1 **Anchored and labile pools of Calcineurin are enabled by a disordered AKAP** 2 **scaffold**

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29

30 **Abstract**

31 Signalling requires precise spatial and temporal regulation of molecular interactions,
 32 which is frequently orchestrated by disordered scaffolding proteins, such as A-kinase
 33 anchoring protein 5 (AKAP5). AKAP5 contains multiple Short Linear Motifs (SLiMs)
 34 that assemble the necessary components, including the phosphatase Calcineurin,
 35 which is anchored *via* a well-characterised PxIxIT SLiM. Here we show, using a
 36 combination of biochemical and biophysical approaches, that Calcineurin also
 37 recognises additional lower-affinity SLiMs C-terminal to the PxIxIT motif. Moreover,
 38 we demonstrate that the assembly is in reality a complex system in which AKAP
 39 SLiMs spanning a wide affinity range act cooperatively to maintain distinct pools of
 40 anchored and more loosely held enzyme, analogous to transcription factor search
 41 complexes on DNA, and compatible with the requirement for both stable anchoring
 42 and responsive downstream signalling. We conclude that the AKAP5 C-terminus is
 43 enriched in lower-affinity/mini-SLiMs that cooperate to maintain a structurally
 44 disordered but tightly regulated signalosome.

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Introduction

Many enzymes involved in signal transduction are promiscuous and act in multiple pathways. Scaffolding proteins are therefore central to signalling since, by tethering signalling components and localising them, they enhance specificity and reaction rates by effectively enhancing local concentrations, and thereby achieve strict spatial and temporal control of catalysis (1, 2). A-kinase anchoring proteins (AKAPs) are a family of intrinsically-disordered proteins (IDPs) that were originally characterized by their ability to scaffold Protein Kinase A (PKA) (3, 4), but have since been shown to assemble multiple components into ‘signalosomes’ that can be targeted to specific receptors or sites (5, 6). Currently there are 14 annotated AKAPs in humans (AKAP1 to AKAP14).

AKAPs are typical of many IDPs in that they interact with their partners *via* Short Linear Motifs (SLiMs) (7, 8). The presence of multiple SLiMs with different specificities in AKAPs enables the combinatorial assembly of a dynamic multi-protein hub. Much of what we understand about disordered signalling complexes is based on the structures of folded partners bound to their peptide SLiMs. The rest of the IDP is either not resolved or is removed to facilitate structural studies. In many cases, the regions between SLiMs are assumed to be inert linkers, simply providing a length-dependent increase in the effective concentration, although predictions from physical models are not always reproduced experimentally, suggesting the reality is more complicated (9). The context in which IDP(R) partner interactions take place can also play an important role (10). The flanking sequences around SLiMs, although disordered even in the complex, often contribute to binding (11). Moreover, there can be complex interplay between SLiMs (7). For example, clustering or overlapping SLiMs can modulate interactions through cooperativity or competition. Further, multiple low-affinity SLiMs binding a single target can cooperate in a multivalent interaction to increase the binding affinity. The cooperativity can take different forms: e.g. allovalency (where multiple epitopes along the ligand bind a single site on

a partner (12)), or ‘fuzziness’ (where multiple interchangeable interaction sites exist on both the ligand and partner (13)).

Here we study the interaction of the ubiquitous phosphatase Calcineurin (also known as PP2B) with the C-terminal region of the scaffolding protein AKAP5 (previously known as AKAP79 in humans and AKAP150 in mice). Calcineurin couples changes in intracellular calcium concentration to the phosphorylation state of several important substrates (14). One example is the NFAT (Nuclear Factor of Activated T cells) family of transcription factors, which are dephosphorylated by Calcineurin, causing their translocation to the nucleus and stimulation of transcriptional programmes (15). Like other phosphatases, Calcineurin dephosphorylates sites with little sequence similarity (16, 17), and primarily recognises substrates or their scaffolding proteins through the interaction with SLiMs, such as the PRIET sequence in NFATc1 (18, 19). PRIET is a canonical Calcineurin recognition motif obeying the consensus sequence PxlIT, a so-called ‘PxlIT’ SLiM. This interaction has a 25 μ M affinity, which is within the optimal window for its signalling function (20). AKAP5 also has a PxlIT SLiM with which it engages Calcineurin, albeit one with a higher affinity – PIAIIT – that is suited to anchoring (0.4 μ M), while not so tight that downstream signalling is inhibited (21). In this study, we examined the AKAP5/Calcineurin interaction from the perspective of the IDP scaffold. Using Surface Plasmon Resonance (SPR) and NMR spectroscopy, we show that binding is far more extensive than the PxlIT alone and we identify additional regions that interact with μ M - mM affinity. We hypothesise that these additional low-affinity sites help to capture and maintain the high-affinity anchored Calcineurin. Moreover, they provide a separate ‘labile but localised’ pool of rapidly dissociating and rebinding Calcineurin, which could be intercepted by proteins such as NFAT that compete for Calcineurin in order to enact optimal downstream signalling.

Results

The AKAP5c scaffold is highly disordered and monomeric

AKAP5 can loosely be divided into three regions (Fig. 1a). Here, we have chosen to focus on the C-terminal region (300-427; AKAP5c), which we define as the region of homology across various species (Supp. Fig. S1) following a large insertion of unknown function in the rodent orthologues. The sequence contains three known SLiMs that bind either enzymes (Calcineurin (22, 23) or PKA (24)) or substrates (TRPV1/4 (25–27)). The enzyme-bound SLiMs adopt defined secondary structures in

Figure 1: Structure, hydrodynamic properties and oligomeric state of AKAP5₃₀₀₋₄₂₇ (AKAP5c). (a) Schematic of AKAP5: the N-terminal region (1-153) is involved in membrane attachment; the middle region (154-299) is recognised by scaffolding proteins of the Membrane associated Guanylate Kinase family; and the C-terminal region (300-427, shown expanded) contains three known SLiMs that bind Calcineurin, PKA and substrates e.g. TRPV1. (b) ¹⁵N HSQC of AKAP5c. (c) Secondary structure propensity (SSP) and Heteronuclear NOE values (colouring of SLiMs as a). (d) Far-UV CD spectrum. (e) Sedimentation velocity continuous c(s) distribution. (f) Live HEK- and CHO-cell split-luciferase assays (NanoBiT®, Promega). AKAP5 = full-length protein, PKA = regulatory PKA subunit (R2A) (positive control), Halo = HaloTag® (negative control). The order of listing indicates the orientation of the split luciferase: <protein>-LgBiT + <protein>-SmBiT.

the complexes: a β -strand in the case of Calcineurin (21) and an α -helix in the case of the docking and dimerization (D/D) domain of PKA (24). There is no structural information on the SLiM reported to bind TRPV1/4 (25, 26).

