Identification and Structural Characterization of Novel A-kinase Anchoring Proteins

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Identificatie en Structuur Karakterisering van Nieuwe A-kinase Anchoring Proteins (met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op vrijdag 14 maart 2014 des middags te 4.15 uur

door
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Promotor Prof. dr. A.J.R. Heck

Co-promotor Dr. A. Scholten

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Chapter 1

General Introduction

1. Modulation of Cell Signaling

Many complex and very diverse organisms populate our world. These organisms often consist not of just one cell but a large array of varying cell types. These cells are all connected and in order to properly function they are constantly communicating. This signaling between cells allows them, and thus the body, to adjust to different environmental, physiological and/or psychological conditions. For instance, the rhythm of the heart will adjust once signals, which convey the message that more oxygen is needed when performing strenuous exercise are sent and processed. Not only heart rhythm but also memory, the functioning of all organs/tissues and other functions of the body are regulated by cellular signaling in order to maintain proper function.

Cell signaling is a multifaceted system consisting of complex, intricate networks of signaling molecules, receptors and transducing proteins which, together, control the cellular activities and their corresponding actions. The signals which are sent and received, act in response to the external and internal stimuli of the cell, i.e. its microenvironment(1). There are three forms of intercellular signaling: Juxtacrine(2), paracrine(3) and endocrine(4).

Juxtacrine signaling requires cell-to-cell contact for transmission of the signal between two cells(2) (Figure 1). The Notch signaling pathway, with its single-pass transmembrane Notch receptor protein, has parts inside and outside of the cell membrane. Once a ligand docks onto the extracellular domain, proteolytic cleavage is activated and the intracellular domain of the Notch protein is released to transduce the signal inwards(5). Notch ligands are transmembrane proteins as well, which require direct contact to activate Notch signaling in an adjacent cell(6).

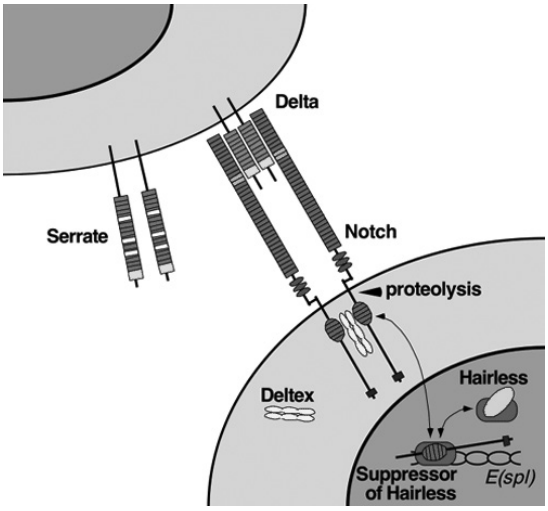

Figure 1 *The Notch signaling pathway is an example of juxtacrine signaling. The transmembrane Notch receptor protein allows docking of a ligand from an adjacent cell. Due to the docking the conformation of the Notch protein alters which induces proteolytic activity of the protein. The intracellular domain is cleaved and sent as a signal (Reproduced from NIH).*

Dopamine, a neurotransmitter, acts in a paracrine fashion(7). This form of signaling requires the target cell to be situated close to the signal-releasing cell(3) (Figure 2). For instance, in the transmission of information through nerves, dopamine is released from presynaptic neurons into the synaptic cleft. The dopamine active transporter, a transmembrane protein on the receiving postsynaptic neuron binds dopamine and actively transports this messenger inwards where it propagates the signal via intracellular pathways. Various diseases and addictive drugs are associated with this pathway, such as for example: cocaine(8) and cancer(9-11).

Endocrine signaling acts via the re-

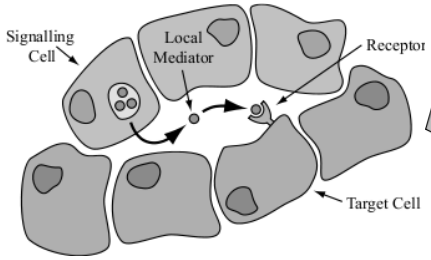

Figure 2 *Paracrine signaling is a form of communication between two nearby cells. There is a certain perimeter of the released ligand which limits the outcome of the signal.*

(http://www.labbookpages.co.uk/research/bioNode.html).

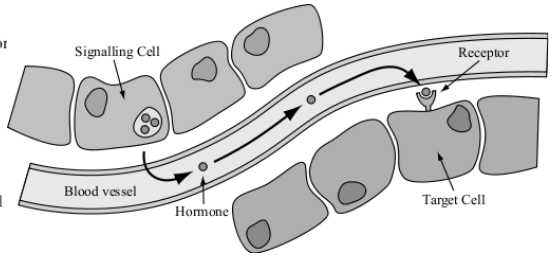

Figure 3 *Endocrine signaling consists of a ligand (a hormone or peptide/protein) being released from a signaling cell. This ligand will then traverse the bloodstream to reach the target cell. Once the ligand has reached the target cell it will bind to the corresponding receptor.*

(http://www.labbookpages.co.uk/research/bioNode.html)

lease of extracellular signaling molecules, such as hormones from glands in the brain and throughout the body to act on distant cells in other organs and tissues (Figure 3). This type of signaling regulates metabolism(12), growth(13) and several tissue functions(14). Two types of hormone receptors exist: Peptide hormone receptors are typically transmembrane proteins, frequently referred to as the G-protein-coupled receptors (GPCR)(15). Steroid hormone receptors are generally intracellular receptors(16). Hormone receptors are also found to fall under paracrine signaling.

Hormone receptors initiate rapid signal transduction pathways(17) which often consist of a linear cascade of enzyme activities. These control the proteins which mediate the physiological response of the cell, and thus ultimately the organ. To transport the molecular signal that binds to a receptor on the cell surface inwards across the membrane, signaling pathways often make use of so-called second messengers(18, 19). In this course of action, the receptor itself, or an associated or proximal enzyme, synthesizes a diffusible second messenger molecule to propagate and amplify the extracellular cue within the cell. For example, cyclase enzymes synthesize cyclic nucleotide second messengers upon steroid hormone mediated activation. Another example is ion channels which allow signaling metal ions (e.g. Ca2+) to enter or leave the cell(20). These messengers activate their effector molecules such as intracellular ion channels and protein kinases. There are three main types of second messenger molecules: Hydrophilic molecules such as cAMP(21), cGMP(22), IP3(23) and Ca2+ (24) which are located within the cytosol; Hydrophobic molecules like diacylglycerol(25) and phosphatidylinositols(26), which are located within the membrane, and; the gases nitric oxide(27) and carbon monoxide(28) that are not prohibited to either environment.

2. cAMP Pathway

GPCRs are integral membrane proteins that consist of a seven membrane spanning region and constitute the largest protein superfamily in mammalian genomes(29) (Figure 4). They are only present in eukaryotes, including yeast, plants and animals(30). Two major sig-

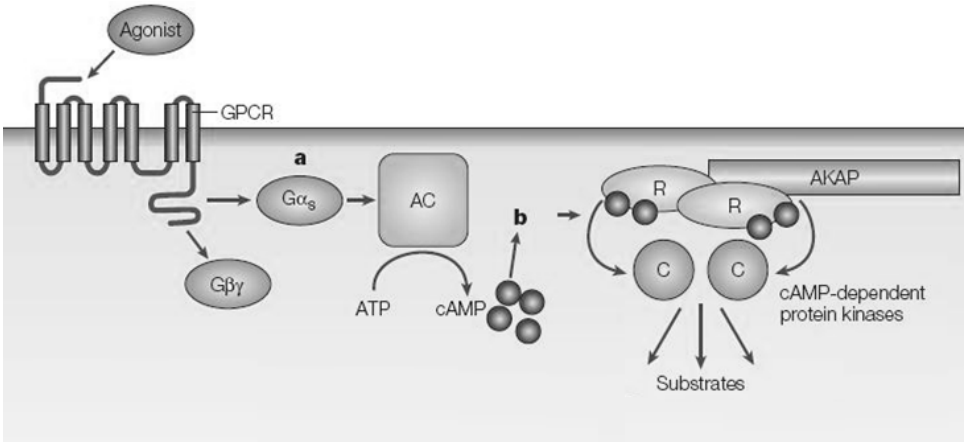

Figure 4 *A peptide/hormone binds to GPCR causing the release of Gα. Gα will bind to AC which transforms ATP into cAMP. The secondary messenger will then bind to the two cAMP binding domains each PKA regulatory subunit contains. This causes a change in the tertiary structure of PKA-R, ultimately releasing the catalytic subunit. Due to the release, PKA-C may then phosphorylate its large array of substrates. However, PKA-C is limited to a certain subset of its substrates due to its relocation via AKAPs (Reproduced with permission[74]).*

nal transduction pathways exploit GPCRs: the phosphatidylinositol 3-Kinase-AKT(31)and cAMP(32) signaling pathways. A ligand, for instance the hormone epinephrine, can bind to the G-protein coupled ß-adrenergic receptor causing a conformational change of the protein resulting in it acting as a guanine nucleotide exchange factor (GEF). This activates the associated G-protein complex (α, β and γ) whose role as a molecular switch regulates downstream signal transduction processes. In the inactive conformation guanosinediphosphate (GDP) is bound and upon activation guanosine triphosphate (GTP) is bound. Upon receptor stimulation, the GTP-loaded Gα subunit is released from the G$\beta\gamma$ dimer and then stimulates nearby adenylyl cyclase (AC), which in turn produces the second messenger, cAMP (3′-5′-cyclic adenosine-mono-phosphate) from ATP(33) (Figure 4). This leads to a local concentration increase of cAMP and the subsequent potential activation of various proteins(34), such as Exchange protein activated by cAMP (EPAC), cyclic nucleotide-gated ion channels and, most established, Protein kinase A (PKA) also known as cAMP-dependent protein kinase (cAK).

3. Protein Kinase A

Protein kinases are enzymes which modify their target protein substrate by covalent addition of a phosphate group from a nucleoside triphosphate (almost always the γ-phosphate of ATP) to side chain residues containing a hydroxyl group (serine, threonine and tyrosine). There are serine/threonine kinases, tyrosine kinases and aspecific kinases(35) that act on all three residues. Kinases are also known to modify other amino acids such as histidine(36) and arginine(37) but these are rare and not observed in eukaryotes. Protein kinases constitute 1.7 % of the entire human genome and ~500 kinases are identified so far(38) (Figure 5). Kinases play a central role in managing various aspects of cell survival and thus are tightly regulated via myriad mechanisms. In spite of the size of this enzyme superfamily,

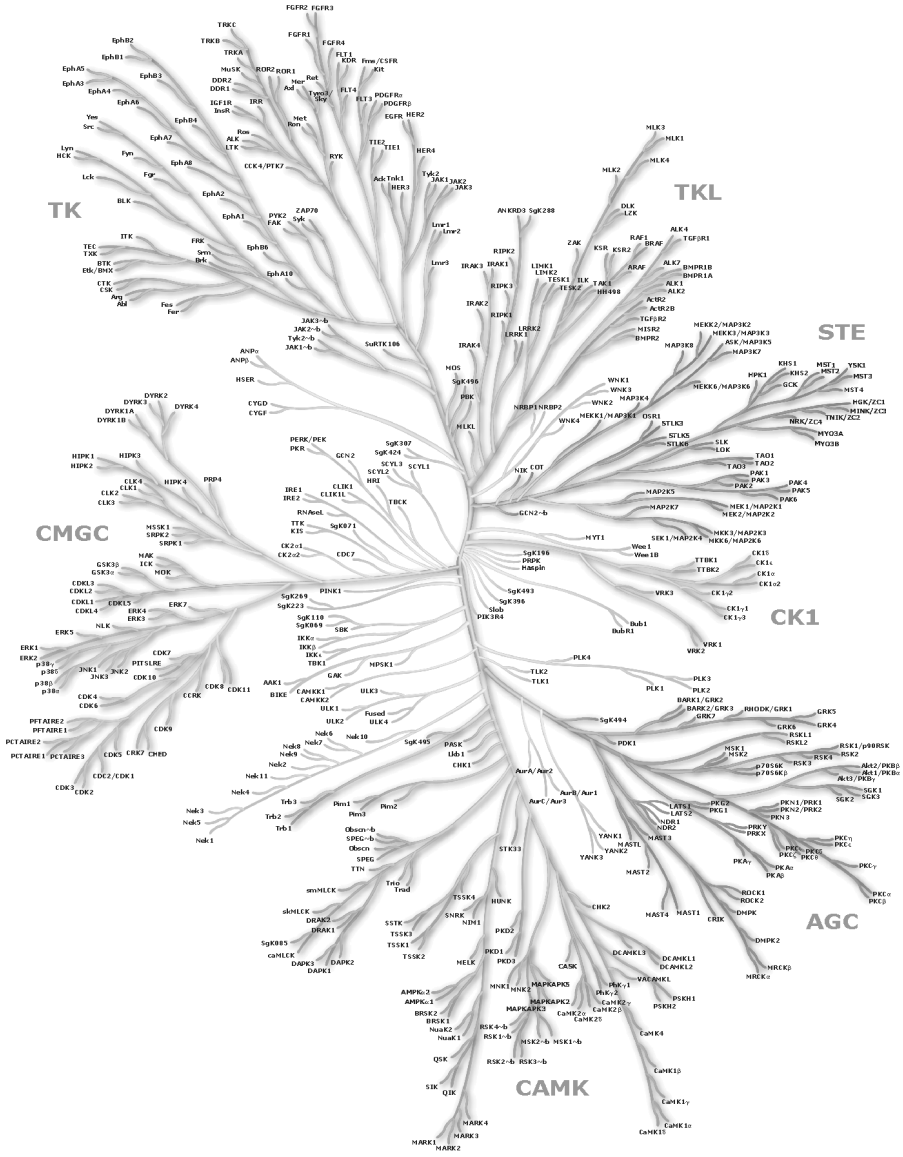

Figure 5 *The entire kinome of kinases of which PKA serves as a model (Reproduced with permission[38]).*

the protein kinases all share a conserved catalytic core. Kinases contain a multi-domain architecture wherein apart from the catalytic kinase domain; there are regulatory domains which modulate kinase activity in response to upstream molecular signaling events as well as targeting domains which recruit the kinase to its subcellular site of action where its substrates reside or mediate its interaction with other signaling proteins(39). ATP is the energy currency of living organisms and is a phosphate group donor in regulation of protein activity through side chain phosphorylation(40). Nearly 30% of the encoded proteins in the human genome contain covalently bound phosphate and abnormal phosphorylation is

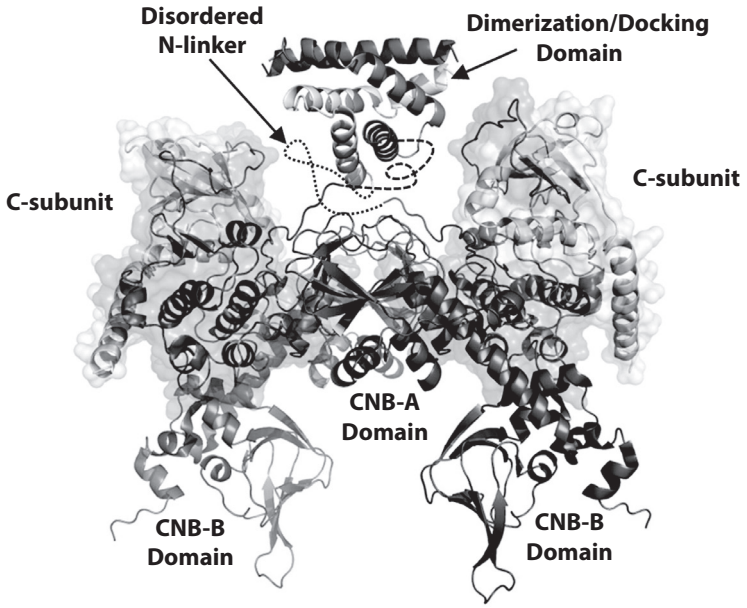

Figure 6 *The quaternary structure of PKA-RIα.*

The regulatory subunits consist of the CNB domains, the disordered linker and the D/D domain. To each regulatory subunit a catalytic subunit is attached. (Reproduced with permission from Susan S. Taylor).

now recognized as a cause and/or consequence of many human diseases(41). Protein phosphorylation is understood to be one of the most common mechanisms controlling enzyme function to regulate intricate cellular machinery(42, 43). It can alter the function of proteins in any conceivable example by decreasing or increasing its biological activity, by stabilizing or targeting the protein for destruction, or by initiating or disrupting protein-protein interactions(44).

Discovered in 1968(45), PKA serves as an important prototype for structure/function relationships of the entire kinase family(46, 47). The catalytic subunit of PKA (C) was the second protein kinase to be discovered(45). Shortly thereafter the regulatory subunits (R) were identified as the major receptors for cAMP in mammalian cells(48). The PKA holoenzyme is a heterotetramer consisting of two interacting regulatory (R) subunits which each bind a C-subunit(49) (Figure 6). The R-subunit has four genetically distinct isoforms(50) (RIα, RIβ, RIIα and RIIβ) whereas the C-subunit has three isoforms (Cα, Cβ and Cγ). Each of the isoforms have specific cell and tissue distributions (Figure 7) and are considered to be non-redundant, although, interestingly, only knockout of PKA-RIα is embryonically lethal(51).

In signaling pathways, protein kinases often function as switches that must be turned on and off. The activity of PKA is uniquely regulated by the inhibitory, cAMP-binding R-subunits. In the absence of cAMP the R-subunit dimer binds with high affinity to the C subunit and renders it inactive. PKA's catalytic activity is induced by the cooperative binding of

two cAMP-molecules per R-subunit, which causes the C subunit to be released and activated to phosphorylate target protein substrates(52). The cAMP binding domain, like cAMP itself, is an ancient motif, conserved in every genome from bacteria to man(45, 53). The dissociative mechanism is unusual for protein kinases, making the extensively studied PKA a rather peculiar and intriguing model kinase.

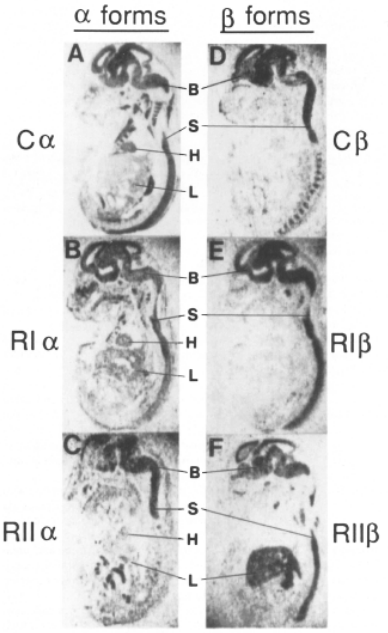

Figure 7 *The tissue distribution of PKA subunits in a 14 days mouse fetus (B – Brain, S – Spinal cord, H – Heart and L – Liver)(Reproduced with permission[51]).*

PKA is ubiquitously expressed throughout in mammalian cells and is essential for memory, cell growth, differentiation, development, metabolism, and hormone responsiveness, as well as many other cellular processes(54). PKA is also associated with many diseases such as malignancies and cardiac myopathies (55-62). The disruption of the normal balance between the PKA isoforms is highly associated with tumorigenesis and tumor growth(63, 64). For example, the Cushing syndrome, an endocrine tumorigenesis, is the result of PKA down regulation(65). The regulation of PKA also makes it interesting as a biomarker for cancer detection and monitoring of therapy. Numerous indirect methods to treat cancers exist as well. For instance, acute lymphoblastic leukemia is treated using glucocorticoids. However, resistance to these chemicals does occur, be it in phase 1 or phase 2 of treatment. On the other hand it has been shown recently that by increasing cellular cAMP levels as a result of down regulating PKA the resistance was halted(66). Due to the many major diseases associated with dysregulation within cAMP signal transduction pathways, specifically PKA and its direct partners are of interest with regard to the molecular understanding of human diseases and the hunt for suitable targets to possibly treat or cure one (or more) of these ailments. This can either be accomplished as a trial and error experiment or a direct investigation into the detailed molecular structure of PKA and its molecular signaling pathways.

4. A-Kinase Anchoring Proteins (AKAPs)

For many years the R subunits were considered to be simply inhibitors of C and receptors for cAMP, but this simplistic view has now changed. Initially it was assumed that the PKA holoenzyme was not confined to a specific location, but dispersed throughout the cell. However, in the light of the growing number of functions attributed to cAMP/PKA-signaling, it was made nearly impossible to efficiently coordinate all of those separate cAMP pathways without a certain space and time constraint. Now the R subunits instead are recognized to be essential for signal integration and not solely as inhibitors. This is due to the discovery of a novel group of proteins about 30 years ago: A kinase Anchoring Proteins

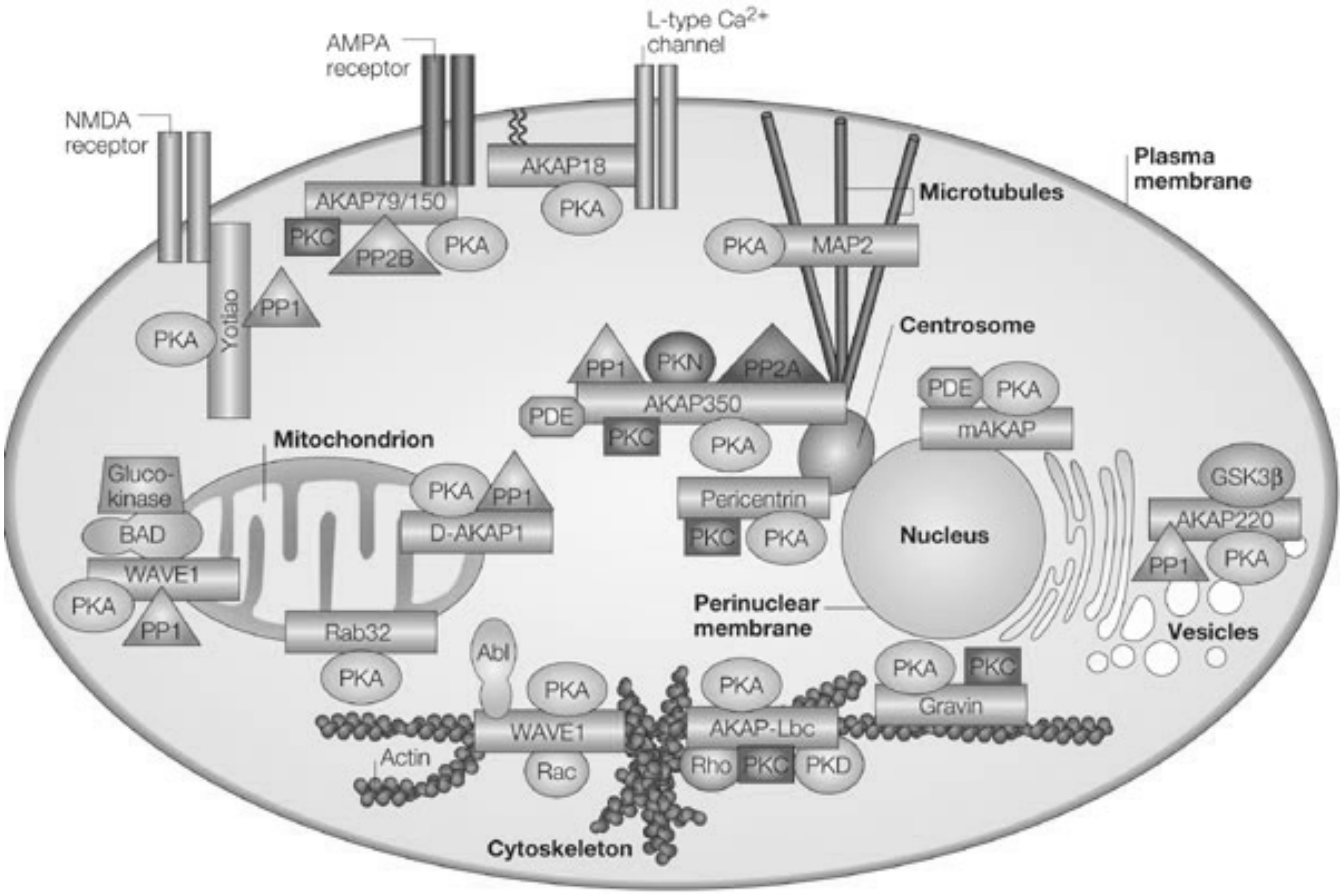

Figure 8 *Depicted are the various locations AKAPs occupy throughout the cell. In addition, their binding partners are shown with whom they form a signaling complex to not solely allow temporal but also spatial signaling (Reproduced with permission[74]).*

(AKAPs). AKAPs act as scaffolding proteins for PKA and bring the kinase close to its substrates(67, 68). Typically, other (related) signaling proteins, such as ACs, phosphatases (PP, remove phosphorylation) and phosphodiesterases (PDE, degrade cAMP signal) also bind to AKAPs, thereby generating a compartmentalized, efficient signaling hub. In this way, PKA action is very efficiently regulated in microdomains by tight modulation of the cAMP concentration via the interplay between ACs (increase) and PDEs (decrease) (Figure 8).

cAMP was originally thought of being freely diffused throughout the cell with an action radius of 220μm(69). Live cell imaging disproved the suggestion of cAMP having such a vast area of coverage. Rich et al. examined the local and global concentration changes of cAMP, respectively, by measuring the influx of fluorescently labeled Ca2+ through CNG channels and the activity of adenylyl cyclases converting [3H]ATP into [3H]cAMP(70). The second messenger accumulated in proximity to the membrane. The global changes, exposed by cAMP production, revealed that the second messenger concentration hit equilibrium(71, 72). This further enhances the fact of not just PKA being attached to the scaffolds presented by AKAPs but phosphatases and other signaling proteins as well. A different experiment using fluorescent resonance energy transfer (FRET) with fluorescently labeled R and C subunits also confirmed this(73).

Ultimately this results in a more controlled manner of the eventual phosphorylation status of the substrate through balancing the action of PKA with nearby PP activity. Because the R subunits link the catalytic moiety to this scaffold, they play an essential role in the subcellular localisation of PKA, and this inevitably is an important factor in determining specificity(74).

As efficiency is induced by strong communication and well-connected routes, A-kinase anchoring proteins provide the solution to the extreme effectiveness with which the cAMP signal transduction pathway conducts its course. In 1984, Lohmann et al., discovered the first AKAP(75). The protein, microtubule-associated protein 2 (MAP2) with a molecular weight of approximately 300kDa, co-purified with RII(75). Since then more than 50 AKAPs have been identified that all bind protein kinase A(76). The multitudes of the AKAPs have high affinity binding with the RII subunits of PKA(77). Some of the A-kinase anchoring proteins specifically bind to RI(78-80), whereas in several cases an AKAP has no specificity and binds to both regulatory subunits(21, 81, 82). The two most common dual specific AKAPs are D-AKAP1 and D-AKAP2(81, 82).

Despite AKAPs being allocated at many different places throughout the cell and human body they all share certain properties:

1) PKA anchoring domain

2) Able to form complexes with other signaling molecules

3) Contain unique localization sequences

Nonetheless, PKA is not traced back exclusively to one location in the cell (Figure 6). Therefore, the A-kinase anchoring proteins which control the location of the kinase must have different locations(83).

Some compartments may contain different AKAPs with different functions. Differentially spliced AKAP isoforms localizing to different locations via different targeting mechanisms have also been described. The plasma membrane is where both AKAP79/150 and AKAP250 bind. Whereas AKAP79/150 binds only to phospholipids on the plasma membrane, AKAP250 contains N-terminal myristoyl modifications which allow binding to the plasma membrane in addition to the specific phospholipid binding sequences which AKAP79/150 has as well. The AKAP18α and AKAP18β isoforms each target the plasma membrane as well, but possibly at different spots, due to them, respectively, having myristoyl and palmitoyl groups(74). Another method of targeting is by splicing of the AKAP. AKAP350 can be sliced into different lengths. Larger isoforms, such as AKAP350 and AKAP450, are able to bind to the centrosome in conjunction with the PACT domain near their C-terminal. The yotiao splice variant of AKAP350, on the other hand, can bind to the cytoplasmic tail of the NR1-subunit of the N-methyl D-aspartate (NMDA) receptor. These alterations lead both to PKA being localised to various destinations and that the regulation of phosphorylation is kept in control(84, 85).

Since kinases, phosphatases, phosphodiesterases and other signaling proteins are able to bind to A-kinase anchoring proteins a single AKAP also has the leeway of having different signaling protein complexes attached to its scaffold. This is foremost due to dynamic localization of the complexes. There are three different mechanisms which allow the variation in formations.

1) Phosphorylation of AKAP

2) Competition of substrates

3) Modification of targeting signal

For instance, WAVE1 can bind to actin allowing this AKAP to be bound to the cytoskeleton. The RII subunit of PKA has an overlapping binding domain with actin, resulting in competition between the two. This can affect where WAVE1 is located(86). AKAP18β, discussed earlier, is reversibly modified by N-terminal palmitoylation, which mediates it being membrane bound or not(87). The dynamic relocalization can cause the AKAP to have a different set of signaling proteins binding to it and thus serve a different function.

5. Scope and outline of this thesis

The work described in this thesis initially focusses on the discovery of a novel PKA-RI specific AKAP: small membrane AKAP (smAKAP). Afterwards we centre on the structural interaction between smAKAP and PKA-RI to reveal the first PKA-RI specific AKAP bound to PKA-RI crystal structure. Interestingly, a novel self-inhibition mechanism was discovered which allows PKA to block its binding to AKAPs under certain restrictions. In order to fully understand the specific limitations associated with binding a study centering on the PKA-RI and PKA-RII interactions with AKAPs was performed.

In **Chapter 2**, recent literature on the structural interface between PKA-RI/RII and AKAPs is reviewed. Most of these structural studies involve either X-ray crystallography, three-di-

mensional NMR, binding affinity assays and various other biochemical methods.

In **Chapter 3**, the discovery and initial characterization of a novel AKAP termed smAKAP is described. Via binding affinity assays and imaging techniques it is shown that smAKAP is PKA-RI specific. The intracellular location of smAKAP at the plasma membrane is shown by means of fluorescence imaging and advanced electron microscopy.

In **Chapter 4**, structural techniques such as hydrogen/deuterium exchange and X-ray crystallography are applied to probe the interaction interface between smAKAP and PKA-RI. Additionally, via a phosphoproteomics study it was shown that in the middle of the A-kinase binding domain of smAKAP there is a putative PKA phosphorylation site. Upon phosphorylation of this site, PKA cannot bind to smAKAP anymore. A mechanistic model on how this disruption occurs is presented.

In **Chapter 5**, a novel bioinformatic tool, THAHIT (THe AKAP/amphipathic Helix Identification Tool), is able to predict PKA-RIα and/or PKA-RIIα binding domains. This software package is based on currently known and well-established PKA-RIα and PKA-RIIα binding motifs. After applying it on all known AKAPs, numerous new PKA-RIα and PKA-RIIα binding domains in these AKAPs were found and/or narrowed down. Several of these were confirmed via conservation (BlastP), in silico docking studies using HADDOCK and in vitro binding studies using fluorescence anisotropy. In addition, several cAMP pull-downs were investigated for potential novel AKAPs using THAHIT. Here we propose a novel very large AKAP: vlAKAP.

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At the Interface of Spatiotemporal Signaling; a Structural Perspective of PKA-AKAP Interactions

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Summary

A-kinase anchoring proteins (AKAPs) form scaffolds that efficiently orchestrate the various cAMP-signalling pathways in a spatial and temporal manner. This is achieved by anchoring cAMP-dependent protein kinase (PKA) to this scaffold, along with a variety of other proteins, including phosphatases, kinases, receptors, ion channels and many other signalling molecules, bringing them in close proximity to their substrates. The functioning of several of these tethered protein complexes has been well documented and reviewed. In the last twenty years there has also been a great leap forward in understanding the molecular basis of the interaction between PKA and AKAPs, through data obtained by means of peptide arrays, binding affinity assays, crystal/NMR structures and various other structural biology methods. Here we present a summary of these studies that cumulatively have provided a greater insight into the structural determinants of these interactions. Particularly the specific determinants that drive the specificity of AKAPs for the different co-occurring isoforms of the PKA regulatory subunit are discussed.

Introduction

Cell signaling is a multifaceted system consisting of complex, intricate networks of signaling molecules, receptors and transducing proteins that together control the cellular activities and their corresponding actions. The cAMP signaling pathway is one of the most elucidated and well described signaling pathways. At the core of this pathway is the enzyme cAMP-dependent Protein Kinase (PKA), which is a tetrameric holoenzyme consisting of a regulatory subunit dimer (PKA-R) and a catalytic subunit (PKA-C) attached to each PKA-R in its inactive state(1-4). Upon binding of cAMP to PKA-R, PKA-C is released initiating the phosphorylation of a myriad of substrates(5). In mammalian systems there are four isoforms of PKA-R: PKA-RIα, PKA-RIβ, PKA-RIIα and PKA-RIIβ, and there are three PKA-C isoforms: PKA-Cα, PKA-Cβ and PKA-Cγ.

cAMP was originally thought of as being freely diffusible throughout the cell with an action radius of 220μm(6). Live cell imaging disproved the suggestion of cAMP having such a vast area of coverage and showed there to be localized pools of cAMP. For many years the PKA-R subunits were considered to be inhibitors of PKA-C and receptors for cAMP, but this simplistic view has now changed. Initially it was assumed that the PKA holoenzyme was not confined to a specific location, but dispersed throughout the cell. However, in the light of the growing number of functions attributed to cAMP/PKA-signaling, it was made nearly impossible to efficiently coordinate all of those separate cAMP pathways without a certain space and time constraint.

Now the regulatory subunits instead are recognized to be essential for signal integration and not solely as cAMP-sensing inhibitors. This is due to the discovery of a novel group of proteins about 30 years ago: A kinase Anchoring Proteins (AKAPs). AKAPs act as scaffolding proteins for PKA and bring the kinase close to its substrates(7, 8). Typically, other (related) signaling proteins, such as adenylyl cyclases, phosphatases and phosphodiesterases also bind to AKAPs, thereby generating a compartmentalized, efficient signaling hub(9). In this way, PKA action is very efficiently regulated in microdomains by tight modulation of the cAMP concentration via the interplay between adenylyl cyclases (cAMP synthesis) and phosphodiesterases (cAMP degradation).

PKA-AKAP Structural Interaction

The function of PKA in the context of several different AKAPs has been well described in previous reviews(10-14). Here we provide a detailed, overview on structural determinants of the PKA-AKAP interaction. We first describe studies that have contributed crucial insight into both sides of the interaction, starting on the AKAP-side, followed by the PKA-R side. We will then treat a relatively novel aspect of the field; the structural/molecular determinants driving the binding of PKA-RI and PKA-RII to their own repertoire of AKAPs to further functionally segregate the versatile functions of cAMP/PKA signaling in space and time. Finally we present an outlook into interesting opportunities for future research to ultimately understand each aspect of cAMP signaling in fine detail.

The AKAP Side: The Amphipathic Helix

In 1989, deletion mapping studies identified a region of MAP2 that mediates association with PKA-RII(15, 16). Carr et al. used a computer-aided analysis of secondary structure on four of the then established AKAPs (MAP2, AKAP79 (human gene: Akap5), AKAP150 (mouse/rat gene: Akap5) and Ht31 (Akap13)) to determine what type of local sub-structure is present. They identified a common region of approximately 14 residues containing many acidic and hydrophobic amino acids. Applying helical wheel projections and secondary structure predictors it was suggested that the AKAP binding regions form an amphipathic alpha helix. By substituting residues at several locations in this region with proline, which interrupts helix formation, interaction with PKA-RII was abolished(17). When residues outside of the binding region were substituted no reduction in affinity to PKA-RII was noted(18). The initial model of the prototype AKAP amphipathic helix was Ht31, now also referred to as AKAP-Lbc(19). A peptide of the PKA binding region (AA493-5150) of Ht31 was created and its conformation was analyzed using circular dichroism which confirmed its native helical structure. Further confirmation was obtained when the synthesized amphipathic helix of AKAP79 was used in competition binding assays. The amphipathic helix of AKAP79 turned out to compete very efficiently with PKA-RII for interaction with AKAP79(18). To probe into the polar and non-polar interfaces of the helix, several single and double site mutations were made in AKAP75(20). This provided the insight that the interaction surface of the helix not only depends upon hydrophobic and polar sidechains but also upon the size of them, i.e., that steric hindrance plays a role as well. For instance, the mutation of I405A drastically reduced the affinity of AKAP75 to PKA-RII, even though alanine is hydrophobic and has high helical potential. Various methods, such as truncations/mutations of the AKAPs, interaction cloning and foremost sequence homology/amphipathic helix detection using computer modeling, led to the discovery of more AKAPs and their PKA binding regions(21-25). An alignment of 15 known AKAPs displayed a more robust consensus domain of the amphipathic helix(26) and its conserved functional properties(27).

dAKAP1 and dAKAP2 are two dual-specific AKAPs, i.e. they have the ability to bind both PKA-RI and PKA-RII isoforms, albeit that PKA-RII binds with a five-fold higher affinity(24, 25). Since only PKA-RII binding AKAPs had been identified at that time, these dual-specific AKAPs formed the first basis for elucidating differences in binding between PKA-RI and PKA-RII. N-terminal and C-terminal truncations of the 27-residue PKA binding domain of dAKAP2 were synthesized by using SPOT synthesis on cellulose membranes (Figure 1A)(28). Binding was evaluated by chemiluminescence of PKA-RI and PKA-RII conjugated with GFP. This experiment suggested that the interaction surface between PKA-RII and dAKAP2 is smaller than the interaction surface between PKA-RI and dAKAP2. This was followed up by peptide substitution arrays of the 27-residue PKA binding domain of dAKAP2 (where each position of the binding domain was substituted by the other 19 amino acids) with PKA-RI and PKA-RII (Figure 1B). These confirmed the large difference in interaction surfaces. The peptide array of dAKAP2 with PKA-RI displayed four contact points containing two hydrophobic amino acids, interspersed by two polar residues, thus forming the amphipathic helix. The interaction between PKA-RII and dAKAP2 seemed to be less specific as it only showed two clear contact points containing fewer hydrophobic residues.

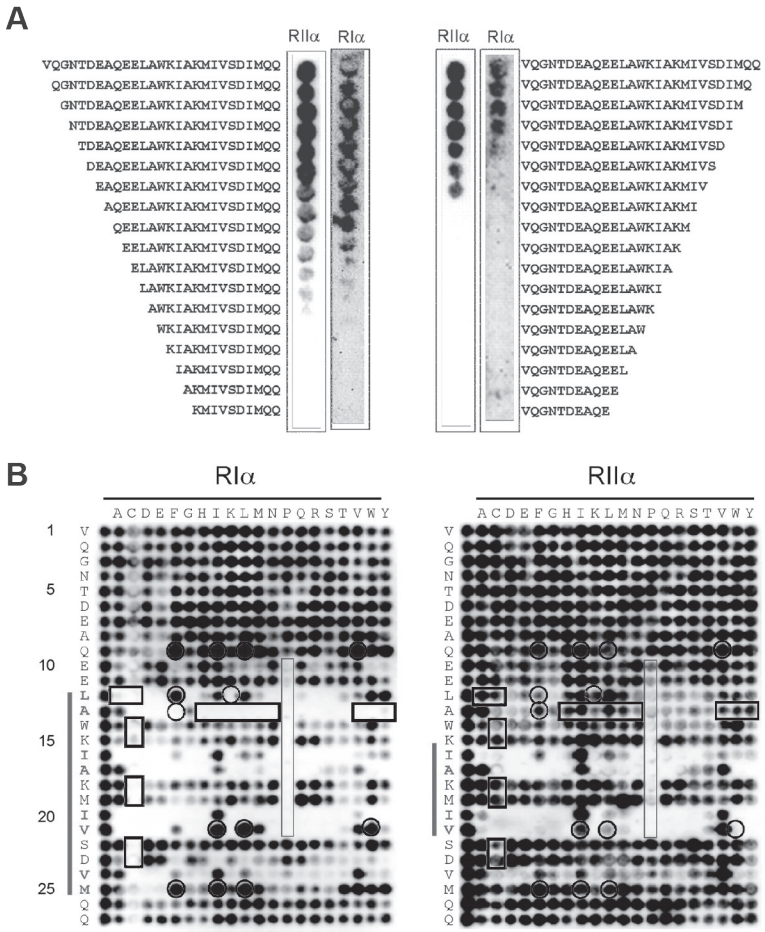

Figure 1 *(A) In order to determine the minimum length of the amphipathic helix of dAKAP2 required for binding to PKA-RI or PKA-RII the N- and C-termini of the peptides were truncated. Binding to PKA-RI is much more restricted due to the apparently larger interaction surface. (B) A peptide substitution array of the amphipathic helix of dAKAP2 also displays stricter requirements for PKA-RI binding (left) compared to PKA-RII binding (right). The line illustrates the key interacting residues. The mutated residues surrounded by boxes show decreased affinity for PKA-RIα while remaining stable for PKA-RIIα. The circles show mutations which decrease the affinity for PKA-RIIα while remaining stable for PKA-RIα. The long vertical rectangle shows decreased affinity for both regulatory subunits. (Reproduced with permission[28])*

Based upon these initial truncations and substitution arrays various single and multiple site mutations were created to design isoform-specific disruptors of PKA. Upon the mutation A635L, dAKAP2 becomes almost 1000-fold PKA-RII specific with a Kd = 2493 ± 409 nM for PKA-RI and a Kd = 2.7 ± 0.1 nM for PKA-RII. Whereas upon the mutations Q631F, V643W and M647F, dAKAP2 became ~100-fold RI-specific with a Kd = 5.2 ± 0.5 nM for PKA-RI and a Kd = 456 ± 33 nM for PKA-RII. Firstly, this shows, by both peptide array and mutation, that PKA-RI does not allow any residue larger than an alanine in the position of A635, postulating sterical hindrance. This is a PKA-RI specificity determining factor whereas the PKA-RII D/D cannot accommodate a large hydrophobic residue at the start or in the middle

of the PKA binding domain of dAKAP2 (Figure 1B).

Based on these and other data a bioinformatics approach was used to design an RII-selective peptide: AKAP-IS(29). Using the MEME algorithm, the strongest PKA-RII binding AKAPs were aligned and then a position-dependent scoring matrix was created, resulting in a consensus sequence of 17 amino acids which displayed an apparent stronger PKA-RII binding than the other PKA-RII specific AKAPs. A peptide array of the consensus sequence was created to optimize the affinity resulting in a Kd for PKA-RI of 227 ± 55 nM and a Kd for PKA-RII of 0.45 ± 0.07 nM. Surprisingly, the large difference between Ht31 and AKAP-IS is not the hydrophobic side but the polar side of the helix. This is due to Ht31 not having solely hydrophilic amino acids on its polar face as well as it having less helical propensity than AKAP-IS. Using a peptide screening approach, a further improved peptide, Super-AKAP-IS, was developed (4-fold higher affinity for PKA-RII and 12.5-fold lower affinity for PKA-RI). This peptide is ~10,000-fold more selective for RII than RI(30). In a similar manner a PKA-RI specific peptide, RI Anchoring Disruptor (RIAD), was created(31).

The PKA Side: Dimerization and Docking Domain

Similar to how deletion map assays were employed to pinpoint the PKA binding region of MAP2, various deletion mutants and chimeric proteins were screened to outline the region of PKA-RII required to bind to MAP2(32, 33). The first 45 residues of PKA-RII were the minimum requirement. The AKAP binding region and dimerization domain were subsequently allocated to these 45 residues and termed dimerization and docking domain (D/D-domain). Interestingly, already in 1988 it was suggested that the D/D-domain had an anti-parallel arrangement based upon intramolecular disulfide bridges (between Cys16 and Cys37) in PKA-RI(34). It must be mentioned that PKA-RII has an aromatic residue in the location of Cys16. A mutant of PKA-RII lacking residues 1-5 still had the ability to form a dimer but was not able to bind AKAPs anymore(35, 36). Upon the mutations of I3A and I5A PKA-RII lost the ability to bind several AKAPs and at least decreased the affinity six-fold for others. If long or bulky hydrophobic residues (such as a leucine or phenylalanine) were introduced the affinity was also decreased 24-fold(36). If polar groups (for instance, aspartic acid) replaced the hydrophobic groups no binding to AKAPs was observed any longer(36). This indicates that these are determinants for AKAP binding. The remaining initial 40 residues are required for dimerization of PKA-RII. Li and Rubin found several key residues (between AA13-36) which inhibited the formation of a PKA-RII dimer(37). A stoichiometric ratio of two PKA-RII protomers and one AKAP amphipathic helix is needed for the PKA-AKAP interaction(19). If no dimerization occurs the AKAPs have no chance to bind either(35).

Surprisingly, the two intermolecular disulfide bridges between Cys16 and Cys37 within PKA-RI are very stable for a cytosolic protein(38). Most likely by being buried slightly within the interface of the subunits the disulfide bridge is protected. In order to determine the strength of this bridge, increasing concentrations of the reducing agent DTT were added. Only upon a concentration of 50 mM or higher, a significant amount of monomers were

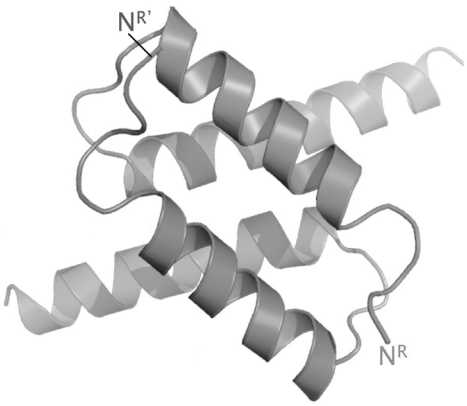

Figure 2 *Via triple-resonance, three-dimensional NMR-structure of the dimerization and docking domain of PKA-RII was elucidated. It shows a clear X-type four-helix bundle with the 5 residue N-terminal β-strands sticking out. (Reproduced with permission[40])*

noted. Even in the presence of 8 M Urea, 50 mM DTT was still required to reduce the disulfide bonds, signifying the strength of the intermolecular bond. To confirm if the monomers are aligned anti-parallel the following mutation was made: C37H. Upon this mutation PKA-RIα (AA12-61) was a monomer, consolidating the anti-parallel arrangement(38).

Secondary/tertiary Structure

An initial interpretation of the D/D domain of PKA-RIIα (AA1-44) was shown via NMR in 1997(39). Here it was revealed that there were two alpha-helices detected for a large part of each monomer (Leu9 through Gln23 and Leu28 through Ala42). This was confirmed via hydrogen/deuterium exchange studies. In addition, two β-turns were proposed (Pro6-Pro7-Gly8-Leu9 and Pro25-Pro26-Asp27-Leu28), with the second β-turn creating a tight hairpin turn between helix I and helix II. Based upon this, Val20 would be near Phe31, creating an intramolecular contact. A structural model of the dimer consisting of a helix-turn-helix motif was predicted to be an anti-parallel arrangement of the two resulting in a four-helix bundle.

The first structure of a dimeric domain, via triple-resonance, three-dimensional NMR, confirmed the models based on the C37H-mutation, CD and NMR data (Figure 2)(40). An X-type four-helix bundle (of the two monomers each consisting of helix-turn-helix motif) was revealed with extensive, well-ordered hydrophobic interactions in its core. The structure can be divided into two functional regions where the initial 23 residues constitute the AKAP binding interface (confirming the earlier discovery of the five initial residues being AKAP binding determinants) and the remaining 21 residues (24-44) contain most of the dimer contacts. Residues forming important dimer contacts include Ile5, Pro6, Leu9, Leu12, Leu13, Tyr16, Val20 and Leu21 in helix I, and Leu28, Val29, Ala32, Val33, Phe36, Thr37 and Leu39 in helix II. Not only does a very strong hydrophobic surface contribute to the dimer but a strong intermolecular hydrogen bond between Asp30 and Arg40 helps stabilize the dimer interface as well. Sequence alignments of the four PKA-R isoforms show a conservation of the hydrophobic dimerization core. One large difference, as earlier noted, is the absence of the intermolecular disulfide bridge in PKA-RII due to the presence of a conserved aromatic residue (α: Tyr; β: Phe) in the position of the PKA-RI cysteine.

The other functional region, the AKAP binding interface, is a solvent-accessible hydropho-

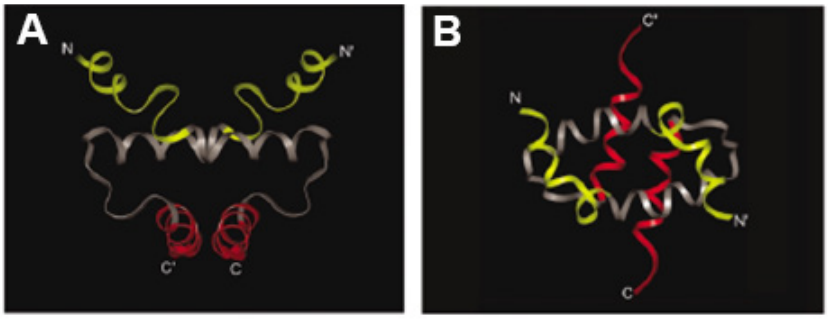

Figure 3 *(A) A side and (B) top view of the D/D of PKA-RIα (AA12-61) displaying a similar X-type four-helix bundle as PKA-RIIα. However, unlike PKA-RIIα the N-termini (highlighted in yellow) do not stick out. (Reproduced with permission[41])*

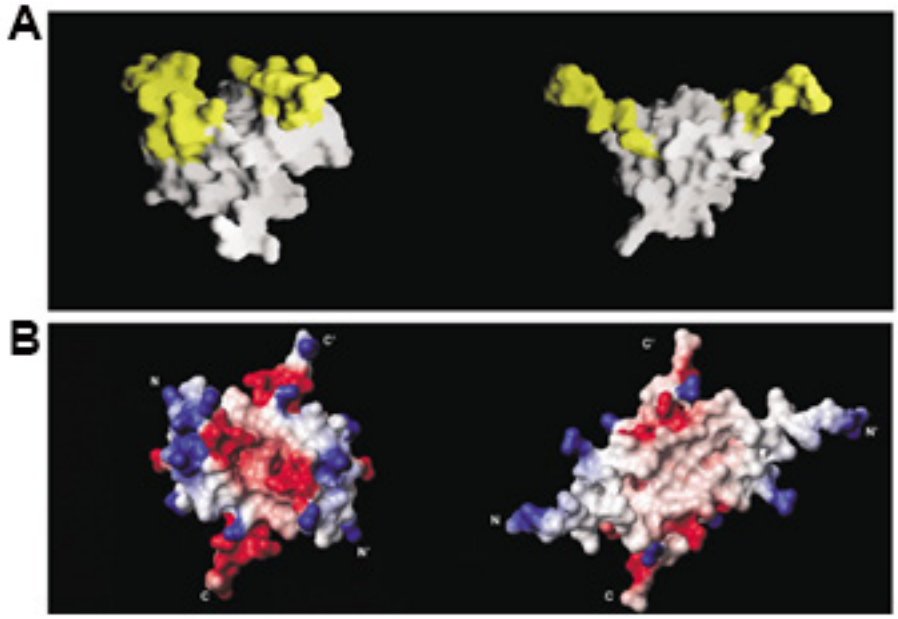

Figure 4 *(A) The isoform specific regions (yellow) of PKA-RIα (left) and PKA-RIIα (right) show that PKA-RIα has a tighter groove. (B) The electrostatic potential (acidic – red; basic – blue) of PKA-RIα (left) is much greater than that of PKA-RIIα (right). This allows greater interaction of the hydrophilic side of the AKB domain with PKA. (Reproduced with permission[41])*

bic surface. It is formed via the anti-parallel array of helix I. A sequence alignment of PKA-RIIα and PKA-RIIβ conveys that residues Ile5, Pro6, Thr10, Thr17, Val18 and Leu21 are strictly conserved and therefore offer a recognition surface for interactions with AKAPs. Additionally, His2, Glu11 and Arg22 possibly provide ionic interactions with PKA-RII binding AKAPs as the polar side of the PKA binding domain of AKAPs often have large polar groups protruding.

Four years later the D/D domain (AA12-61) of PKA-RIα was solved (Figure 3)(41). This D/D domain is held together as a dimer via the intermolecular disulfide bridges, thus covalent dimers, whereas the PKA-RIIα subunits are non-covalent dimers(42). However, both

PKA-RIα and PKA-RIIα share elements of secondary structure, i.e., helices I and II(40, 42). Similarly to PKA-RIIα, the D/D domain of PKA-RIα consists of an X-type four helix bundle which encompasses an extended hydrophobic dimerization interface as well as a hydrophobic AKAP binding surface(41). Residues important for the dimer interface are present in both helices I and II. Unlike PKA-RIIα, which has a β-strand upstream of helix I, PKA-RIα has an α-helix upstream of helix I. This could very well lead to the isoform specificity as these regions interact with the AKAP as well. For instance, Ile3 and Ile5 of PKA-RIIα, once mutated, decreased the affinity to various AKAPs(36). Another striking difference between PKA-RIα and PKA-RIIα is the electrostatic surface potential surrounding the cleft into which the AKAP helix docks(41) (Figure 4). There are many charged residues around this site in PKA-RIα when compared to PKA-RIIα leading to an even bigger and more selective interaction surface as it allows multiple intermolecular interactions with the hydrophilic face of the amphipathic helix.

Structural details of the PKA-RIIα AKAP Interaction

The first structural details of two PKA-R/AKAP complexes: PKA-RIIα (AA1-44) with Ht31 (AA493-515) and AKAP79 (AA392-413) were reported in 2001(43). The structural properties of the D/D domain of PKA-RIIα in the complexes are very similar to free PKA-RIIα(40); the domain structure is still defined as an antiparallel, X-type four-helix bundle motif with the 5-residue long N-terminal β-strand sticking out (Figure 2). When either AKAP peptide binds, ~10% (800 Å2) of the total surface area of the D/D becomes solvent inaccessible. This primarily occurs in helix I, however, in line with the earlier mentioned mutation studies, it also includes residues Ile3 and Ile5 which are located in the N-terminal β-strand(35). The structures of these complex ratify that the solvent-accessible groove formed by helix I is the AKAP recognition surface in the D/D-domain(43). As one might expect upon binding, changes in the structure will occur. However, from the overlay of free D/D and the complex, almost no visible changes are present in both the AKAP binding surface and the dimerization domain. The only two minute changes are those of Leu13 and Leu9 occupying slightly different locations in space. Thus, it seems that the PKA-RIIα D/D delivers a preformed, hydrophobic, solvent-accessible groove as an interface for AKAP binding. As well, there are only small structural changes in the N-terminal β-strand with it moving slightly towards the AKAP.

Both amphipathic helices of Ht31 and AKAP79, lie diagonally, at 45°, across the helix I plane when bound (Figure 5). Even though the sequence of both peptides is not conserved, the arrangement of the hydrophobic face (Residues 2, 5, 6, 9, 10, 13, 14 and 17) of each peptide is similar. The structure suggests that a palindromic sequence of the critical hydrophobic residues, centered at positions 9/10, would create an AKAP with the highest affinity for PKA-RIIα. This is also corroborated by the peptide substitution arrays in the Hamuro et al. and Gold et al. articles(28, 30). The surface topography of the D/D elects for several long-branched hydrophobic residues in the center of the AKAPs PKA binding domain.

Later, two additional complexes consisting of PKA-RIIα and AKAP-IS and dAKAP2 were

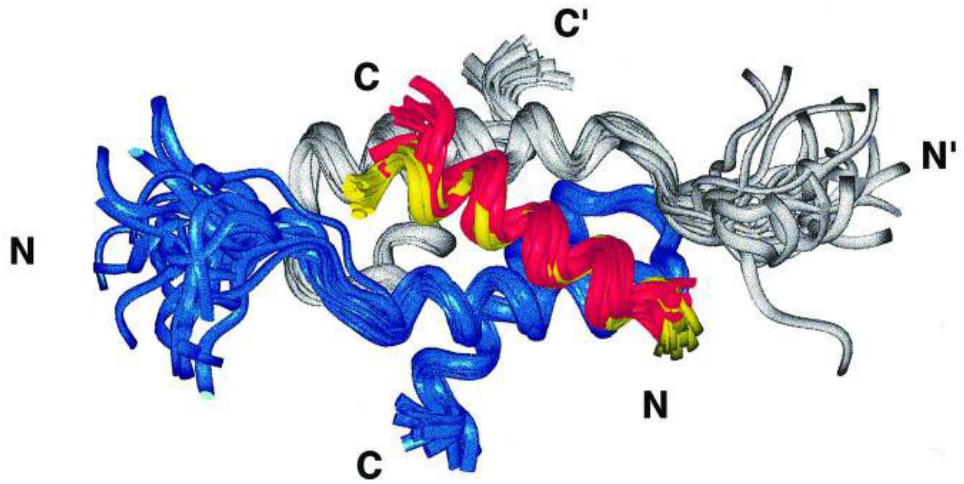

Figure 5 *The superposition of the regulatory subunit (AA1-44) dimer (Blue/Gray) bound to Ht31 (AA493-515) (red) and AKAP79 (AA392-413) (yellow). Both AKAPs bind to the regulatory subunits with identical regions and comparable helical structures. The N-termini of both regulatory subunits are disordered as expected. (Reproduced with permission[43])*

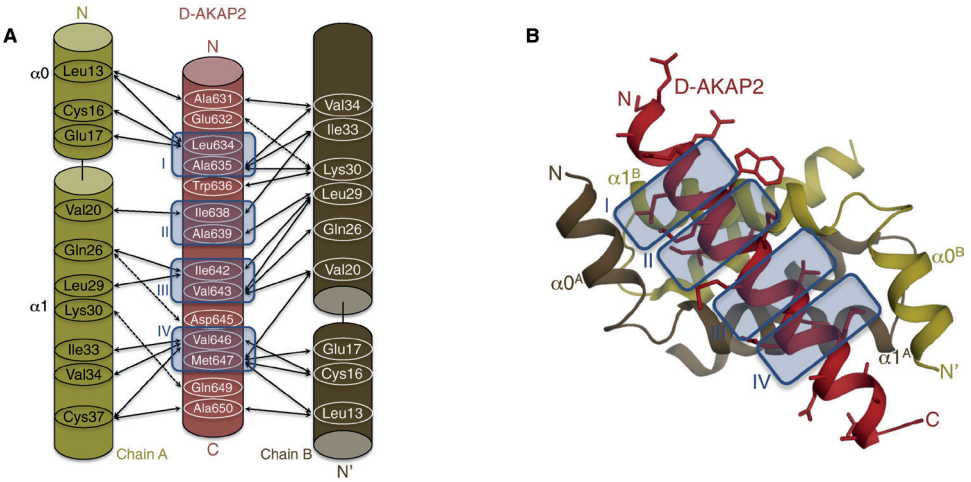

Figure 6 *(A) Shown here is the structure of dAKAP2 peptide bound to PKA-RIαD/D. Depicted are the four binding pockets (blue boxes) which each contain two residues of the hydrophobic side of the AKB domain. The interactions between the sidechains of dAKAP2 (red) and PKA-RIα (turquoise/green) monomers (arrows) show a large interaction domain compared to PKA-RIIα. (B) The overall structure showing the interaction between dAKAP2 and PKA-RIα. The interacting sidechains of PKA-RIα are exposed to exhibit the interface of binding. (Reproduced with permission[45])*

crystallized(30, 44). The amphipathic helix rotated slightly in each due to the presence of different residues. Notably, the N-terminal β-strand relatively repositions itself depending upon the hydrophobic interface of the amphipathic helix. The complex containing AKAP-IS had several salt bridges: Glu6 (AKAP) – Arg22 (PKA-RIIα), Asp14 (AKAP) – Thr10 (PKA-RIIα), Asn15 (AKAP) – Thr10 (PKA-RIIα) and Gln19 (AKAP) – Gln14 (PKA-RIIα). These salt bridges assisted in increasing the helical potential of AKAP-IS. As well, the intramolecular hydrogen bond between Asn15 and Gln19 supported the α-helical conformation(30).

PKA-RIα and AKAP Structural Interaction

Only two PKA-RIα:AKAP structures have been solved compared to the four PKA-RIIα:AKAP structures(45). However, these structures contribute to a very good understanding of what makes the PKA-RIα:AKAP interaction different and what drives the specificity of PKA-RI and PKA-RII for their own palette of AKAPs. The first structure was of the dual specific dAKAP2 amphipathic helix with PKA-RIαD/D(45). Similar to the PKA-RII structures, only one helix binds diagonally upon the X-type antiparallel four-helix bundle making extensive hydrophobic and polar interactions.

Only residues from α0 and α1 contribute to the interactions between PKA and dAKAP2 (Figure 6A). There are four main regions or pockets at the interface: pockets I-IV (Figure 6A/B). Pocket I, consisting of Leu13A, Cys16A, Glu17A, Val34B and Ile33B, is occupied by Leu634 and Ala635. Due to residues Val34B and Ile33B, which are tightly packed, only an alanine can occupy the second spot of pocket I. This correlates with the peptide array data discussed earlier(28), where mutating the alanine to a leucine decreased the affinity to PKA-RIα 50-fold whereas the affinity to PKA-RIIα did not change(28). