for RII α (30 Å).

Sequence alignments indicate that the need for all three anchor points in the AKAP helix for specific binding to RI α may not be as crucial as for binding to RII α (Figure 3E). The AKAPs specific for RI α often use only a set of two anchor points in different combinations as observed for AKAP_{CE}, smAKAP, SKIP and PAP7. The same is recognized for most of the dual specific AKAPs aligned in frame for RI α binding, such as AKAP5, Ezrin, AKAP4

(FSC1A), AKAP11 and MAD2D, with the exception of AKAP10 (D-AKAP2) and AKAP1, which make use of three anchor points. AKAP3 and AKAP4 (FSC1B) employ only the most highly conserved anchor point II for RII α binding, suggesting a slightly different binding compared with D-AKAP2 binding of RII α .

Loss of anchor points in AKAP18 crucially reduces RII binding *in vitro*

For evaluation of the influence of individual amino acids on the interaction between AKAP18 β and full-length RII α subunits of PKA, we substituted each amino acid residue between Asp⁴⁹ and Glu⁷⁴ of AKAP18 β with alanine. Polypeptides (with 30 amino acids) representing the wild-type sequence or peptides carrying the substitutions were spot synthesized and overlaid with radioactively labelled RII α subunits (Figure 4). Substitutions of any of the amino acid residues engaged in hydrophobic interactions with an alanine did not substantially affect the binding. Binding was reduced by a maximum of 25 %, e.g. by Leu⁵⁹Arg (Figure 4A). Only the introduction of kinks into the helix through substitution of two hydrophobic amino acid residues with prolines abolished the binding (Figure 4A). Individual alanine substitutions of the anchor points Asp⁴⁹, Gln⁷⁰ and Glu⁷⁴ modestly reduced the binding. However, if two of these anchors were replaced by alanine, the binding decreased by approximately 60 %. Moreover, the loss of all three amino acid residues reduced binding to 40 % compared with the interaction of the wild-type sequence (Figure 4B). Thus these three residues are key determinants of the AKAP18 β -RII α interaction. We also substituted amino acids between Asp⁴⁹ and Glu⁷⁴ individually with aspartate and carried out an RII overlay (Figure 4C). The introduction of the negative charge had generally more pronounced effects on the interaction compared with the alanine substitutions. Replacement of Ser⁵⁶, Leu⁵⁹, Val⁶⁰, Ala⁶³, Val⁶⁴ or Ala⁶⁷ reduced binding by at least 60 %. A reduction by approximately 40 % resulted from substitutions, with aspartate, of the hydrophobic residues Leu⁵², Leu⁵⁵ or Val⁶⁸, which are involved in the binding of the N-termini of the RII α -D/D domain. The single substitutions of anchor points II (Gln⁷⁰) and III (Glu⁷⁴), with the negatively charged aspartate or with alanine alone, hardly affected the interaction (Figures 4B and 4C). These experiments confirm that substitution of an individual anchor point with either an alanine or an aspartate does not effectively interfere with the interaction, i.e. a minimum of two anchor points are sufficient to maintain a stable AKAP-RII α interaction.

To estimate the relative binding affinity of the triple-Ala-AKAP18 β (D49A/Q70A/E74A) to the D/D domain of PKA compared with the wild-type (wt) AKAP18 β , an *in silico* approach using the Prime/MM-GBSA module of the Schrödinger suite (Schrödinger Release 2015-1 – see <http://www.schrodinger.com/citations>) was used to calculate the G_{bind} (kJ/mol). For the AKAP18 β -wt-RII α -D/D complex the calculation of G_{bind} gave – 179.9 kJ/mol, whereas the loss of three bonds in the triple-Ala-AKAP18 β led to a decreased G_{bind} of – 167.6 kJ/mol. From these values the calculated dissociation constant K_D of triple-Ala-AKAP18 β is 140-fold larger than that of AKAP18 β . This supports computationally the reduction in binding affinity of triple-Ala-AKAP18 β and, together with the peptide spot analyses, confirms the importance of the hydrophilic anchor point interactions of AKAP18 β and the RII α -D/D domain.

ITC measurements were performed to evaluate the binding strength of AKAP18 β (43–83) and the triple anchor point substitution AKAP18 β (43–83)D49A/Q70A/E74A. One AKAP18 β (43–83) molecule binds to one RII α -D/D dimer with an estimated K_D of 0.6 nM $\pm$ 0.2 nM, using a one-site binding model (Figure 5A). This value is close to K_D values determined in Biacore measurements for the interaction of a peptide (25 amino acids) representing the PKA-binding domain of AKAP18 δ to RII α (0.4 nM) [10,35]. Binding of AKAP18 β (43–83)D49A/Q70A/E74A to the D/D domain was dramatically different. The titration could be fitted only with a sequential binding model and allowed an estimation of the binding constants for the two binding phases of K_{D1} = 0.4 nM and K_{D2} = 22.8 nM (Figure 5A). Presumably, the AKAP18 β (43–83)D49A/Q70A/E74A helix cannot immediately anchor to the D/D domain, resulting in multiple different binding modes. These observations underline our findings in the peptide spot analyses and the *in silico* calculations that, as soon as AKAP18 β lacks two or three anchor points, there is a drastic reduction in binding strength to the RII α -D/D dimer.

To determine the kinetics for the interaction of RII α with wild-type AKAP18 β (43–83) and the triple mutant, surface plasmon resonance measurements were performed. RII α was captured on an 8-AHA-cAMP chip and the AKAP18 β variants were injected at a flow rate of 100 μ l/min. For the association, the AKAP18 variants were injected for 60 s. Both variants displayed similar association behaviours (Figure 5B, inset). To obtain accurate dissociation phases, the dissociation phases were recorded for 2 h and dissociation rate constants of 3.1×10^{-5} /mol per s and 1.5×10^{-4} /mol per s were calculated for wild-type and mutant protein, respectively. A 10-fold faster dissociation rate of the triple mutant compared with the wild type was revealed (Figure 5B). Thus it appears that the anchor points affect the dissociation of AKAP18 from rather than the association with RII α .

Loss of anchor points in AKAP18 γ and smAKAP reduces binding of regulatory subunits of PKA in cells

Next, we examined whether the loss of anchor points affects the interaction of a full-length AKAP18 with RII subunits in cells. AKAP18 γ , which encompasses the region of AKAP18 β that was co-crystallized with the DD domain of RII α , and which is longer than AKAP18 β (326 vs 104 amino acids), was expressed in HEK293 cells as a Flag-tagged protein. The Flag-tagged AKAP18 γ was immunoprecipitated with anti-Flag magnetic beads and co-immunoprecipitated RII α and RII β were detected using Western blotting (Figure 6A). The anchor point substitutions in AKAP18 γ (D293A/Q314A/E318A) correspond to those in AKAP18 β (D49A/Q70A/E74A). These substitutions reduced the interactions of AKAP18 γ with both RII subunits, whereby the interaction with RII β was more strongly affected than that with RII α .

As an example for a full-length, RI-specific AKAP, we examined whether the loss of the predicted anchor points in smAKAP (see Figure 3E) affects its interaction with RI α subunits (Figure 6B). Indeed, co-immunoprecipitation studies confirmed that the interaction of smAKAP carrying alanine in the anchor point positions (Asp⁷²Ala/Gln⁷⁶Ala) with RI α is strongly reduced compared with the interaction of the wild-type smAKAP with RI α .

CONCLUSIONS

Our current analyses are in line with our previous study of the AKAP18 δ -RII α interaction, in which we used molecular modelling in combination with RII overlays over polypeptides of 25 amino acids that covered the RII-binding domain of AKAP18 δ [35,43]. These studies had defined six hydrophobic interactions (Leu⁵², Leu⁵⁹, Val⁶⁰, Ala⁶³, Val⁶⁴ and Ala⁶⁷ of AKAP18 β). The core PKA-binding domains of the rat AKAP18 δ and the human AKAP18 α , - β and - γ are identical in sequence. Thus the new complex structure presumably represents the PKA-binding mode of all AKAP18 isoforms. Our previous analysis of interactions of AKAP18 δ with membrane lipids identified clusters of positively charged amino acids on the surface of the molecule that can interact with negatively charged lipids [54]. In addition, a recent modelling study suggested a pentameric organization of AKAP18 γ in complex with two RII and two C subunits of PKA [55]. Furthermore, a 3D structure of around 200 amino acids N-terminally from the RII-binding domain of AKAP18 γ is available [56]. Thus a picture of the AKAP18 structure is emerging. However, a full-length 3D structure of any of the AKAP18 isoforms with full-length RII subunits of PKA is lacking.

In summary, the novel AKAP18 β -RII α -D/D complex structure exhibits pivotal hydrophilic anchor points important for recognition of PKA RII subtypes that were hitherto unknown. Targeting these amino acid residues can lower the binding affinity or prevent the interaction. Thus, these anchor points provide a unique opportunity for specific pharmacological interference and may act as the basis for the design of specific inhibitors that will allow us to ascribe functions to this specific AKAP-PKA interaction. Our sequence alignments and biochemical analyses suggest the presence of different sets of anchor points in different AKAPs. These anchor points may generally influence the binding affinity between AKAPs and R subunits of PKA, and may thus also lead to new strategies for pharmacological targeting of other AKAP-PKA interactions. This is so far not possible because only global disruptors of AKAP-PKA interactions are available [4].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AKAP	A-kinase-anchoring protein
AKB	A-kinase-binding domain

D/D	dimerization/docking
ITC	isothermal titration calorimetry
LIC	ligation-independent cloning
PDE	phosphodiesterase
PKA	protein kinase A
SLS	static light scattering

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Figure 1. Model of the D/D domain of human regulatory RII α subunits of PKA bound to the AKB of AKAP18 β

(A) The X-type four-helix bundle of the RII α -D/D dimer is composed of helices A, B and A', B' coloured in blue and salmon, respectively. Two AKAP18 β -RII α -D/D complexes involving chains I, J and G, H for RII α -D/D, with the corresponding AKAP18 β helix chains Q, P, respectively, were superimposed to derive a model with the maximum length of AKAP18 β (coloured in yellow). For clarity only chains G and H of RII α -D/D in combination with the AKAP18 β helix chains Q, P are shown. The side view along the AKAP18 β helix demonstrates the binding of AKAP18 β by the helices A and A' of the RII α -D/D dimer. (B) Surface representation of the hydrophobic cavity of the RII α -D/D dimer with the bound AKAP18 β helix shown in yellow. Hydrophobic residues of the RII α -D/D dimer are shown in grey, polar residues in green, and negatively and positively charged residues in red and blue, respectively.

Figure 2. Detailed presentation of the binding of AKAP18 β to a RII α -D/D dimer

(A) Two-side view of the AKAP18 β -RII α -D/D-binding interface. The nine turns (I–IX) of the AKAP18 β amphipathic helix (yellow) bind with their hydrophobic sides to the helices A and A' (blue and salmon, respectively) of the RII α -D/D dimer. Besides the hydrophobic core region, crucial additional salt bridges (dotted lines) stabilize the interface. Only the residues involved in complex formation are shown. **(B)** Schematic presentation of the binding interface between AKAP18 β and a RII α -D/D dimer. Solid and dashed lines describe the polar and hydrophobic interactions, respectively. The conserved hydrophobic patches are indicated as grey boxes and numbered from the N- to the C-terminus.

Figure 3. AKAP anchor points I-III for interaction with D/D domains of regulatory subunits of PKA

The monomers of the RII α -D/D dimer are depicted in blue and salmon cartoons and the AKAP helices as ribbons. The anchor points in the AKAP helices, specific for RII α and RII β binding, are shown as yellow and red spheres, respectively. The complementary anchor points at the D/D domains are displayed as green and blue spheres for RII α and RII β , respectively. (A) The AKAP18 β helix, containing the three anchor points, bound to RII α -D/D, is shown in yellow. The short AKAP79 (AKAP5) with one anchor point is shown in (B) and the longer AKAP-Lbc (AKAP13) with no anchor points is presented in (C), both in grey. (D) Superimposition of D-AKAP2 (AKAP10) depicted as a grey ribbon in complex with RII α -D/D (blue and salmon cartoons) on to the complex of D-AKAP2 (black ribbon)

bound to RI α -D/D (green cartoon). The approximate position of anchor points II and III for the D-AKAP2–RII α -D/D complex are modelled on to the D-AKAP2 helix of the D-AKAP2–RII α -D/D complex. The positions were estimated based on the observed locations in AKAP18 β and on the sequence alignment shown in (E). **(E)** Alignment of the PKA-binding domains and adjacent sequences of the indicated AKAPs. The five conserved hydrophobic patches forming the core of the interactions between AKAPs and D/D domains are depicted as grey boxes. Amino acid residues matching the hydrophobic core are emphasized in bold letters. The anchor point positions I, II and III, specific for RI α and RII α binding, are marked with yellow and red boxes, respectively. AKAP18 β residues involved in RII α binding are highlighted with an asterisk. The dual-specificity AKAPs are aligned to either the RI- or the RII-binding frame.

Figure 4. Hydrophilic anchor points are essential for the interaction of AKAP18 β with regulatory RII α subunits of PKA, and hydrophobic bonds constitute the backbone

Polypeptides of 30 amino acids representing the wild-type (wt) sequence of the PKA-binding domain of AKAP18 β and peptides with (A) individual alanine substitutions, (B) combinations of alanine substitutions or (C) individual aspartate substitutions in the indicated positions were spot synthesized and overlaid with radioactively labelled RII α subunits. Binding of RII α to the peptides was detected by autoradiography. In positions 50, 63 and 67 the wild-type sequence contains alanine, and therefore is shown only once in (A). Representative spots from three independent experiments are shown. Quantification in (B) represents the means $\pm$ S.E.M.s from three independent experiments. For statistical analyses the Student's unpaired *t*-test was applied.

Figure 5. Substitution of the anchor points in AKAP18 β reduces the binding affinity and increases the dissociation rate from RII α

(A) The upper panels show the raw ITC data and the bottom panels show the change in molar heat as a function of the molar ratio of wild-type AKAP18 β (43–83) and AKAP18 β (43–83/D49A/Q70A/E74A) to RII α -D/D(1–44) (left and right panels, respectively). The data of three independent measurements were fitted with the model of one set of sites and yielded a mean $K_D = 0.6 \pm 0.2 \text{ nM}$, $n = 0.98$ sites, $H = -2.255\text{E}4 \pm 171.2$,

$S = -33.4 \text{ cal/mol per } ^\circ$ for the wild-type protein. A fit for the ITC data for AKAP18 β (43–83/D49A/Q70A/E74A) was possible only with a sequential binding model; dissociation constants for the two binding phases were estimated from four independent experiments to be $K_{D1} = 0.4 \text{ nM}$ and $K_{D2} = 22.8 \text{ nM}$. (B) Surface plasmon resonance analysis of the interaction of RII α subunit and AKAP18 β . RII α , 290 resonance units (RU), was captured on an 8-AHA cAMP sensor chip. Wild-type AKAP18 β (43–83) (black lines) and the AKAP18 β triple mutant (blue lines) were injected in concentrations ranging from 0.2 nM to 146 nM.

The association phases of the peptides (0.2–49 nM) were monitored for 60 s, illustrated in the inset. The dissociation phase of the highest concentration tested (146 nM) was monitored for 2 h and separately fitted to determine the dissociation rate (red dashed lines).

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Figure 6. The loss of the anchor points in full-length AKAP18 γ and the RI-specific smAKAP decreases interactions with R subunits of PKA in cells

(A, B) The indicated Flag-tagged, full-length, wild-type AKAPs (WT) and the variants lacking the anchor points were expressed in HEK293 cells. AKAP18 γ lacks the anchor points for RII (AAA) and smAKAP for RI (AA) subunits. The AKAPs were immunoprecipitated (IP) with anti-Flag magnetic beads. The AKAPs and R subunits were detected by Western blotting (IB) using anti-Flag, anti-RI α , anti-RII α and anti-RII β antibodies. For statistical analyses the Student's *t*-test was applied.

Table 1

Data collection and refinement statistics

AKAP18 β -RII α -D/D	
Data collection	
Beamline	BESSY 14.1
Wavelength (Å)	0.9184
Space group	P2 ₁
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	56.9, 121.0, 57.1
α , β , γ (°)	90.0, 93.1, 90.0
Resolution (Å) ^a	41.5–2.63 (2.79–2.63)
<i>R</i> _{meas} (%) ^a	13.0 (58.2)
$\langle I/\sigma(I) \rangle$ ^a	10.6 (2.5)
Completeness (%) ^a	99.4 (99.0)
Redundancy ^a	3.2
Refinement	
Resolution (Å)	2.63
No. of reflections	21,760
<i>R</i> _{work} / <i>R</i> _{free}	0.222/0.268
No. of atoms	
Protein	5,319
Ligand/ion	6
Water	60
Mean <i>B</i> factor (Å ²)	36.8
RMSDs	
Bond lengths (Å)	0.007
Bond angles (°)	1.120

^aData in highest resolution shell are indicated in parentheses.