To fully understand the role of disorder in the assembly of signalling complexes by scaffolding proteins, it is important to understand the level and type of disorder present. The term “intrinsic disorder” (28) is used to describe all proteins that populate transient structures through to those that approach true random coils (i.e. those with no structural preferences and uniformly fast backbone dynamics). Two spectroscopic techniques were deployed to assess and delineate regions of nascent structure and to probe the backbone dynamics in AKAP5c: nuclear magnetic resonance (NMR) and circular dichroism (CD). The ^{15}N -HSQC spectrum (Fig. 1b) displays the low $^1\text{H}^{\text{N}}$ chemical-shift dispersion (0.7 ppm) and narrow line widths typical of disordered proteins. A near complete assignment of the backbone H, C, N and C^{β} chemical shifts was possible (Supp. Fig. S2), from which the secondary structure propensity (SSP (29)) could be calculated (Fig. 1c). SSP scores in AKAP5c are close to zero with alternating stretches of weak α - or β -propensities. For comparison, SSP scores are ca. +1 or -1 in stable α - and β -structures, respectively. It is interesting to note that the propensities of the Calcineurin- and PKA-binding SLiMs (red and yellow, respectively, in Fig. 1c) reflect the stable structures adopted in the bound states ($\text{SSP}_{\text{mean}} = -0.116 \pm 0.038$ and $+0.079 \pm 0.037$, i.e. weak β and α , respectively). Backbone dynamics were probed by $\{^1\text{H}\}^{15}\text{N}$ heteronuclear NOE (HNOE; Fig. 1c), which reveals motions on a time-scale faster than overall tumbling (HNOE < ca. +0.6). HNOEs were fairly uniform throughout AKAP5c (disregarding the highly mobile N- and C-termini). The average value was -0.20 ± 0.29 , with no single residue exhibiting a value > +0.1, and all SLiMs falling within one standard deviation of the mean. This is consistent with a highly dynamic polypeptide chain. CD spectroscopy also showed AKAP5c to be predominantly disordered (Fig. 1d): the signature large negative ellipticity at 200 nm was dominant, although there were

indications of some secondary structure (as evidenced by the weak shoulder at 222 nm) that is likely to be transiently populated given the results from NMR.

We next investigated the hydrodynamic properties of AKAP5c by sedimentation velocity analytical ultracentrifugation (SV AUC, Fig. 1e). AKAP5c sedimented slowly ($s_{20,w} = 1.27$) due to hydrodynamic friction resulting from its extended nature (frictional ratio $f/f_0 \sim 1.78$). The fitted mass (~ 15.6 kDa) was close to the calculated monomer mass (14.5 kDa). The overall picture of AKAP5c is therefore one of a highly dynamic and monomeric chain. However, there are reports that AKAP5 can dimerise (30, 31). Of particular relevance to this study is the observation of K333-K333 and K328-K328 intermolecular crosslinks (30), which are in the TRPV1/4 interaction SLiM immediately N-terminal to the Calcineurin binding site. Conversely, Nygren *et al.* (32) concluded that the AKAP5/Calcineurin complex is monomeric by SEC-MALS. Calcineurin is a heterodimer of A and B subunits, which are not reported to oligomerise further (14), although we observed a weak propensity for further dimerisation by SV AUC (Supp. Fig. S3).

Given AKAP5 forms complex assemblies within the cell, we investigated whether full length AKAP5 dimerises in a cellular context (HEK293T and CHO) with a highly sensitive live-cell luminescence assay (NanoBiT®, Promega) that uses a split-luciferase reporter engineered to give reduced background. A strong complementation signal was seen between PKA and AKAP5, however, AKAP5 did not give a self-complementation signal in these assays that was significantly different from the HaloTag® negative control (Fig. 1f). This does not preclude AKAP chains being brought into spatial proximity *in vivo* by interactions with multimeric receptors, e.g. tetrameric TRPV1 (33).

169 Calcineurin binding extends beyond the PxlIT site

170 The Calcineurin anchoring site in AKAP5c is at ³³⁷PIAIIIT³⁴³, a near-consensus PxlIT
 171 SLIM (18, 19), in which ³³⁸I takes the position of the consensus proline. In the crystal
 172 structure, ³³⁷P is not engaged and van der Waals contacts extend ca. five residues C-
 173 terminal of the PxlIT site (21). To investigate the involvement of more distal flanking
 174 regions, we titrated Calcineurin into ¹⁵N-labelled AKAP5c and followed binding by ¹⁵N
 175 HSQC (Fig. 2a). After one molar equivalent of Calcineurin had been added, many
 176 peaks had decreased significantly in intensity, including those from residues
 177 ³³⁸IAIIIT³⁴³ (shown boxed), although the intensity loss was not complete. The changes
 178 were purely to the peak intensities; no significant chemical-shift changes were seen,
 179 nor did the peaks get broader, indicating that the interaction was slow on the
 180 chemical-exchange timescale. No new peaks appeared, indicating slow overall
 181 tumbling of the complex (Calcineurin is 80 kDa), rendering the residues in the region
 182 engaging with Calcineurin invisible. The relative intensity changes when mapped
 183 onto the sequence extend approx. 24 residues C-terminal beyond the PIAIIIT site,
 184 with further broad dips around residues 390 and 410 (Fig. 2b). Calcineurin binding
 185 thus not only extends well beyond the PIAIIIT SLIM (which could be due to weak and
 186 transient interactions extending the footprint) but also involves distal regions of the
 187 C-terminus of AKAP5c.

Figure 2: Interactions of AKAP5c with Calcineurin and Calmodulin by ¹⁵N HSQC NMR. ¹⁵N-labelled AKAP5c (black) titrated sequentially with Calcineurin (Cn; magenta), Ca²⁺ (cyan) and Calmodulin (CaM; yellow), followed by HSQC. (a) HSQC of AKAP5c before and after addition of Cn. Peaks from non-proline residues in ³³⁷PIAIIIT³⁴³ are boxed with arrows. (b) Intensity changes relative to ¹⁵N-AKAP5c alone. Molar ratios of 1:1 Cn:AKAP, 1:20:1 Cn:Ca²⁺:AKAP and 1:20:1:1 Cn:Ca²⁺:CaM:AKAP are shown.

Calcium and Calmodulin do not significantly alter binding

Calcineurin has a regulatory domain that renders it enzymatically dormant in the absence of Ca^{2+} and Calmodulin. Increases in Ca^{2+} concentration lead to partial activation of Calcineurin, through loosening association of the regulatory domain and exposure of the Calmodulin binding region. Further binding of Ca^{2+} -Calmodulin results in full activation (15, 34). In addition to stimulating Calcineurin activity, this exposes a second SLiM-binding region, that interacts with binding partners containing a $\pi\phi\text{LxVP}$ motif (π = polar, ϕ = hydrophobic) in a Ca^{2+} -Calmodulin dependent manner (35). Although the focus of this study is on the dormant complex in which the $\pi\phi\text{LxVP}$ binding pocket in Calcineurin is occluded by the regulatory domain, we wanted to establish whether Ca^{2+} or Ca^{2+} -Calmodulin binding alters the interaction with PIAIIT or the new interactions found above.

We first titrated the pre-formed AKAP/Calcineurin 1:1 complex with Ca^{2+} , followed by Calmodulin. No changes were seen on addition of Ca^{2+} to a large excess (20:1; Fig. 2b). AKAP5c/Calcineurin binding is therefore calcium-independent. On addition of Calmodulin to 1:1:1 Calmodulin:Calcineurin:AKAP in excess Ca^{2+} , no changes were seen in the region of PIAIIT, indicating that PIAIIT-binding of Calcineurin was unaffected, in line with pull-down experiments from other laboratories (30, 32). However, further peak attenuation was observed in the region C-terminal but distal to the PIAIIT site, spanning residues 390-417 (Fig. 2b). In this case, peaks visibly broadened and shifted, indicating intermediate-to-fast exchange on the chemical shift time scale (unlike for Calcineurin binding, in which the peaks disappeared), suggesting that Calmodulin binds to the same site. Consistent with this, a separate titration of AKAP with Ca^{2+} and Calmodulin alone showed larger chemical shift changes for the same peaks along the same trajectories, indicating a larger mole fraction of CaM bound in the absence of Calcineurin (Supp. Fig. S4). Calmodulin therefore appears to compete with Calcineurin for binding to residues 390-417, a region ca. 50 residues from the canonical PxlIT site. This region is known to form an amphipathic helix on binding PKA, and contains two clusters of bulky

hydrophobic residues separated by a stretch of polar residues, which is a feature of Calmodulin binding (36), although the sequence was not predicted to be Calmodulin-binding by a database search (Calmodulin Target Database (<http://calcium.uhnres.utoronto.ca/ctdb/>)). We could not confirm whether Calmodulin also bound Calcineurin in the canonical manner, but the pattern of shifting and broadening of peaks continued beyond a 1:1 molar ratio of Calmodulin, which is compatible with binding to two sites.

PxIxIT is not required for binding to non-canonical sites

Given the involvement of PxIxIT-flanking and distal regions of AKAP5c in binding to Calcineurin, we investigated binding to AKAP5c in which the PIAIIIT site had been deleted, AKAP5c Δ PIAIIIT. The titration with Calcineurin (Fig. 3a) was carried out as previously. From the overlay of the intensity ratios (Fig. 3b), it is clear that the

Figure 3: Interactions of AKAP5c Δ PIAIIIT with Calcineurin by ^{15}N HSQC NMR. (a) ^{15}N -labelled AKAP5c Δ PIAIIIT alone (black; positions of deleted $^{337}\text{PIAIIIT}^{343}$ residues shown boxed with arrows). AKAP5c Δ PIAIIIT titrated with Calcineurin (Cn) to 1:1 Cn:AKAP (green). (b) Intensity changes relative to ^{15}N -AKAP5c Δ PIAIIIT alone. Corresponding WT data (magenta) reproduced from Fig. 2b. Grey boxed regions 1-3 indicate Cn binding sites in AKAP5c Δ PIAIIIT, with the corresponding amino acid sequences shown above. (c) Titrations of the 1:1 AKAP5c Δ PIAIIIT:Cn complex with PxIxIT and $\pi\phi\text{LxVP}$ peptides (0 to 10 molar equivalents shown).

immediate footprint around the PIAIIT site (residues 330-350) is lost, as expected. However, intensities more distal to the PIAIIT site still showed significant decreases in intensity, in the same regions and even more pronounced than in WT AKAP5c. Three regions with pronounced dips in intensity were seen, ³⁶¹QFLIS³⁶⁵, ³⁹⁰TLLIET³⁹⁵ and ⁴⁰⁴IQLSIEQLVN⁴¹³, which we termed secondary sites 1, 2 and 3, respectively.

Binding to these sites therefore does not depend on the presence of the PIAIIT site. Moreover, there is some evidence for competition with PIAIIT since deletion of PIAIIT appears to enhance binding to secondary sites. An important question not resolved by these experiments is whether PIAIIT and the secondary sites bind to the same or different sites on Calcineurin. In order to address this, the 1:1 ¹⁵N-AKAP5c_{ΔPIAIIT}/Calcineurin complex was further titrated with a PIAIIT-containing peptide (EPIAIITDTE; Fig. 3c). The peptide proved an effective competitor; ¹⁵N-AKAP5c_{ΔPIAIIT} peak intensities were almost fully restored after addition of three molar equivalents of the peptide (Fig. 3c, top). Given the similar hydrophobicity of the secondary sites to the πφLxVP motif, the experiment was repeated with a πφLxVP peptide (DDQYLAVPQH (35)), which was ineffective even upon addition of ten molar equivalents (Fig 3c, bottom). The recognition site for secondary sites 1, 2 and 3 is thus the PxlIT-binding groove in Calcineurin and not the πφLxVP binding site.

The secondary site sequences each contain three or more bulky hydrophobic residues and include isoleucine, which together are likely to underpin binding to the PxlIT-binding groove. Therefore, a further AKAP mutant was tested: AKAP_{ILVF->SA}, in which the PIAIIT site was present, but all the hydrophobic I, L, V and F residues in the three secondary sites were mutated to S or A to give ³⁶¹QASAS³⁶⁵, ³⁹⁰TASAET³⁹⁵ and ⁴⁰⁴AQSSAEQSAN⁴¹³. Following reassignment of the chemical shifts, the protein was again titrated with Calcineurin (Fig. 4a). Similar to the WT, large reductions in intensities were observed in and around the PIAIIT site (Fig. 4b). This was more pronounced in AKAP_{ILVF->SA} indicating higher occupancy of the site by Calcineurin, consistent with removal of competing secondary sites. No dips were seen at

secondary sites 2 and 3, (intensities were similar to the unbound AKAP) and site 1 was partially attenuated (overlap with the PxlIT footprint made the effect less clear). The secondary sites therefore each constitute additional hydrophobic SLiMs that despite not being canonical PxlIT sites, are nevertheless recognised by the PxlIT-binding pocket on Calcineurin. One small extra region of non-PIAIIIT interaction was apparent in AKAP_{ILVF->SA} in the absence of the secondary sites: ³⁷¹VGVF³⁷⁵, which contains three bulky hydrophobic residues but no isoleucine, and there were also weaker dips corresponding to regions containing just one or two hydrophobic residues: ³⁷⁸NGFED³⁸², ³⁸⁶EQYET³⁹⁰, ³⁹⁸SLVKN⁴⁰² and ⁴²⁴NLLQ⁴²⁷ (shown by asterisks in Fig. 4b). We hereon refer to these as ‘mini-SLiMs’ in order to differentiate them from the primary and secondary sites.

Non-canonical sites sequester additional Calcineurin

The detection of three secondary Calcineurin-binding SLiMs led us to speculate about their relative affinity compared to PxlIT, and whether there is interplay between them. Modified AKAP constructs, Cys-AKAP5c, Cys-AKAP5c_{ΔPIAIIIT} and Cys-

AKAP5_{CILVF->SA} were chemically biotinylated for N-terminal-specific immobilisation on a streptavidin surface so that Calcineurin binding could be assessed by SPR. Calcineurin binding to AKAP5c (Fig. 5a) was clear but complex: the response curves (1) had no clear plateau and (2) displayed evidence of multiple binding sites with both fast and slow kinetics in both the association and dissociation phases. In addition, a fraction of the Calcineurin remained bound at long times. The complexity was reduced somewhat in the curves for AKAP5_{CILVF->SA} (Fig. 5b), which showed a clear saturation plateau that was absent in the WT, and complete dissociation. For AKAP5_{ΔPIAIIIT} (Fig. 5c), the curves showed a clear plateau and complete dissociation, but weaker overall binding. Also noteworthy was that the binding curves for AKAP5_{CILVF->SA} and AKAP5_{ΔPIAIIIT} did not sum to reproduce the AKAP5c binding curve,

Figure 5: cAKAP/Calcineurin binding by surface plasmon resonance. Calcineurin injected over a streptavidin surface pre-immobilised with (a) AKAP5c, (b) AKAP5_{CILVF->SA} or (c) AKAP5_{ΔPIAIIIT}. Calcineurin concentrations were 2-fold dilutions from 2.56 μM (AKAP5c and AKAP5_{CILVF->SA}) or 10 μM (AKAP5_{ΔPIAIIIT}). (d) Fits of 'steady-state' response vs Calcineurin concentration used to obtain estimates of $K_{d,app}$ and R_{max} . (e) Surface site complexity modelled in Evifit (37): distribution plots of k_{off} vs K_d obtained by fitting the data from a-c using a regularization and Bayesian approach (see Supp. Fig. S5 for further details).

i.e. $A \neq B + C$, despite their comprising separately the two kinds of binding site present in the WT. This was particularly apparent in the dissociation phase, where the WT showed a sizeable fraction of Calcineurin was essentially permanently bound, while no long-lasting binding of Calcineurin was observed with either mutant. The binding to the PxlIT and the secondary sites is therefore cooperative.

An estimate of the K_d could be obtained from fitting the signal (RU) at the end of the association period vs Calcineurin concentration (Fig. 5d). The values obtained can only be estimates for AKAP5c and AKAP5c $_{\Delta PIAIIT}$ due to the lack of saturation ('steady-state') of the former and the lack of high-concentration data for the latter. Nevertheless, the trend in apparent K_d is clear: AKAP5c binds Calcineurin about twice as tightly compared to AKAP5c $_{ILVF \rightarrow SA}$, ($K_d \sim 0.3$ vs $0.6 \mu M$) while both PIAIIT-containing AKAP constructs bind Calcineurin with at least ten-fold higher affinity than AKAP5c $_{\Delta PIAIIT}$, which has a K_d of $\sim 10 \mu M$. Estimation of the total surface capacity for Calcineurin (R_{max}) is also instructive as it gives information on the stoichiometry of binding under saturating conditions. From extrapolation of the binding curves, it appears that AKAP5c can accommodate ca. 50% more Calcineurin than AKAP5c $_{ILVF \rightarrow SA}$ (62 vs 42 RU; Fig. 5d). The secondary sites may account for this 'extra' 20 RU capacity, since R_{max} for AKAP5c $_{\Delta PIAIIT}$ is (very approximately) 12 RU.

Multivalent ligands such as AKAP5c are intrinsically heterogeneous, and thus deviation from ideal pseudo-first order binding kinetics is not unexpected. Such systems require a different analysis strategy if they are to be properly characterised. A suitable alternative approach has been developed by Schuck and colleagues ('Evilfit'; (37)), which uses modern regularization methods combined with a Bayesian approach to add features that are essential to explain the observed data to the single species model. Such features could be populations of lower-affinity or poorly reversible sites. With this approach, it was possible to obtain excellent fits to the raw SPR data (Supp. Fig. S5), and to extract k_{off} vs K_d distribution plots (Fig. 5e). The complexity seen for Calcineurin binding to AKAP5c was deconvolved into 3 or 4 classes of surface sites: one or possibly two very similar sites with K_d 10-100 nM,

another with K_d 1-10 μM , and a final class with a very slow k_{off} that presumably accounts for the lack of full dissociation observed even at long times (i.e. poor reversibility). The number of surface site classes was reduced in AKAP5_{C_{ILVF}->SA} to one main site with K_d 100 nM-1 μM . The distribution was simpler again for AKAP5_{C_{ΔPIAIIIT}} which displayed a single class of sites with K_d 10-100 μM (the true value is likely to lie at the lower end of this range given the value obtained from the equilibrium fit in Fig. 5d (~10 μM) and the fact that a high fraction bound was seen by NMR at a concentration of 100 μM (Fig. 3b)). These results support a picture in which AKAP5c acts as a multivalent surface capable of binding one or more molecules of Calcineurin at different sites. There are also strong indications that the PIAIIIT and secondary binding sites bind cooperatively. Firstly, the K_d seems to vary by as much as an order of magnitude dependent on the presence or absence of the other class of site (Fig. 5e). For two-site binding in the absence of cooperativity, the overall observed association constant will be $K = K_A + K_B$. Given the apparent overall K_d s are $K_{d,\text{WT}} = 0.26$, $K_{d,\text{ILVF} \rightarrow \text{SA}} = 0.55$ and $K_{d,\Delta\text{PIAIIIT}} \sim 10 \mu\text{M}$ (Fig. 5d), the K_a s are $K_{a,\text{WT}} \sim 3.8$, $K_{a,\text{ILVF} \rightarrow \text{SA}} \sim 1.8$ and $K_{a,\Delta\text{PIAIIIT}} \sim 0.1 \mu\text{M}^{-1}$. Clearly, $3.8 > 1.8 + 0.1$, indicating positive cooperativity. Secondly, there appears to be a ‘long-lived’ (slow k_{off}) bound population that is only significant when both sites are present (Fig. 5a&e). This latter phenomenon is reminiscent of the re-capture behaviour seen for bivalent antibodies, which also show anomalously slow dissociation, or ligands rebinding to receptors at cell surfaces (38).

Discussion

From our experiments, it is clear that the Calcineurin binding site in AKAP5c is far more extensive than PIAIIIT, involving additional SLiMs and ‘mini-SLiMs’ in the C-terminal region that interact with μM -mM affinity: non-consensus SLiMs containing as few as three bulky hydrophobic residues appear able to bind Calcineurin with low-to-mid μM affinity (AKAP5_{C_{ΔPIAIIIT}}; Fig. 5d&e), and mini-SLiMs containing only one or two hydrophobic residues produce detectable binding by ¹⁵N-HSQC (Fig. 4b). These

344 additional sites cooperate to increase both the effective affinity, and potentially the
 345 binding capacity of the scaffold. The effective affinity is likely to be raised through
 346 both an increase in the number of productive encounters given the high density of
 347 SLiMs, and through rebinding mechanisms that reduce the diffusion of Calcineurin
 348 away from the scaffold since the molecule is likely to be intercepted by another
 349 binding site rather than diffusing away. We note that a recently developed model for
 350 competitive two-site binding showed that the bound population at one site can
 351 influence kinetics of binding at the other where they are connected by a diffusion
 352 pathway (39). This latter point is borne out directly in our experiments by the lack of
 353 complete dissociation of Calcineurin on AKAP5c seen by SPR (Fig. 5a). The 'labile but
 354 localised' pool of rapidly dissociating, diffusing and rebinding Calcineurin can
 355 presumably also be captured by downstream effectors such as NFAT despite their
 356 mid μM affinities (Fig. 6).

Figure 6: Non-canonical SLiMs could facilitate both anchoring and formation of a labile Calcineurin pool that enables efficient transfer to effectors. AKAP5c captures and retains Calcineurin using its consensus PIAIIT (red arrow) and secondary sites (red rectangles), building anchored and signalling pools of bound Calcineurin (blue and green, respectively). Calcineurin binds with lower-affinity to the secondary sites, enabling effective competition by weaker PxlIT motifs on downstream effectors, e.g. the PRIET SLiM in NFAT, and optimal signalling.

These new insights are made possible by the combination of NMR experiments and a non-traditional approach to SPR data fitting that is validated by strategic mutagenesis. The experimental study of molecular recognition by disordered proteins can be very challenging due to the rapid increase in complexity inherent in multivalent binding. Competition, cooperativity and multiple binding modes often limit the study to global behaviour only (in which mechanistic information may be lost), or dissection into simpler pairwise interactions, treating the intervening sequences as inert (which may incur the loss of important information on e.g. cooperativity). SPR data on such systems is often high quality in both signal-to-noise and reproducibility, while being deemed “unfittable” due to the ill-posed problem of decomposing a distribution of exponentials. However we find that the combination of regularization and Bayesian approaches (37), designed for the deconvolution of mass transport and surface heterogeneity, is also very effective at decomposing multivalent interactions. The resulting distributions may be subsequently validated by competition and mutagenesis, as we have done here, alongside mapping by NMR, which is both residue-specific and sensitive to a wide range of interaction affinities (40).

In addition to the secondary sites and mini-SLiMs we establish in this study, further evidence for weak, non-canonical engagement of Calcineurin with hydrophobic residues is seen in the existing PxlXIT peptide/Calcineurin X-ray crystal structures, in which the reverse face of the PxlXIT site provides an additional potential encounter point (Supp. Fig. 6a). In the structures, one with PVIVIT (20) and one with the AKAP sequence PIAIIIT (21), the stoichiometry in the asymmetric unit is two Calcineurin heterodimers to one peptide, with the second heterodimer binding to the opposite side of the PxlXIT site, out of register by one residue towards the C-terminus of AKAP5. The peptide thus forms an axis of pseudosymmetry. In both cases, the first heterodimer that engages the consensus elements of the PxlXIT sequence has a larger buried surface area and a greater number of hydrogen bonds. The opposite side displays VxV or Axl, to which a second heterodimer is bound in the

crystal, using the same hydrophobic PxIxIT-binding groove, but with significantly lower affinity, since no evidence for a 2:1 stoichiometry was seen in solution by size exclusion chromatography or SEC-MALS (20, 21). However, the presence of hydrophobic residues in the two 'x' positions may serve as an additional capture surface on PxIxIT, even if unstable. Their presence may (partially) account for the fact that in our experiments, mutation of the three distal secondary sites in AKAP5_{CILVF->SA} did not restore 'pure' pseudo first-order binding characteristic of simple bimolecular collisions (Fig. 5b & e). Considered as a group, PxIxIT affinities span a wide range, and the fine-tuning of PxIxIT affinities by altering the 'x' positions presumably facilitates evolution into either anchoring or signalling roles. It is interesting to note that during selection for competitive peptide inhibitors of PxIxIT binding there was a clear enrichment for hydrophobic residues (V, I and L) in the two x positions (41), while many of the low affinity substrates favour charged or polar amino acids in these positions that would be incompatible with the non-polar groove on Calcineurin e.g. PRIET in NFATc1 (25 μ M; Supp. Fig. 6b).

Our findings also demonstrate that SLiM recognition in AKAP5c is non-exclusive: both Calcineurin and Calmodulin recognise features of the canonical PKA SLiM and therefore all three are in competition for the same site (Fig. 2 & 3). Interactions of one SLiM with many partners have been documented (42), and due to the substitution-tolerant nature of SLiMs coupled with the conformational plasticity of IDPs, may be a common feature of disordered scaffolds. In this example, PKA has a higher affinity for AKAP5c than Calcineurin (and presumably Calmodulin). However, the *in vivo* concentration of cellular PKA is sub- μ M (43) whereas concentrations of Calcineurin are generally much higher (e.g. 1% of the total protein concentration in brain (44)), and further concentrated at the membrane or cytoskeletal elements (45). A slowly diffusing pool of highly concentrated Calcineurin (or Calmodulin), fed by an intrinsically high cellular presence and maintained by other local SLiMs, will have a high effective concentration and therefore be

competitive. Moreover, due to the high abundance of AKAPs, there is likely to be an excess of anchoring sites compared to the kinase (46).

Parallels can be drawn between this ‘labile but localised’ pool of Calcineurin that supplies the canonical anchoring site with the ways transcription factors (TFs) bind DNA. TFs recognize their binding site with high affinity, but also bind, albeit with low affinity, to non-canonical DNA and *via* mechanisms such as sliding, jumping and monkey bar strand transfer reduce the dimensionality of the search (47, 48). The recognition of IDPs containing multiple SLiMs of different affinities for a partner is likely to share similar properties, for which the term ‘allovalency’ has been coined (12, 49).

Here we uncover several Calcineurin interaction motifs spanning a range of affinities from nM-mM in a short region of a scaffold protein. At least three hydrophobic patches bind the Calcineurin PxlIT groove with μ M affinity, which is similar to Calcineurin’s affinity for many of its PxlIT-containing substrates. The patches in AKAP5c are conserved across relevant AKAPs (Supp. Fig. 1b) and do indeed appear to bestow a biologically useful function: to enhance capture and anchoring, while maintaining a more mobile pool of Calcineurin than would be present if the anchoring protein contained one ultra-high affinity SLiM. The mM-affinity partial/mini-SLiMs may act together to further conserve the hydrophobic ‘capacitance’ of the region responsible for Calcineurin capture.

Conclusion

The scaffolding protein AKAP5 contains a well-characterised PxlIT-motif Calcineurin binding site. We characterise at least three additional binding sites of lower affinity C-terminal to the PxlIT site and propose that these additional sites create a ‘labile but localised’ pool that supplies Calcineurin to both the canonical anchoring site and to downstream effectors such as NFAT. We conclude that in AKAP5, the disordered regions between anchoring SLiMs, rather than being inert tethers, are enriched in

lower-affinity/mini-SLiMs that act together to maintain a stable but responsive signalosome.

Materials and Methods

Constructs

pcDNA™3.1 V5-His TOPO containing full length human AKAP5 with V5 and His tags was obtained. AKAP5_{C_{ILVF}->SA} was generated by gene synthesis (GeneArt; ThermoFisher). pOP5T was created from pOP5TT (Hyvönen *et al.*, unpublished), by deletion of thioredoxin leaving a TEV-removable N-terminal octa-His tag. pET15b CnA CnB, which contains human PPP3CA and PPP3R1 (50) was obtained from Addgene (11787). Plasmid pQE30 CaM containing rat Calmodulin (protein sequence 100% identical to human) was obtained from Dermot Cooper, Department of Pharmacology, University of Cambridge. NanoBiT vectors were obtained from Promega.

Cloning was carried out using standard molecular biology techniques. The coding sequence for AKAP5c was PCR amplified from pCDNA3.1 hAKAP and inserted between the BamHI and HindIII sites of pHAT3 using restriction cloning. All other cloning was carried out using a modified SLIC protocol based on (51). The appropriate plasmid and insert sequences (see above) with 15 bp complementary overhangs, or 12 bp for deletion of the PIAIIIT site were amplified using Q5 polymerase (NEB). For unique Cys mutants, a Cys codon was added to the forward primer immediately after the GS in the Thrombin or TEV site. Final constructs were:

pHAT3 AKAP5c	Human AKAP5 300-427 with a Thrombin-cleavable His ₆ tag
pHAT3 AKAP5c_{ΔPIAIIIT}	Human AKAP5 300-427 Δ337-343 (PIAIIIT) with a Thrombin-cleavable His ₆ tag
pOP5T AKAP5c_{ILVF->SA}	Human AKAP 300-427 F362A, L363S, I364A, L391A, L392S, I393A, L406S, I408A, L411S, V412A with a TEV cleavable N-terminal His ₈ tag
pOP5T Cys-AKAP5c	Human AKAP 300-427 with a TEV cleavable N-terminal His ₈ tag and an N-terminal Cys
pOP5T Cys-AKAP5c_{ΔPIAIIIT}	Human AKAP 300-427 Δ337-343 (PIAIIIT) with a TEV cleavable N-terminal His ₈ tag and an N-terminal Cys
pOP5T Cys-AKAP5c_{ILVF->SA}	Human AKAP 300-427 F362A, L363S, I364A, L391A, L392S, I393A, L406S, I408A, L411S, V412A with a TEV cleavable N-

	terminal His ₈ tag and an N-terminal Cys
pOP5T CaM	Rat Calmodulin with a TEV cleavable N-terminal His ₈ tag
pBiT1.1-C AKAP5	Full-length human AKAP5-LgBiT
pBiT2.1-C AKAP5	Full-length human AKAP5-SmBiT
NanoBiT™ Negative Control Vector	HaloTag®-SmBiT
LgBiT-PRKAR2A Control Vector	LgBiT-PRKAR2A (RIIA regulatory subunit of PKA)

Protein sequences are presented in full in Supplementary Information.

Protein expression and purification

All proteins were expressed in *E.coli* BL21(DE3) or BL21 Star (DE3) cells transformed with the appropriate plasmid. For unlabelled proteins 2 L baffled flasks containing 500 ml of AIM LB or AIM 2YT autoinduction medium (Formedium) supplemented with 50 µg/ml carbenicillin (and 1 mM CaCl₂ for Calmodulin) were inoculated with 1% starter culture in LB medium and grown overnight at 30 °C with shaking at 170 rpm. Labelled proteins were expressed similarly, but in M9 minimal medium containing (as appropriate) 0.4 g/L ¹⁵N ammonium chloride, 4g/L uniformly labelled ¹³C glucose as the sole nitrogen and carbon sources and supplemented with 5% Celvone complete medium with the appropriate isotopic labelling. Cells were grown to an OD₆₀₀ of 0.7 before induction with 0.5 mM IPTG, and grown overnight at 23 °C.

AKAP and Calmodulin constructs expressed from either pHAT3 or pOP5T were purified as follows: cells were resuspended in IMAC buffer A (20mM Tris pH 8.0, 0.5 M NaCl, 10 mM imidazole) with SIGMAFAST EDTA free protease inhibitor cocktail. Cells were lysed using an Emulsiflex and clarified lysate was heated at 70 °C for 20 min. Precipitated protein was removed by centrifugation and the supernatant further purified by Ni-NTA affinity chromatography. Bound proteins were eluted with IMAC buffer B (A containing 400 mM imidazole) and dialysed overnight into 20 mM Tris pH 8.0 using 7 kDa cut-off membrane, the His tag was removed with thrombin (pHAT3) or TEV (pOP5T), uncut protein removed by passing over Ni-NTA resin, and proteins further purified using a 6 ml Resource Q column (Cytiva) eluted with a linear NaCl gradient 0-400 mM. DTT (2 mM) was present in all steps after the initial Ni-NTA purification of proteins containing a unique cysteine. Proteins were concentrated and buffer exchanged into the appropriate buffer before storage at -80 °C.

Calcineurin was purified similarly, except that rather than heating, it was precipitated from clarified lysate by addition of ammonium sulphate to 45% saturation on ice before being resuspended in IMAC buffer A, and the His tag on the A subunit was not removed. After elution from the Resource Q column, Calcineurin was concentrated to ~ 5-10 mg/ml and further purified by size exclusion chromatography using an S200 16/600 column (Cytiva) in 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM DTT and stored at -80 °C.

Peptides

Peptides (EPIAIIITDTE and DDQYLAVPQH) were purchased from Biomatik and dialysed extensively against the appropriate buffer before use.

Biotin labelling

Proteins were labelled using EZ-Link™ Maleimide-PEG2-Biotin (ThermoFisher) in 10 mM PIPES pH 6.5, 150 mM NaCl according to the manufacturer's instructions.

Concentration measurements

Protein and peptide concentrations were determined spectrophotometrically using a NanoDrop One^C instrument (ThermoFisher) at 205 nm for the AKAP constructs (due to their low ϵ_{280}) and 280 nm for Calcineurin and Calmodulin. Extinction coefficients were calculated using <https://spin.niddk.nih.gov/clore/Software/A205.html> based on Anthis & Clore, 2013 (52).

Nuclear Magnetic Resonance

NMR measurements were made on ¹⁵N- or ¹³C,¹⁵N-labelled proteins (~100 μM) in 10 mM PIPES pH 6.5, 150 mM NaCl (NMR buffer) and 10-15% ²H₂O. Experiments were recorded at 25 °C on Bruker DRX600 or 800 spectrometers. Assignments were obtained using a conventional triple-resonance approach (HNCA, HN(CO)CA, HNCO, HNCACB, HN(CO)CACB) alongside TOCSY-¹⁵N-HSQC, NOESY-¹⁵N-HSQC and

HNN/HN(C)N experiments (53, 54). Data were processed using AZARA (v.2.7, © 1993-2021; Wayne Boucher and Department of Biochemistry, University of Cambridge). Triple resonance experiments were recorded with 25% nonuniform sampling, using Poisson-gap sampling (55), and reconstructed using the Cambridge CS package and the CS-IHT algorithm (56). Assignments were made using CcpNmr Analysis v. 2.4 (57). Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS). Heteronuclear NOE values were obtained at 600 MHz with either 4 s of ^1H saturation using a 120° pulse train or a 4 s delay prior to the first ^{15}N pulse (58). Complexes with Calcineurin were first prepared at the defined stoichiometries in 10 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM DTT and then dialysed into NMR buffer. Peptides were added from concentrated stocks to minimise dilution effects. Intensity ratios were calculated using fitted peak heights in Analysis. Chemical-shift differences were calculated using $\Delta\delta = [(\Delta\delta^{\text{H}})^2 + (0.15 \times \Delta\delta^{\text{N}})^2]^{1/2}$ (40).

Far-UV Circular Dichroism Spectroscopy

Experiments were performed at 0.1 mg/ml over a 185-260 nm range at 25 °C in 10 mM PIPES pH 6.5 and 150 mM NaF and in 1 mm path-length cuvettes. Spectra were acquired using an AVIV 410 spectrometer in 1 nm wavelength steps, averaged over three accumulations and baseline-corrected using buffer before smoothing, using the manufacturer's software. Millidegree units were converted to mean residue ellipticity (MRE) with units $\text{deg cm}^2 \text{dmol}^{-1} \text{res}^{-1}$ using $\text{MRE} = \text{millideg.} / \{(\text{no. residues} - 1) \times c \times l \times 10\}$, where c = molar concentration and l = path length in cm.

Analytical Ultracentrifugation

Sedimentation velocity was measured in an Optima XL-I centrifuge (Beckman Coulter). For AKAP5c, standard 12 mm double-sector Epon centrepieces with sapphire windows contained 400 μl of AKAP5c at 1 mg/mL or buffer (10 mM sodium phosphate pH 6.5, 150 mM NaCl). Interference data were acquired at 40 krpm using an An60 Ti four-hole rotor, at 20 °C. A multi-component sedimentation coefficient

$c(s)$ distribution was obtained from 100 scans collected over 22 h by direct boundary modelling of the Lamm equation using Sedfit v.15 (59). The density and viscosity of the buffer ($\rho = 1.0055$ g/ml; $\eta = 1.01881$ cP) and the partial specific volume of the protein (0.72918 ml/g) were calculated using Sednterp (60). For Calcineurin, 12 mm double-sector Epon centrepieces with sapphire windows contained 400 μ l of Calcineurin at 0.4 mg/mL or buffer (10 mM PIPES pH 6.5, 150 mM NaCl, 5 mM DTT). Interference data were acquired at 50 krpm using an An50 Ti eight-hole rotor, at 20 °C. A multi-component sedimentation coefficient $c(s)$ distribution was obtained from 20 scans collected over 5 h by Sedfit, as above. The density and viscosity of the buffer ($\rho = 1.0054$ g/ml; $\eta = 1.02631$ cP) and the partial specific volume of the protein (0.73431 ml/g) were calculated by Sednterp, as above.

Cell culture

HEK293T cells were cultured in 10 cm tissue culture treated dishes grown at 37 °C in a 5% CO₂ atmosphere in HEPES buffered DMEM/F12 1:1 (Merck) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. Cells were maintained in logarithmic growth phase and passaged upon reaching ~80% confluence (every 2-3 days).

Split-Luciferase Reporter Assays (NanoBiT)

Split luciferase reporter assays were carried out using the Promega NanoBiT system. Briefly, cells were diluted in HEPES buffered DMEM/F12 1:1 (Merck) supplemented with 10% fetal bovine serum and 2 mM L-glutamine to 50,000 cells/ml and plated at a density of 5000 cells per well (100 μ l) in 96-well white clear-bottom tissue culture treated plates (Corning 3610). Cells were grown for 24 h before transfection. Cells were transiently transfected with 50 ng each of the appropriate plasmids using FuGENE HD (Promega) at a FuGENE DNA ratio of 3:1. After transfection cells were grown for a further 24 h before assay. Plates were left to equilibrate at room temperature for 20 minutes before addition of NanoGlo live cell substrate (Promega) according to the manufacturer's

instructions. After mixing at 800 rpm for 10 s, luminescence was assayed in a PHERAstar microplate reader (BMG LABTECH) at 25 °C using bottom optics and an averaging time of 10 s.

Surface Plasmon Resonance

Experiments were performed on a Biacore T200 instrument (GE) at 25 °C. Biotin-Cys-AKAP5c, Biotin-Cys-AKAP5_{C_{ILVF}->SA} and Biotin-Cys-AKAP5_{ΔPIAIIIT} were immobilised on separate channels of an SA sensor chip (Cytiva). In order to optimise the analyte response signal-to-noise ratio, 12 RU were immobilised for AKAP5c and AKAP5_{C_{ILVF}->SA}, and 55 RU for AKAP5_{ΔPIAIIIT}; analyte responses for AKAP5_{ΔPIAIIIT} were subsequently re-scaled by a factor of 12/55 for comparative purposes. Binding to AKAP5c and AKAP5_{C_{ILVF}->SA} was determined in 10 mM PIPES pH 6.5, 150 mM NaCl, 0.05% v/v Tween20, at ten Calcineurin concentrations (0, 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 nM). Binding to AKAP5_{ΔPIAIIIT} was determined at nine Calcineurin concentrations (0, 78, 156, 313, 625, 1250, 2500, 5000 and 10000 nM). All concentrations were run in duplicate. The contact time, dissociation time and flow rate were 300 s, 1200 s and 30 μL min⁻¹, respectively. The chip surface was regenerated with 50 mM NaOH, 1 M NaCl for 1 or 2 x 60 s. Data were processed using the manufacturer's BIAevaluation software and further analysis was carried out in EVILFIT (37).

Data availability

Chemical-shift assignments for AKAP5c (see also Supp. Fig. S2) have been deposited in the BMRB with accession number XXXX.

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