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AKAP18 Contains a Phosphoesterase Domain that Binds AMP

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Protein kinase A anchoring proteins (AKAPs), defined by their capacity to target the cAMP-dependent protein kinase to distinct subcellular locations, function as molecular scaffolds mediating the assembly of multicomponent complexes to integrate and organise multiple signalling events. Despite their central importance in regulating cellular processes, little is known regarding their diverse structures and molecular mechanisms. Here, using bioinformatics and X-ray crystallography, we define a central domain of AKAP18δ (AKAP18^{CD}) as a member of the 2H phosphoesterase family. The domain features two conserved His-x-Thr motifs positioned at the base of a groove located between two lobes related by pseudo 2-fold symmetry. Nucleotide co-crystallisation screening revealed that this groove binds specifically to adenosine 5′-monophosphate (5′AMP) and cytosine 5′-monophosphate (5′CMP), with the affinity constant for AMP in the physiological concentration range. This is the first example of an AKAP capable of binding a small molecule. Our data generate two functional hypotheses for the AKAP18 central domain. It may act as a phosphoesterase, although we did not identify a substrate, or as an AMP sensor with the potential to couple intracellular AMP levels to PKA signalling events.

Keywords: AKAP; PKA; scaffold; AMP; phosphoesterase

Introduction

Synthesis of the intracellular second messenger cAMP by adenylyl cyclase stimulates activation of cAMP-binding proteins such as cyclic nucleotide-gated channels[1] and Epac guanine nucleotide exchange factors,[2] with the principal intracellular target being protein kinase A (PKA).[3] Although cAMP is diffusible, rises in cAMP concentration are restricted to microdomains of the cell.[4] A-kinase anchoring proteins (AKAPs) organise signalling within cAMP microdomains by anchoring multiple signalling proteins,[5] which in addition to PKA and other signalling proteins include the enzymes responsible for cAMP synthesis[6] and breakdown.[7] Recent investigations of AKAPs challenge the view of scaffold proteins as inert platforms that merely colocalise discrete subsets of proteins.

Despite considerable structural and functional diversity, all AKAPs share three common properties: an amphipathic helix to anchor the PKA holoenzyme through interaction with the N-terminal docking/dimerisation (D/D) domain of its regulatory (R) subunits[8–10]; targeting elements to particular subcellular locations; and the ability to bind other signalling proteins.[5] Subcellular location of AKAPs is often dynamic; for example, AKAP79 is released from the cell membrane following rises in calcium concentration and activation of calmodulin.[11] The ability of AKAPs to bind multiple proteins provides a mechanism to integrate different second messenger signals; for example, AKAP-Lbc inputs signals from both protein kinase C and PKA to activate protein kinase D.[12] AKAPs may also modulate the properties of bound proteins; for example, 3-phosphoinositide protein kinase 1 is activated upon engaging mAKAP[13] and Yotiao can directly increase the

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Abbreviations used: AKAP, A-kinase anchoring proteins; PKA, protein kinase A; CMP, cytosine monophosphate; MANT, 2′- (or 3′-)-*O*-(*N*-methylanthraniloyl); NLS, nuclear localisation sequence; CNP, cyclic nucleotide phosphoesterase.

current passing through the heart potassium channel IK_5.[14] Consistent with their role in organising signalling complexes, AKAPs are involved in important biological processes; for example, AKAP79 targets PKA and protein phosphatase 2B for respective phosphorylation and dephosphorylation of a glutamate receptor critical in hippocampal synaptic plasticity,[15,16] and mAKAP anchors an array of signalling enzymes[17] for regulation of various aspects of heart function including calcium release from the sarcoplasmic reticulum.[18]

Many AKAPs are expressed as alternatively spliced isoforms. The AKAP15/18 gene encodes four splice variants (Supplementary Fig. S1) that range in size from 15 to 37 kDa. These anchoring proteins utilise distinct targeting motifs to determine specific subcellular locations.[19–21] Membrane targeting of the α and β variants is mediated by lipid modification of sites at the extreme amino terminus,[22,23] whereas the larger δ variant is predominantly cytoplasmic.[24] Under certain circumstances, the AKAP18γ variant is believed to reside in the nucleus.[25] Nonetheless, all four variants contain an R subunit-binding helix, which binds both PKA regulatory subunit isoforms,[19–21] and a putative leucine zipper motif that anchors at least the α isoform to L-type calcium channels.[26] AKAP18α plays a role in the brain, where it directs PKA toward neuronal sodium channels,[27] allowing their dopaminergic modulation.[28] More recently, the δ isoform has been shown to anchor PKA for both phosphorylation of aquaporin-2 at Ser256 following vasopressin stimulation in the kidney[29] and phosphorylation of phospholamaban in the heart.[30] AKAP18δ has also been reported to bind to PDE4D isoforms through elements within its central region.[31]

Knowledge of the three-dimensional structures of proteins provides crucial insights into protein function unavailable from other observations. However, structural information for AKAPs has to date been limited to studies of the AKAP–PKA interaction.[8–10] For a variety of reasons, AKAPs are challenging targets for crystallographic investigation. AKAPs are often large proteins that can be difficult to express in bacteria. Generally, AKAP sequences are thought to be of low complexity, containing few discrete globular folded domains. Additional problems arise because most AKAPs simultaneously interact with multiple binding partners, so heterologous expression in their absence may destabilise protein structure and increase protein degradation.

To define features in AKAPs that might be amenable to structural analysis, we analysed mammalian AKAPs by a bioinformatics approach to identify putative globular domains. Here, we present the identification and structure determination of a central domain (~200 residues) common to AKAP18γ/δ (termed $AKAP18^{CD}$). Our results, the first crystal structure of an AKAP domain, reveal a structural resemblance to the 2H phosphoesterase family, proteins that harbour a pair of conserved His-x-Thr motifs. Members of this family include RNA ligases and cyclic nucleotide phosphoesterases. Characterisation of the domain revealed that $AKAP18^{CD}$ specifically interacts with 5′AMP and cytosine monophosphate (CMP), but lacks a range of phosphoesterase activities. These data suggest that either AKAP18δ acts as a phosphoesterase for an unidentified substrate or functions as an AMP sensor, thereby coupling intracellular AMP levels with PKA-mediated phosphorylation processes.

Results

AKAP18δ incorporates a 2H phosphoesterase domain

The longest splice variant of each known mammalian AKAP (20 proteins) was analysed using the secondary structure prediction program JPRED[32] and the protein tertiary structure recognition program PHYRE[33] (Table 1). Regions of discrete non-repetitive secondary structure were predicted by JPRED, for example, a C-terminal region in AKAP95 (approximately residues 360–692) and throughout the short AKAP, AKAP28. Protein fold recognition analysis indicated regions of potential architectural similarity to proteins of known structure. Domains detected in some AKAPs had previously been characterised (e.g., the RhoGEF domain of AKAP-Lbc), whereas in other AKAPs, novel domain definitions using PHYRE were strongly supported by high (>40%) sequence identity with the identified structural homologue (e.g., the Sec7 domain in BIG2). Structural analysis of these regions would be less likely to yield much in the way of new functional information. Of more interest was the detection of structural homology within AKAPs to proteins of known fold, not clearly evident from sequence analysis alone. These included the long AKAP18 isoforms γ and δ, identified as incorporating a central domain likely to resemble members of the 2H phosphoesterase family, despite the absence of significant sequence similarity (17%) (Table 1).

To address the structural and functional properties of AKAP18δ, three constructs for bacterial expression of rat AKAP18δ were designed corresponding to full-length protein, an N-terminal truncation (residues 76–357) and the limited central domain (76–292), termed $AKAP18^{CD}$ (Supplementary Fig. S1). The central domain was purified by glutathione affinity and gel filtration chromatography, whereas the longer proteins eluted in the void volume on gel filtration and were not investigated further. $AKAP18^{CD}$ was crystallised by hanging-drop vapour diffusion, and the structure was determined at 1.8 Å resolution by single isomorphous replacement with anomalous scattering (SIRAS). Data collection, phasing and refinement statistics are listed in Table 2.

$AKAP18^{CD}$ adopts a globular α–β-type architecture consisting of four α-helices and eight β-sheets, with approximate dimensions of 50 Å×40 Å×30 Å. The domain is bilobal with two four-stranded anti-

Table 1. Bioinformatic analysis of mammalian AKAPs

AKAP[a]	Accession no. (total amino acids)	Secondary structure (SS) prediction by JPRED[b]	Fold recognition by PHYRE[c] (estimated certainty)
D-AKAP1 (AKAP1)	AAH00729 (903)	Diffuse mixed SS elements throughout	KH domain 605–675 (100%), Tudor domain 760–810 (95%)
AKAP-KL (AKAP2)	NP_001004065 (948)	Dense mixed SS throughout	–
AKAP110 (AKAP3)	NP_006413 (853)	Dense mixed SS throughout	–
AKAP82 (AKAP4)	CAI41561 (854)	Dense mixed SS throughout	–
AKAP150 (AKAP5)	AAB07887 (714)	160–640 no SS	Porin transmembrane β-barrel 255–525 (20%)
mAKAPα (AKAP6)	NP_004265 (2319)	480–615 no SS, 740–1250 helical only, 1250–2319 mixed SS	Spectrin repeats 750–1250 (100%)
AKAP18δ (AKAP7)	AAR06859 (353)	1–70 no SS	Region 90–290 similar to *P. horikoshii* protein similar to 2′-5′ RNA ligase (100%), *T. thermophilus* 2′-5′ RNA ligase (100%), Appr>r CNP (90%)
AKAP95 (AKAP8)	AAH37270 (692)	360–692 predicted to have dense and predominantly helical SS	–
AKAP450 (AKAP9)	CAB40713 (3908)	Mixed SS up to 150, remaining sequence coiled coil	Aligns with long helical region of colicin1α throughout sequence (100%)
D-AKAP2 (AKAP10)	EAW50913 (662)	Mixed SS throughout	RGS domain 220–360 and 370–510 (both 100%)
AKAP220 (AKAP11)	EAX08676 (1901)	Mixed SS throughout	Sir4 coiled-coil dimerisation motif 480–640 (25%)
Gravin (AKAP12)	NP_005091 (1782)	Mixed SS throughout	Myc box interacting protein 1620–1760 (20%)
AKAP-Lbc (AKAP13)	NP_006729 (2817)	Mixed SS throughout, coiled coil 2350–2400 and 2570–2690	Ankyrin repeat 170–220 (45%), C1 domain 1790–1840 (95%), DH/PH domain 1975–2350 (100%)
AKAP28 (AKAP14)	NP_848928 (197)	Mixed SS throughout	–
BIG2	NP_006411 (1785)	Predominantly helical SS, no SS 210–360	Sec7 domain 635–840 (100%), armadillo repeats 10–600 (100%)
MAP2	NP_002365 (1827)	Mixed SS throughout with exception of no SS 1480–1600	–
PAP7	AAN60219 (528)	Mixed SS throughout with exception of no SS 10–65	Acyl-CoA-binding domain 85–175 (100%)
Pericentrin B	AAP46636 (3336)	Predominantly coiled-coil throughout	Aligns with long helical region of colicin1α throughout sequence (100%)
RSP3	AAK26432 (418)	Mixed SS throughout	–
WAVE1	NP_001020107 (559)	280–490 no SS	Syntaxin6 25–75 (25%)

The longest isoform of each mammalian AKAP was systematically investigated for regions of discrete nonrepetitive secondary structure using the secondary structure prediction program JPRED and for regions of potential structural similarity to proteins of known structure by the fold recognition program PHYRE. C1, cysteine-rich phorbol-binding domain; DH, Dbl homology; KH, K homology; PH, Pleckstrin homology; RGS, regulator of G-protein signaling.

[a] Gene nomenclature committee names are in parentheses.

[b] Regions of greater than 50 amino acids with no predicted secondary structure are listed.

[c] Alignments with estimated precision of 10% or below were discounted-for AKAPs >1200 residues, the sequence was broken into blocks of 1200 amino acids with 400-residue overlaps.

parallel β-sheets at its core related by a pseudo 2-fold rotational symmetry (Fig. 1a). A deep, water-lined groove lies between the two lobes and penetrates approximately 15 Å into one face of the protein. The strands β2 and β5 run antiparallel to each other at the base of this groove (Fig. 1a), and each harbours a His-x-Thr motif that defines the 2H phosphoesterase family[34] and is conserved in AKAP18γ/δ across species (Fig. 1b).

The side chains of residues Arg219 and Thr220 (arginine-containing 'R' loop, connecting α3 to β5, Fig. 1a) are not visible in the electron density map and the main-chain atoms of these residues are relatively mobile ($\langle B \rangle_{\text{main chain}}$ of 40.4 Å^2 for residues 219 and 220 compared to overall $\langle B \rangle_{\text{main chain}}$ of 30.0 Å^2). This region lies at one entrance to the groove, adjacent to another flexible loop region (residues 179–182, connecting β3 and β4) that also possesses relatively high *B*-factors ($\langle B \rangle_{\text{main chain}}$ of 37.8 Å^2).

AKAP18δ binds 5′AMP and 5′CMP specifically

Because 2H phosphoesterases catalyse reactions involving nucleotides, we tested the nucleotide-binding capability of AKAP18δ by co-crystallising AKAP18^{CD} in the presence of a variety of nucleotides. In each instance, a complete data set was collected at higher than 2.5 Å resolution, and the presence of nucleotide was detected by determining the structure by molecular replacement. Significantly, electron density characteristic of nucleotide was visible in $F_o - F_c$ difference maps when AKAP18^{CD} was co-crystallised in the presence of

Table 2. Crystallographic data collection and refinement statistics

Data set	AKAP18^{CD}	Selenium-substituted AKAP18^{CD}	AKAP18^{CD}–5′AMP complex	AKAP18^{CD}–5′CMP complex
Collection and phasing statistics				
X-ray source	ESRF ID14.3	ESRF ID14.2	ESRF ID14.3	ESRF ID14.3
Space group	$P3_1$	$P3_1$	$P3_1$	$P3_1$
Unit cell (Å)	$a=b=42.42$, $c=96.91$	$a=b=42.61$, $c=98.34$	$a=b=42.23$, $c=97.72$	$a=b=42.97$, $c=97.97$
Z	1	1	1	1
Wavelength (Å)	0.9794	0.9330	0.9794	0.9794
Resolution (Å)	1.80	2.10	1.50	2.25
Observations (N)	49,322 (7318)	136,308 (16,456)	84,120 (12,321)	38,757 (5420)
Unique reflections (N)	18,073 (2654)	11,263 (1426)	31,159 (4543)	9559 (1391)
Completeness (%)	99.7 (99.9)	96.9 (85.4)	99.7 (100.0)	99.5 (98.4)
R_{merge}	0.043 (0.272)	0.052 (0.281)	0.039 (0.323)	0.091 (0.297)
$I/\sigma(I)$	17.7 (3.1)	38.5 (9.1)	18.2 (2.5)	13.6 (5.2)
Anomalous completeness (%)	–	96.0 (83.4)	–	–
Anomalous multiplicity	–	5.7 (5.3)	–	–
DelAnom correlation between half-sets	–	0.805 (0.520)	–	–
Selenium sites (N)	–	5	–	–
Refinement statistics				
Resolution range (Å)	97.13–1.80		97.59–1.50	98.06–2.25
Reflections (N)	17,152		29,583	9095
No. of nonhydrogen atoms	1710		1863	1689
Working R value	0.206		0.161	0.225
Free R value (N reflections)	0.238 (921)		0.209 (1570)	0.261 (460)
Deviation from ideality				
Bond lengths (Å)	0.010		0.015	0.015
Bond angles (°)	1.252		1.590	1.721
B-factor averages (Å^2)	31.3 (total), 36.6 (water molecules)		20.2 (total) 30.5 (water molecules) 18.5 (5′AMP)	19.3 (total) 17.9 (water molecules) 32.3 (5′CMP)
Ramachandran values (%) (preferred/allowed/generously allowed/disallowed)	91.6/8.4/0/0		92.2/7.8/0/0	93.9/6.1/0/0

Figures for the highest resolution shell are in parentheses.

either 5′AMP or 5′CMP. However, there was no electron density suggestive of nucleotide after co-crystallisation with a range of other nucleotides, including 3′AMP, guanosine 5′-monophosphate (5′GMP) and thymidine 5′-monophosphate (5′TMP), as listed in Fig. 2a. Co-crystallisation with decreasing concentrations of 5′AMP indicated that the ligand binds with a dissociation constant (K_d) in the mid-micromolar range (Fig. 2a).

The affinity constant for 5′AMP was more accurately quantified by equilibrium fluorescence binding using the AMP derivative 2′- (or 3′)-*O*-(*N*-methylanthraniloyl) adenosine 5′-monophosphate (MANT-AMP). MANT fluoresces at 448 nm upon excitation near 335 nm, which allows protein binding to be detected by FRET upon excitation at 290 nm. A plot of the difference in fluorescence in the presence and absence of AKAP18^{CD} over a range of MANT-AMP concentrations reveals the fraction of AKAP18^{CD} that is bound to MANT-AMP at a given concentration. As the concentration of AKAP18^{CD} (25 μM) is low, the K_d for MANT-AMP can be approximated by fitting the data to a hyperbolic function. Iterative rounds of least square fitting modelled the data shown in Fig. 2b to the function:

$$\Delta F = 92.8[\text{MANT-AMP}]/(194 + [\text{MANT-AMP}])$$

to give a K_d of 194±30 μM ($n=3$). This value agrees with the results of the 5′AMP co-crystallisation titrations, where electron density was visible for bound 5′AMP when at 670 μM but not at 40 μM. Immobilisation of different AKAP18δ fragments to 5′AMP-coupled agarose demonstrated that the central domain is necessary for binding 5′AMP (Fig. 2c).

To understand the molecular basis for preferential binding to 5′AMP/CMP rather than 5′GMP/TMP, structures of AKAP18^{CD} in complex with 5′AMP and 5′CMP were refined at 1.5 and 2.25 Å resolution, respectively. Data collection and refinement statistics for these complex crystal structures are listed in Table 2. In both complexes, nucleotide binds within the deep groove of the AKAP18δ central domain with the phosphate moiety coordinated by the two His-x-Thr motifs, and the base moiety contacted by residues from the β3/β4 and R loops (Fig. 3).

Coordination of the identical ribose and phosphate moieties of 5′AMP and 5′CMP by AKAP18^{CD} is very similar. At the base of the groove, the two His-x-Thr motifs dominate interactions to the nucleotide phosphate group *via* a network of direct and water-mediated hydrogen bonds (Fig. 4a). The two threonine residues (134 and 226) of the His-x-Thr motifs donate direct hydrogen bonds to phosphate oxygen atoms, whereas His224 of the second His-x-Thr motif donates a hydrogen bond to the ester oxygen. Water-mediated hydrogen bonds to phosphate oxygen atoms are provided by the side-chain amine of Lys229 and by main-chain amide

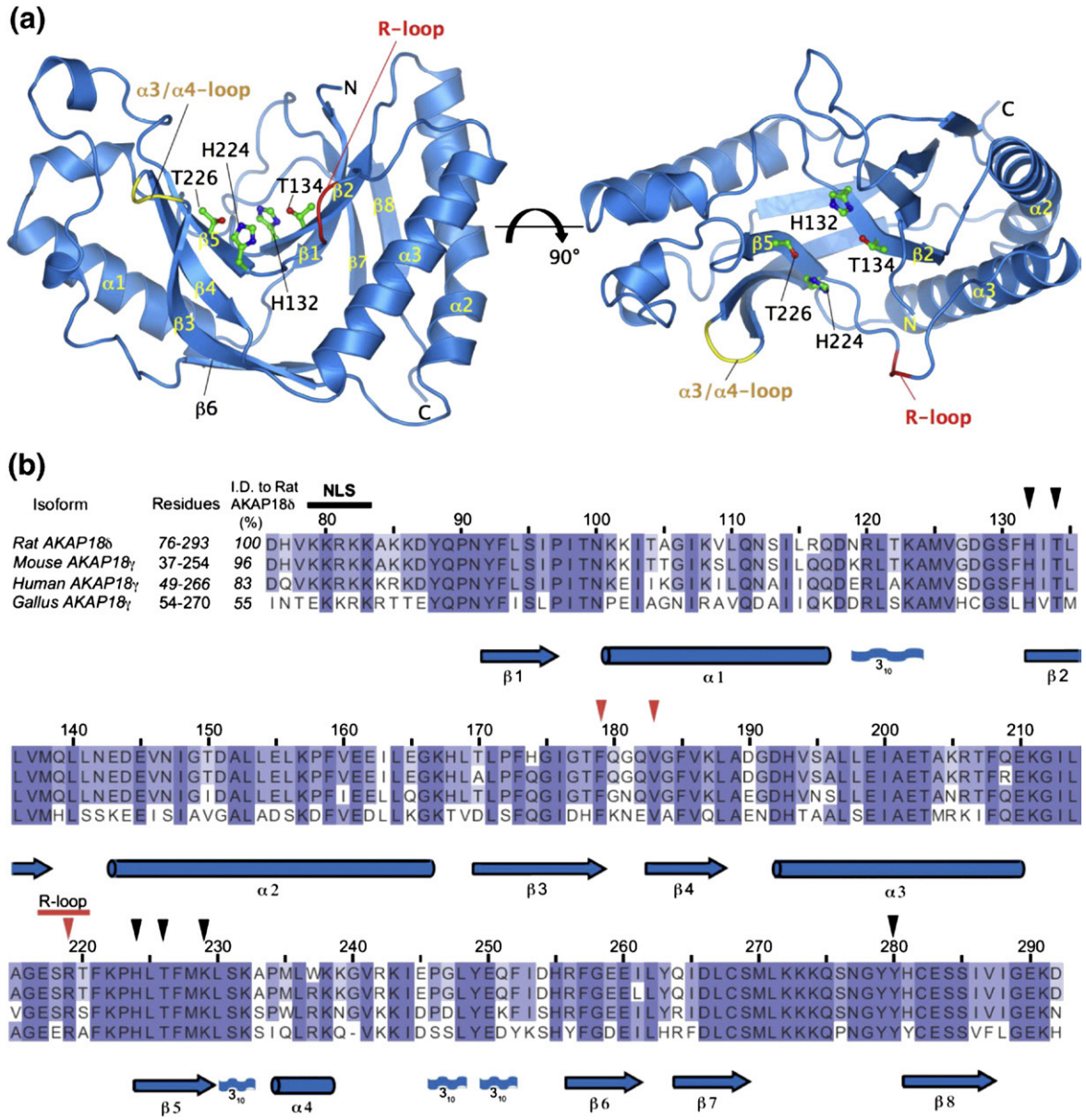


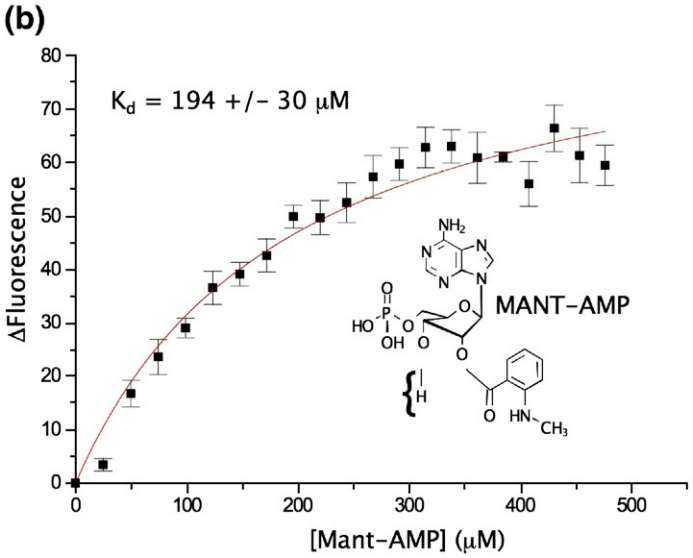
Fig. 1. Structural overview. (a) Ribbon representation of the AKAP18δ central domain. The His-x-Thr motifs, N and C-termini, secondary structure elements and R and α3/α4 loops are labelled. (b) Multiple sequence alignment of mammalian AKAP18 homologues of various species. Residues are coloured by percentage identity. The positions of the R loop and NLS are indicated. Triangles denote the positions of conserved residues lining the binding groove that either directly contact or form water-mediated hydrogen bonds with the phosphate (black) and adenosine (red) moieties of 5′AMP in the complex crystal structure.

(Thr134) and carbonyl (His132) groups. The only interaction to the ribose moiety is a van der Waals contact with Val183 (Fig. 4a). This residue, like the majority of the residues lining the groove, is invariant in AKAP18 across all species (Fig. 1b). The ribose hydroxyl groups of 5′AMP are solvent accessible (Fig. 3b).

The base moieties of both 5′AMP and 5′CMP are sandwiched between the side chains of Phe179 and Arg219, at one entrance to the groove (Fig. 4b and c). Arg219 forms a cation–π interaction with the adenine moiety of 5′AMP (Figs. 3b and 4b) and with the cytidine moiety of 5′CMP (Fig. 4c), an interaction known to stabilise protein–DNA complexes, while Phe179 forms a π-stacking interaction on the opposite side of the base. The binding of nucleotide stabilises and immobilises the loops harbouring Phe179 (β3/β4 loop) and Arg219 (R loop), with concomitant ordering of the side chains of Arg219 and Thr220. Two water-mediated protein-base hydrogen bonds are also visible in the 5′AMP complex structure: adenine position N6 to the main-chain carbonyl of Thr220 and position N7 to the main-chain amide of Lys222 (Fig. 4b). Residues involved in the coordination of phosphate and adenosine moieties of 5′AMP/CMP are respectively labelled with black and red triangles in the sequence alignment (Fig. 1b).

(a)

Nucleotide	Concentration	Electron density
5' AMP	5 mM	Yes
5' CMP	5 mM	Yes
5' GMP	5 mM	No
5' TMP	5 mM	No
3'–5' cAMP	5 mM	No
Rp-cAMPS	5 mM	No
ATP	5 mM	No
ADP	5 mM	No
2'–3' cCMP	5 mM	No
2'–3' cNADP	5 mM	No
2'–3' cCMP & 3' AMP	Both 5 mM	No
3' AMP	5 mM	No
5' AMP	670 μM	Yes
5' AMP	40 μM	No
5' AMP	10 μM	No



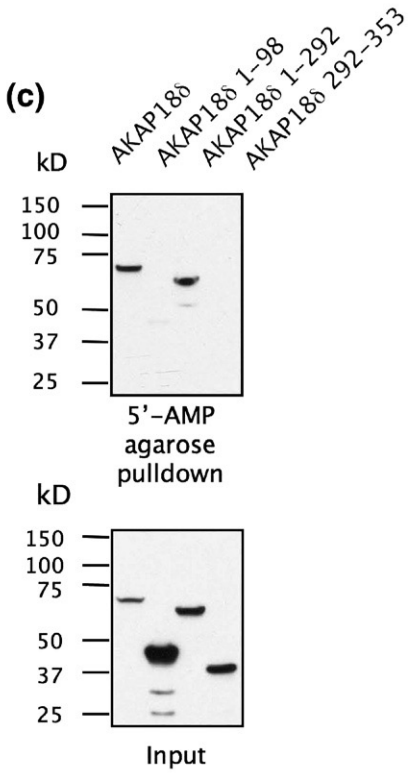


Fig. 2. Nucleotide-binding properties of the AKAP18δ central domain. (a) Table showing results of co-crystallisation of $AKAP18^{CD}$ with a range of nucleotides at varying concentrations. (b) The fluorescence of 25 μM $AKAP18^{CD}$ alone and MANT-AMP alone was subtracted from that of MANT-AMP in the presence of 25 μM $AKAP18^{CD}$. ΔFluorescence (arbitrary units) is plotted as a function of total MANT-AMP concentration. The chemical structure of MANT-AMP is shown. (c) Anti-V5 epitope Western blot showing binding of V5 epitope-tagged AKAP18δ constructs to 5′AMP-agarose beads. Isolated regions N (1–98)- and C (292–353)-terminal to the central domain did not bind to the beads.

The coordination of 5′AMP and 5′CMP suggests that a contact between the backbone carbonyl of Thr220 and the group attached to C6 (purine, Fig. 4b) or C4 (pyrimidine, Fig. 4c) of the base provides base specificity for AKAP18δ. In the 5′AMP complex, the amine in this position is 3.5 Å from the carbonyl of Thr220 (Fig. 4b); the equivalent distance is 4.1 Å for the 5′CMP complex (Fig. 4c). Both are close enough that substitution of carbonyl for amine, as in guanine and thymidine, would cause a repulsive interaction with the carbonyl of Thr220, accounting for the specificity of AKAP18δ for 5′AMP and 5′CMP.

We performed further co-crystallisations to explore nucleotide specificity. ATP, ADP and NADP did not associate with $AKAP18^{CD}$. These results may be rationalised from the coordination observed for the mononucleotides 5′AMP and 5′CMP; there is no space for the additional phosphate group of a dinucleotide. The cyclic nucleotides 3′-5′ cAMP, its nonhydrolysable analogue Rp-cAMPS, and the cyclic nucleotides cyclic 2′-3′ cytosine monophosphate (2′-3′ cCMP) and cyclic 2′-3′ nicotinamide diphosphate (2′-3′ cNADP) also failed to bind when present at 5 mM in the crystallisation solution (Fig. 2a). Any of these cyclic nucleotides would be unable to satisfy the precise network of hydrogen bonds coordinating the phosphate and ribose moieties in the 5′AMP complex (Fig. 4a). The nucleotide-binding properties that we observed in the crystal are likely to be a true reflection of the binding capabilities of AKAP18δ, as the highly conserved groove region is not influenced by crystal lattice contacts.

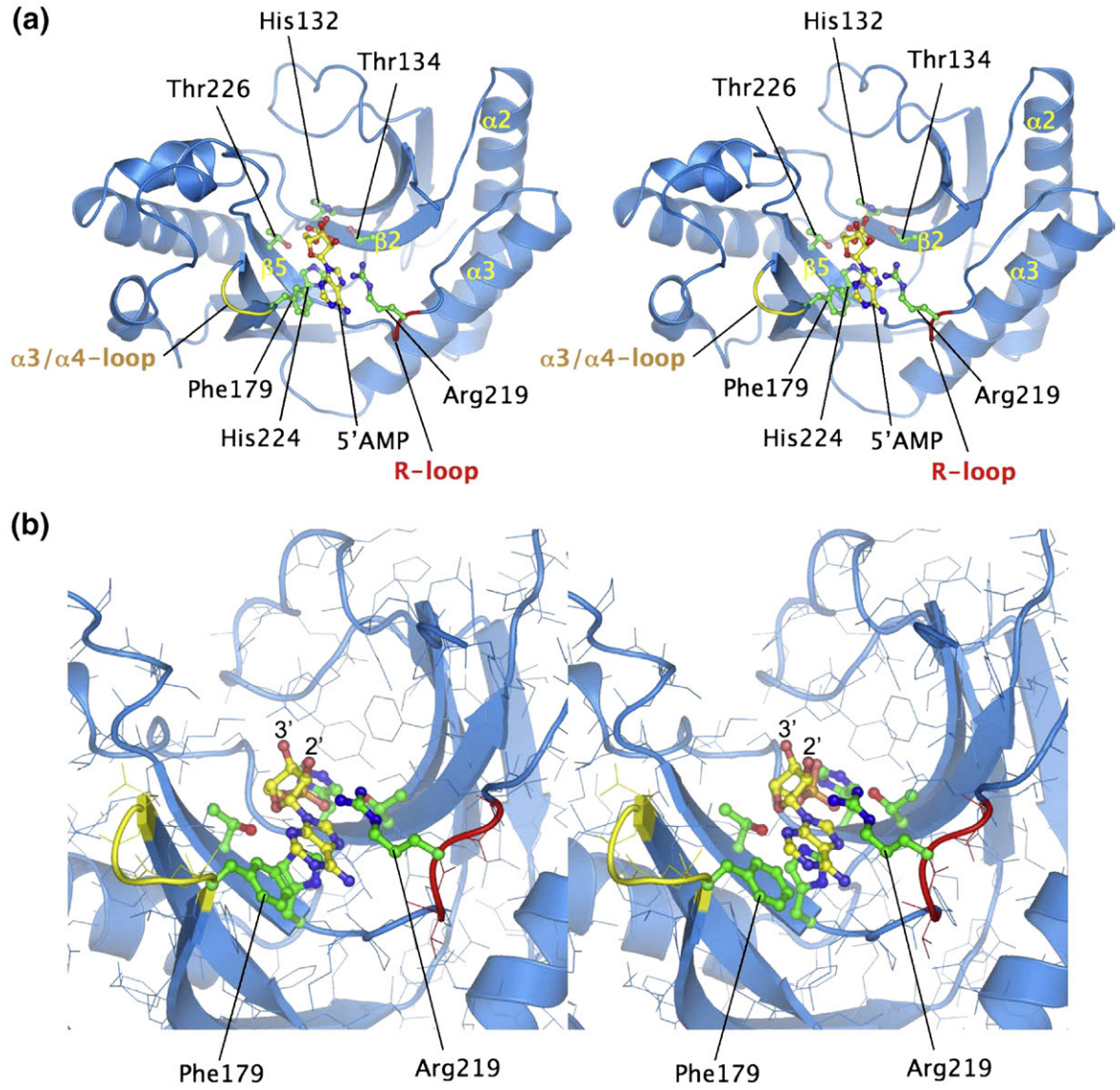


Fig. 3. AKAP18δ–AMP complex. (a) Stereo views of the AKAP18δ central domain in complex with 5′AMP (yellow). The positions of the His-x-Thr motifs and Phe179 and Arg219, which sandwich the adenine moiety of 5′AMP, are indicated. (b) Close-up of AMP bound to AKAP18^{CD} with all atoms shown demonstrating accessibility of the O2′ and O3′ hydroxyl groups.

AKAP18^{CD} resembles bacterial 2′-5′ RNA ligases

The DALI† server[35] was used to search for structural homologues of AKAP18^{CD} (Supplementary Table S1) (Fig. 5). As expected, the domain aligned with 2H phosphoesterase proteins. The closest superpositions were to bacterial 2′-5′ RNA ligases from *Pyrococcus horikoshii*[36] and *Thermus thermophilus*.[37] The domain also superposed well with *Arabidopsis thaliana* 1′-2′-cyclic nucleotide 2′-phosphodiesterase,[39] although less well to goldfish[40] and human[41] brain 2′-3′-cyclic nucleotide 3′-phosphodiesterases.

The existence of the 2H phosphoesterase protein superfamily has been recognised for some time,[34] but the functions of the various superfamily members remain poorly understood. The 2′-5′ RNA ligases catalyse the ligation of half-tRNA molecules with 2′-3′-cyclic phosphate and 5′-hydroxyl termini (Fig. 6a). *A. thaliana* 1′-2′-cyclic nucleotide 2′-phosphodiesterase hydrolyses ADP-ribose 1″,2″-cyclic phosphate (Appr>r), a product of the tRNA splicing reaction (Hoffman *et al.*, 2000) (Fig. 6b). Brain 2′-3′-cyclic nucleotide 3′-phosphodiesterase remains an enigma—its physiological substrate is still unknown despite considerable investigation of its biological roles. Although the protein is capable of hydrolysing the cyclic phosphodiester of the unphysiological molecule cyclic 2′-3′ NADP, its role as a phosphodiesterase is controversial. The 2H phosphoesterase superfamily comprises 10 subfamilies, with four major clades.[42] AKAP18δ is assigned to the eukaryotic LigT subfamily comprising eight mem-

† http://www.ebi.ac.uk/dali/

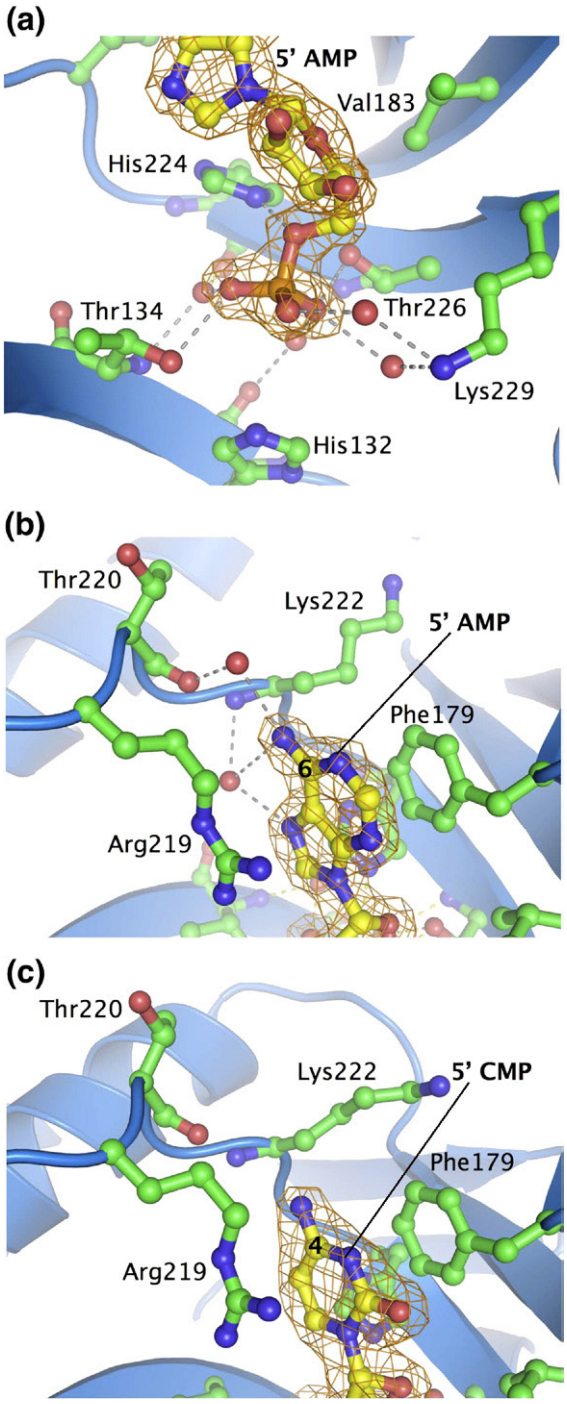


Fig. 4. Structural basis for nucleotide-binding specificity. Close-ups of the AKAP18δ binding groove in complex with mononucleotides (yellow), centred on the phosphate moiety of 5′AMP (a) and the base moieties of 5′AMP (b) and 5′CMP (c). Hydrogen bonds are indicated by dotted lines; $F_o - F_c$ electron density is contoured at 3σ.

bers. One of these, the human protein activating co-integrator 1, which assembles into a four-subunit transcription co-activator complex,[43] bears little similarity to AKAP18δ except for the central His-x-Thr motifs. Interestingly, some superfamily members are coupled to UBA and SH3 domains, suggesting a possible function in signal transduction.

Structural alignment of the AKAP18δ binding groove with homologous 2H phosphoesterases (Fig. 5) indicates that numerous highly conserved residues are shared between AKAP18δ (Fig. 5a) and 2′-5′ ligases from *P. horikoshii* (Fig. 5b)[36] and *T. thermophilus* (Fig. 5c).[37] Equivalent residues for both His-x-Thr motifs, Phe179, Val183 and Tyr280 are positioned within the 2′-5′ RNA ligase-binding grooves (Fig. 5b and c). Lys229 in AKAP18δ is replaced by arginine in the RNA ligases, a conservative substitution, and Val137 is replaced by phenylalanine (Fig. 5b and c). Arg219 in the AKAP18δ R loop is replaced by either glycine (Fig. 5b) or lysine (Fig. 5c) in the 2′-5′ RNA ligases. Apart from the His-x-Thr motifs, AKAP18^{CD} is less similar to *A. thaliana* 1′-2′-cyclic nucleotide 2′-phosphodiesterase.[39,38] Specifically, there are no counterparts to Arg219 and Phe179 (Fig. 5d). Finally, the AKAP18δ-binding groove bears little similarity to 2′-3′-cyclic nucleotide 3′-phosphodiesterases.[40,41]

AKAP18δ lacks a range of cyclic nucleotide -phosphoesterase activities

All known 2H phosphoesterase catalytic reactions involve nucleophilic attack of a cyclic phosphate, although the product of the reaction varies depending on the attacking group and linkage positions of the substrate phosphodiester. A putative mechanism for the *T. thermophilus* 2′-5′ RNA ligase has been proposed (Fig. 6a).[37] His130 functions to activate the 5′ hydroxyl of a tRNA half-molecule for nucleophilic attack onto the cyclic phosphate of a second tRNA half-molecule. Thr41, Thr132 and Arg135 are proposed to anchor the cyclic phosphate, while His39 protonates the 2″-oxygen of the cyclic phosphate and promotes P–O bond cleavage. In this instance, the product of the reaction is the linkage of two RNA half-molecules *via* a 2′-5′ phosphodiester bond.[37] Since residues lining the AKAP18δ groove (Fig. 5a) are conserved with 2′-5′ RNA ligase (Fig. 5c), the AKAP18δ–5′AMP complex crystal structure serves as a model to elaborate the proposed catalytic model.[37] It is likely that Phe80 and Val88 of the ligase (equivalent to Phe179 and Val183 of AKAP18δ), respectively, coordinate the base and ribose moieties of the 3′ tRNA half-molecule (Fig. 6a). Furthermore, Arg135 (Lys229 of AKAP18δ) is suitably positioned to allow for stabilisation of a pentaphosphate transition state (Fig. 6a). This geometry suggests that Phe44 (Fig. 5c), on the other side of the binding groove, is likely to be involved in coordinating the 5′ tRNA half-molecule.

In AKAP18δ, the presence of both His-x-Thr motifs could enable activation of a water or 5′-hydroxyl group for nucleophilic attack and protonation of a leaving oxygen group to complete the catalytic reaction. Interestingly, His132, the putative acid (Fig. 6a), is conserved and not involved in contacts with 5′AMP (Fig. 4a). Potentially important distinctions between the AKAP18δ and 2′-5′ RNA ligase binding grooves are the positions of phenylalanine and lysine/glycine (Figs. 5c and 6a) in the

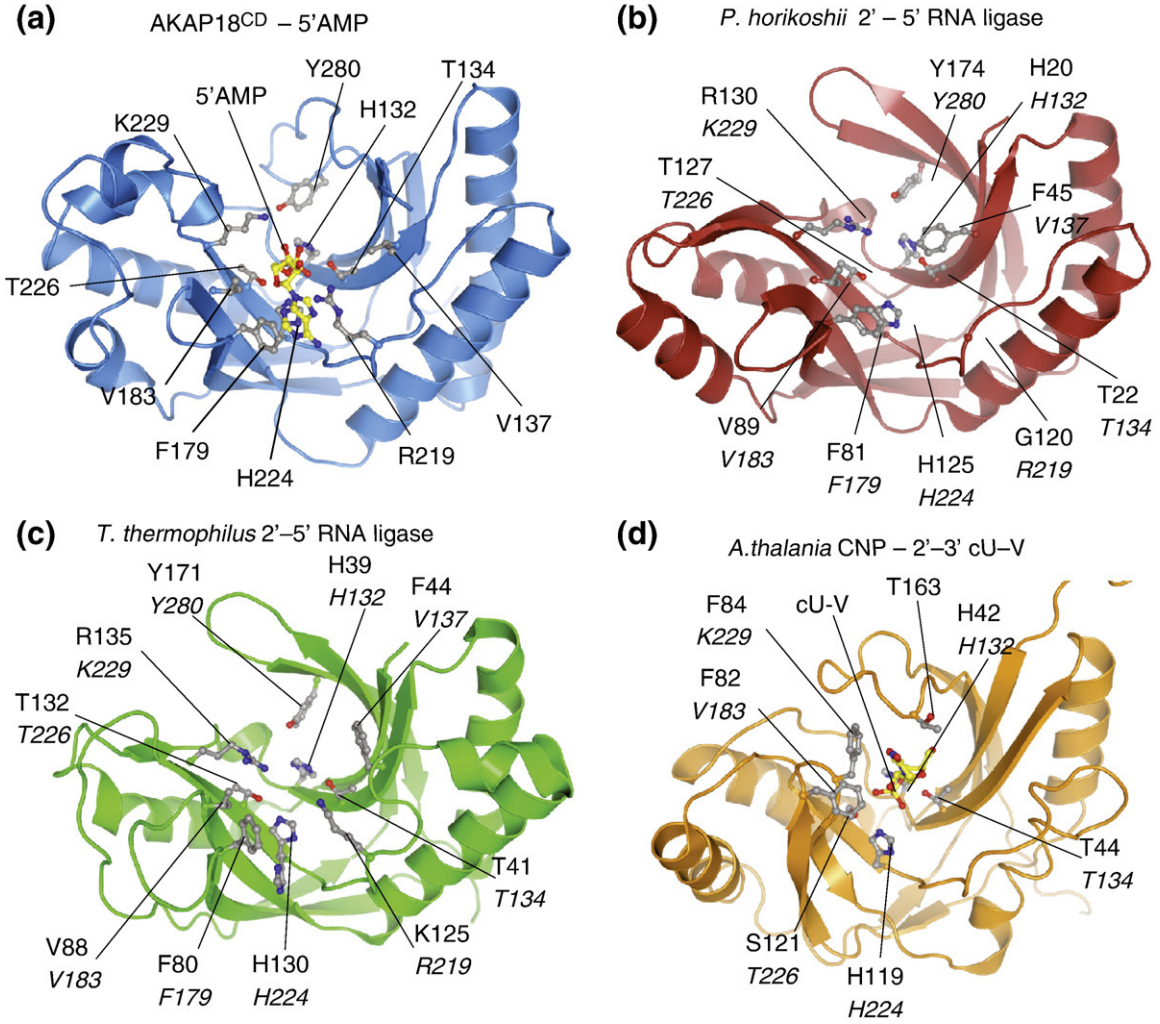


Fig. 5. Comparison with 2H phosphoesterase proteins. Equivalent views of the binding groove in four 2H phosphoesterase proteins after superpositions of the conserved His-x-Thr motifs. (a) AKAP18δ (blue) in complex with 5′AMP (yellow). (b) *P. horikoshii* 2′-5′ RNA ligase [Protein Data Bank (PDB) ID 1VDX].[36] (c) *T. thermophilus* 2′-5′ RNA ligase (PDB ID 1IUH).[37] (d) *A. thaliana* 1′–2′-cyclic nucleotide 2′-phosphodiesterase in complex with cyclic 2′-3′ uridine vanadate (PDB ID 1JH7).[38]

ligases at the equivalent positions to Val137 and Arg219, respectively, of AKAP18δ (Figs. 5a and 6c). Residues of AKAP18δ remote from the binding groove share less than 15% identity with the 2′-5′ RNA ligases. The catalytic mechanism of *A. thaliana* 1′-2′-cyclic nucleotide 2′-phosphodiesterase[39,38] is similar, although in this instance water is activated for nucleophilic attack and the cyclic phosphate forms ester bonds at 1′-2′ (Fig. 6b). The crystal structure of *A. thaliana* cyclic nucleotide phosphoesterase (CNP) in complex with cyclic 2′-3′ uridine vanadate[38] (Fig. 5d) reveals that, in a manner similar to that of the AKAP18–AMP complex (Fig. 5a), the His-x-Thr motifs are responsible for coordinating the oxyanion moiety of the nucleotide, although in this instance the phosphate is replaced by vanadate and is cyclised between the 2′-3′ ribose positions. The base and ribose moieties of cyclic 2′-3′ uridine vanadate are coordinated differently, in comparison to 5′AMP in complex with AKAP18, which enables the cyclic vanadate to be accommodated (Supplementary Fig. S2). In both complexes, phenylalanine residues, although located differently (Phe84 in the *A. thaliana* CNP, Fig. 5d; Phe179 in AKAP18, Fig. 5a), are important for coordinating the base moiety.

In an attempt to test the possibility that AKAP18δ can function as a 2′-5′ RNA ligase, crystallisation of AKAP18^{CD} was performed in the presence of two nucleotides which, according to the reaction mechanism illustrated in Fig. 6a, would be expected to bind to AKAP18^{CD} when present at high concentration. Co-crystallisations were performed with 5 mM 3′AMP to mimic the tRNA 3′ half-molecule (Fig. 6d), and with 5 mM 2′-3′ cCMP to mimic the tRNA 5′ half-molecule (Fig. 6e). Neither nucleotide bound to AKAP18^{CD} (Fig. 2a). These results are inconsistent with the notion that AKAP18δ functions as a 2′-5′ RNA ligase. Furthermore, there is no existing evidence that AKAP18δ binds RNA or is involved in any form of RNA processing.

Given the ability of AKAP18δ to bind 5′AMP, a product of 3′-5′ cAMP hydrolysis, the capacity of

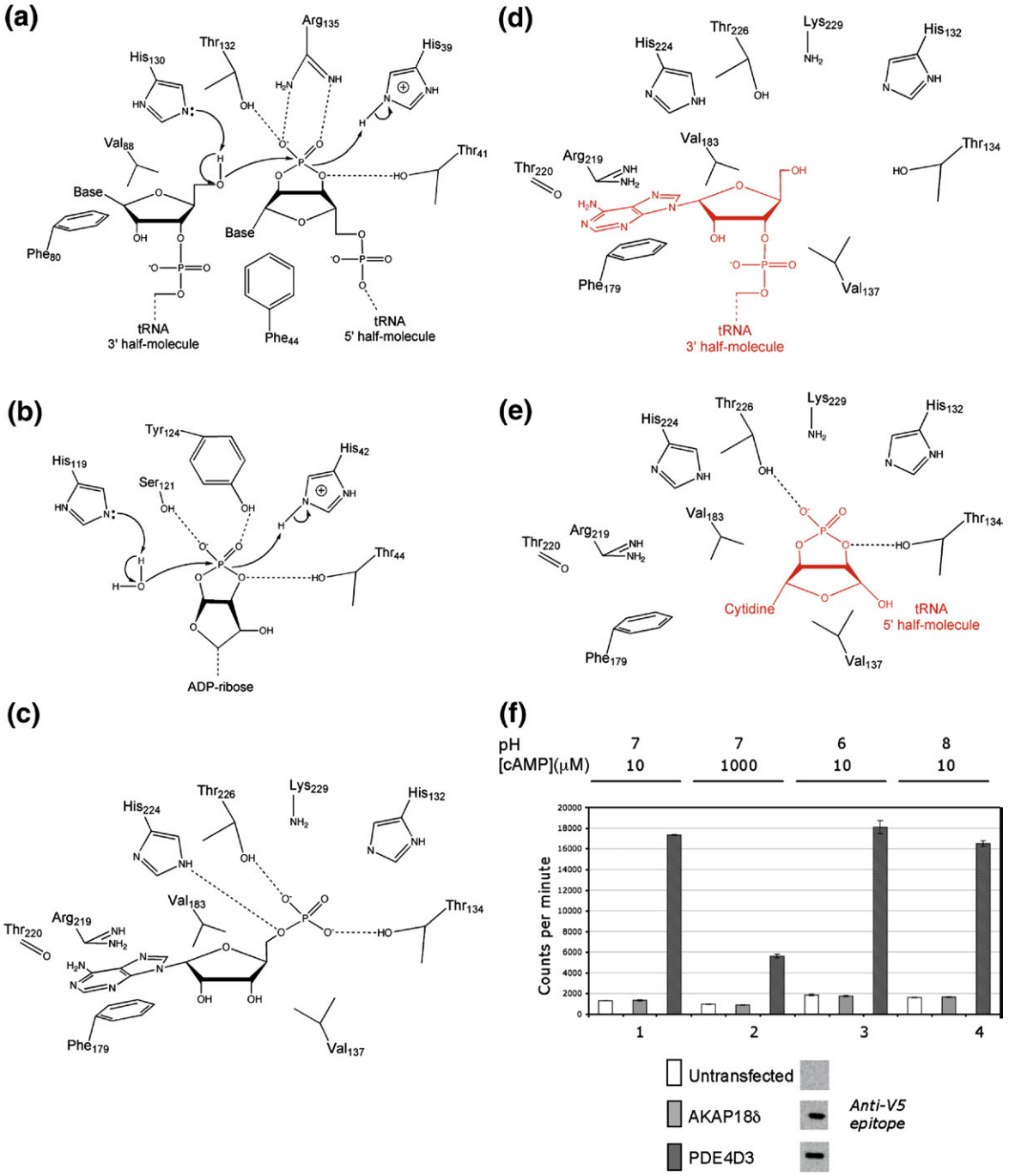


Fig. 6. Catalysis by 2H phosphoesterase proteins. Putative catalytic mechanisms for 2′-5′ RNA ligase (a) and *Arabidopsis* CNP (b) are illustrated. The position of equivalent residues in the AKAP18δ–binding groove and their role in coordinating 5′AMP are illustrated (c). Only direct protein–AMP hydrogen bonds are indicated in the AKAP18^{CD} complex. Binding of 3′AMP and cyclic 2′-3′ CMP to AKAP18δ was attempted to test the potential of AKAP18δ to act as a 2′-5′ RNA ligase, the rational being that these molecules would mimic 3′ (d) and 5′ (e) tRNA half-molecules, respectively. (f) AKAP18δ was assayed for cAMP phosphodiesterase activity. Assays were performed using immunoprecipitated V5 epitope-tagged full-length AKAP18δ (light grey bars), with different concentrations of cAMP (lanes 1 and 2) and at different pH (lanes 1, 3 and 4), in each instance including negative (untransfected, white bars) and positive (PDE4D3, dark grey bars) controls. AKAP18δ activity did not differ significantly from the negative control in any condition.

2H phosphoesterases to hydrolyse phosphodiesters with different ribose linkages, and the role of AKAP18 in anchoring PKA that responds to 3′-5′ cAMP, we tested the hypothesis that the AKAP18δ central domain might function as a 3′-5′ cAMP phosphodiesterase. However, AKAP18δ displayed no cAMP or cGMP phosphodiesterase activity regardless of the cyclic nucleotide concentration or pH tested (Fig. 6f), a finding consistent with the lack of binding of 3′-5′ cAMP in the crystal (Fig. 2a).

Discussion

In this study we have, to our knowledge, performed the first systematic bioinformatic analysis to define domain structures within the AKAP superfamily. Using protein fold recognition approaches, we identified domains within AKAPs that had not been recognised previously. Some novel domain definitions are supported by a high degree of sequence similarity to structural homologues, whereas for others, the degree of sequence similarity is not statistically significant. We defined one such domain of AKAP18γ/δ as belonging to the 2H phosphoesterase family. Crystallographic analysis of $AKAP18^{CD}$ confirmed this prediction, providing the first structural information concerning a functional domain within an AKAP. Nucleotide binding experiments indicate that $AKAP18^{CD}$ specifically interacts with 5′AMP and 5′CMP.

Our studies indicate two possible biological functions for the central domain of AKAP18δ. Its resemblance to tRNA ligases and CNPs of the 2H phosphoesterase family suggests that it either hydrolyses or ligates cyclic nucleotide phosphodiesters; however, we could not detect catalytic activity associated with the protein. The other possibility is that AKAP18δ acts as a 5′AMP effector. 5′AMP functions as an energy sensor to mediate cell signalling, with increased AMP concentrations triggering the activation of both AMP kinase[44] and glycogen phosphorylase.[45] 5′AMP stimulates AMP kinase with an EC_{50} in the low micromolar range,[46] whereas glycogen phosphorylase is converted to the activated conformation with an EC_{50} of ~75 μM in the absence of glucose 6-phosphate.[47] In contrast, the affinity of $AKAP18^{CD}$ for AMP is slightly lower. Its affinity for 5′AMP is likely to be similar to that of MANT-AMP (K_d of 194 μM), as the 2′ and 3′ ribose hydroxyl groups, modified in MANT-AMP, are accessible, unhindered by protein contacts (Fig. 3b).

If we assume that the central domain of AKAP18δ acts as a 5′AMP sensor, how might 5′AMP propagate an effect on binding to the protein? Structural rearrangements on engaging 5′AMP are restricted to ordering of the R loop and a small shift in position of the loop harbouring Lys229 (Fig. 7a). Binding of 5′AMP also alters the shape and charge of the protein surface. Both hydroxyl groups of the 5′AMP ribose moiety are available for interaction, and nucleotide binding neutralises a region of positive charge at the base of the binding groove. A region comprising highly conserved residues (CR2), including residues Asp266-Gln275, lies adjacent to the binding groove (Figs. 1a and 7b) and could potentially be involved in AKAP18δ-mediated intermolecular interactions.

5′AMP binding might affect AKAP18δ activity in multiple ways. The conformational change and altered surface properties accompanying AMP binding could modulate the protein's cellular localisation. One possibility is an influence on the nuclear localisation of the protein, reminiscent of ligand-mediated nuclear targeting of steroid hormone receptors.[48] AKAP18γ/δ comprises a conserved nuclear localisation sequence (NLS)[49] immediately N-terminal to the central domain (Fig. 1b).[25] Mutation of the NLS prevents nuclear localisation of AKAP18γ expressed in HEK293 cells.[25] The NLS sequence, although present in $AKAP18^{CD}$, was not visible in the electron density. The most N-terminal residue with discernible electron density is Tyr88 whose C^{α} atom is positioned 12 Å from the ribose 2′-hydroxyl group in the AKAP18δ–5′AMP complex structure (Fig. 7a), consistent with the possibility that the NLS could interact with elements involved in forming the AKAP18δ–5′AMP complex. There are other targeting mechanisms that could be subject to 5′AMP regulation: AKAP18δ is present on aquaporin 2-bearing vesicles in the kidney,[29] although the basis of this targeting is unknown. Alternatively, 5′AMP could affect the interaction of AKAP18δ with an associated protein. Recently, the AKAP18δ central domain was shown to interact with PDE4D3/9;[31] however, we could not detect an influence of 1 mM AMP on AKAP18δ interactions with either PDE4D6 or the PKA regulatory RII subunits (data not shown).

The phosphoesterase domain presented here is the first structure of a domain within an AKAP and is the first demonstration of an AKAP with the ability to bind a small molecule. The 5′AMP complex structure provides insight into the mechanism of 2′-5′ RNA ligases and should prove useful in understanding other members of the 2H phosphoesterase superfamily. These studies generate two functional hypotheses for AKAP18δ. First, the protein may act enzymatically as a phosphoesterase, although we did not identify a substrate. Alternatively, AKAP18δ may play a role as an AMP sensor, thereby coupling a metabolite effector protein with the primary intracellular receptor for cAMP (PKA). Our findings demonstrate the use of a combined bioinformatic–structural approach for uncovering new properties in proteins, and reveal another layer to the sophistication of AKAPs.

Materials and Methods

Protein expression and purification

Three constructs of rat AKAP18δ (IMAGE ID 7315112) spanning the full-length protein (1–353), central domain (76–292, $AKAP18^{CD}$) and an N-terminal truncate (76–353) were cloned into pGEX6P1 and expressed as PreScission™-cleavable GST-fusion protein (GE Healthcare) from *Escherichia coli* BL21 Codon Plus® (DE3) cells (Stratagene). Cells were induced at OD_{600nm} = 0.5 with 0.5 mM IPTG and harvested after 16 h at 18 °C. Cells were lysed by sonication and following centrifugation the supernatant fraction was applied to 5 ml Glutathione Sepharose™ beads (GE Healthcare). Bound recombinant protein was cleaved from the beads overnight with PreScission™ protease (GE Healthcare), and applied to a Superdex 75 gel filtration column (GE Healthcare) in 15 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM DTT. The central domain

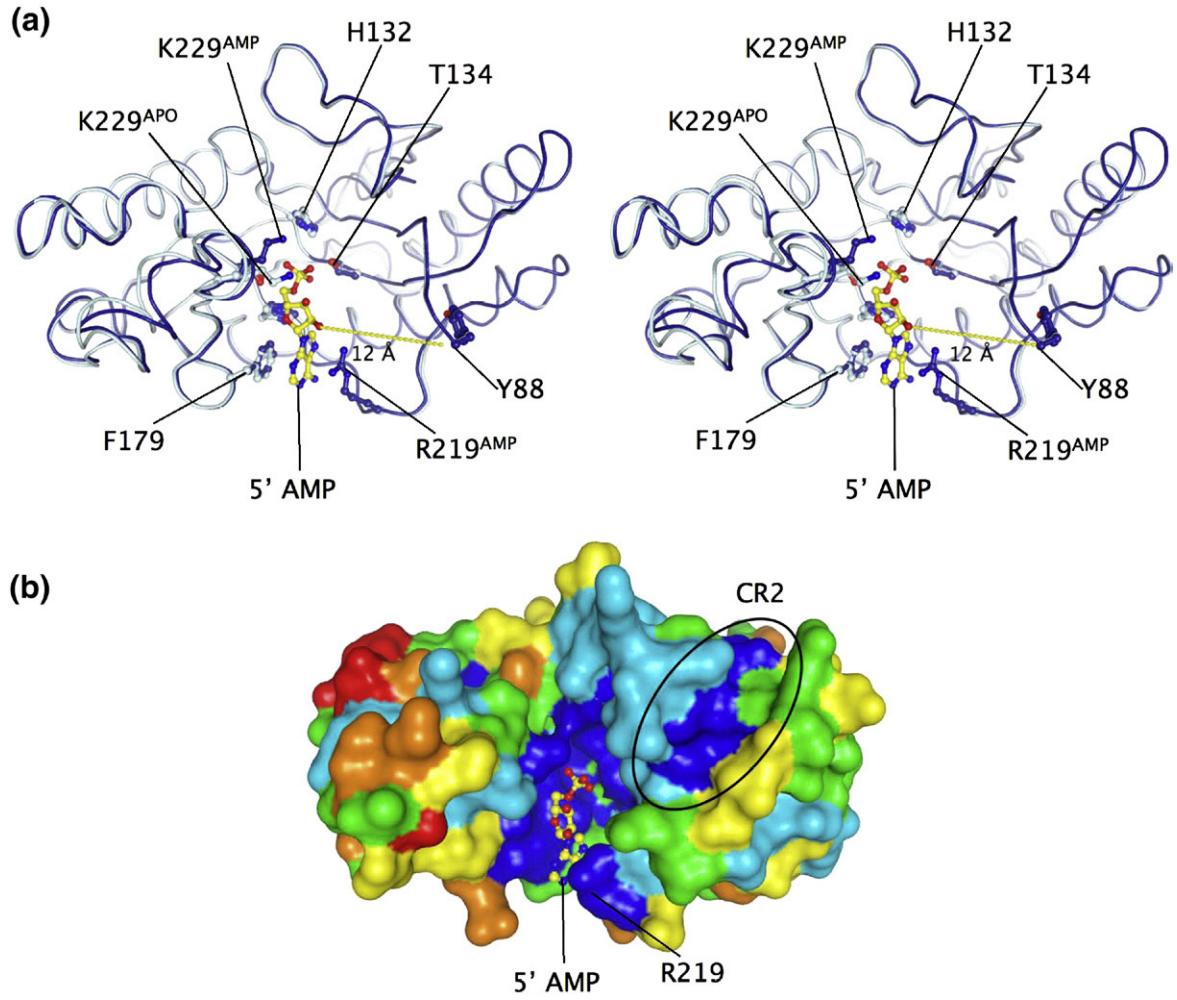


Fig. 7. Potential as a 5′AMP sensor. (a) Stereo views showing structural differences between the AKAP18δ central domain in the apo state (light blue, superscript ^{APO}) and in complex with 5′AMP (dark blue, superscript ^{AMP}). The distance between C^{α} of the first residue visible in the electron density at the N-terminus of AKAP18δ (Tyr88) and the ribose 2′-OH group of 5′AMP in the complex structure is indicated, 5′AMP is coloured yellow. (b) Surface representation showing sequence conservation by colour (red is least conserved, blue is most conserved) on AKAP18δ in complex with 5′AMP. The position of Arg219 is indicated to orient the viewer. A highly conserved region adjacent to the nucleotide-binding groove is circled.

eluted as a single peak on gel filtration. Selenomethionine-substituted AKAP18^{CD} was produced in B834 (DE3) and purified as the native protein. Mouse PDE4D6 (90–518) (IMAGE ID 23274169) was produced by the same protocol. His-tagged AKAP18δ (76–353) was expressed in *Sf*9 insect cells using the Bac-to-Bac Baculovirus Expression System (Invitrogen).

Crystallography

The best diffracting crystals grew in 0.1 M Tris–HCl, pH 8, 10% polyethylene glycol (PEG) 8000, 7 mM DTT by hanging-drop vapour diffusion at 14 °C. Crystals were harvested after 1 week. For cryoprotection, crystals were immersed for 2 min in 0.1 M Tris–HCl, pH 8, 10% PEG 8000, 7 mM DTT, 75 mM NaCl and 25% glycerol, and then flash frozen at 100 K. Native and selenomethionine-substituted data were collected at European Synchrotron Radiation Facility. Crystallographic programs were accessed *via* the CCP4 suite;[50] data were processed using MOSFLM and scaled and merged in SCALA (see Table 2 for details). Phases and initial electron density maps were calculated by SIRAS, using SHELX, and automatic model building was performed using ArpWarp before final rounds of manual model building and refinement using COOT[51] and REFMAC. Nucleotide co-crystallisation experiments were performed by including the specified concentration of nucleotide in both the precipitant solution and cryobuffer. Crystals were harvested after 1 week, and structures were solved by PHASER molecular replacement with, and REFMAC refinement against, the apo structure. The presence of bound nucleotide was determined by inspection of the difference $F_o - F_c$ electron density map in COOT. Complexes with 5′AMP and 5′CMP were further refined with REFMAC. Nucleotide and side chains for residues 219–220, invisible in the apo structure, were visible in the difference $F_o - F_c$ electron density maps, so modelled in COOT before final refinement using REFMAC. Molecular representations in figures were created using the PyMOL Molecular Graphics System‡ and BioDraw 10.0 (Merck).

‡ DeLano Scientific, http://www.pymol.org

cAMP phosphodiesterase assays

V5 epitope-tagged constructs of AKAP18δ and PDE4D3 were transiently expressed in HEK293 cells. Immunoprecipitates were washed three times (20 mM Tris–HCl, pH 7.4) before incubation at 30 °C for 10 min with 10 μM [^{3}H]cAMP in 20 mM Tris–HCl, pH 7.4, and 10 mM $MgCl_2$. The reaction was stopped by boiling for 2 min and [^{3}H]5′AMP was further hydrolysed to [^{3}H]adenosine by incubation with snake venom nucleotidase (Sigma). Finally, [^{3}H]cAMP was removed by anion exchange (Biorad AG 1-X2) before scintillation counting. To assay cAMP activity in GST pull-downs, the [^{3}H]cAMP SPA Enzyme Assay (GE Healthcare) was performed, following the manufacturer's guidelines.

Equilibrium fluorescence measurements

A Cary Eclipse fluorescence spectrophotometer (Varian) was used to measure fluorescence at 448 nm after excitation at 290 nm with increasing concentrations of MANT-AMP (Biolog) in the presence and absence of purified AKAP18^{CD}. A plot of the difference in fluorescence (ΔF) in the presence and absence of AKAP18δ ($F_{\mathrm{MANT}} - F_{\mathrm{MANT+AKAP18\delta}}$) over a range of MANT-AMP concentrations reveals the fraction of AKAP18δ that is bound to MANT-AMP at a given concentration. As the concentration of AKAP18δ (25 μM) is low, the K_d for MANT-AMP binding can be approximated by fitting the data to a hyperbolic function according to the equation:

$$\Delta F = B_0[\text{MANT-AMP}]/(K_d + [\text{MANT-AMP}])$$

where B_0 is the maximum change in fluorescence. ΔF was plotted as a function of [MANT-AMP] and fit to a hyperbolic function by least squares using Origin software in order to determine the K_d.

Pull-down assays

Pull-down assays were performed using GST-fusion proteins immobilised to glutathione Sepharose fast flow beads (GE Healthcare), and with 5′AMP–agarose (Sigma). For GST fusion pull-downs, GST-fusion protein was immobilised to the beads and washed with (500 mM NaCl, 25 mM Tris–HCl, pH 7.5, 2 mM DTT, 0.5 mM EDTA). Protein samples were then mixed with the beads in low salt buffer (150 mM NaCl, 25 mM Tris–HCl, pH 7.5, 2 mM DTT, 0.5 mM EDTA, 0.05% Tween 20). After washing away nonspecifically bound protein in low salt buffer, bound protein was detected by Western blotting, or cAMP phosphodiesterase assay in the case of PDE4D6. Pull-down with immobilised GST alone was routinely included as a negative control for pull-downs using GST fusion proteins.

Protein Data Bank accession numbers

Atomic coordinates have been deposited in the PDB with accession codes 2VFY (APO), 2VFK (5′AMP complex) and 2VFL (5′CMP complex).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2007.11.037

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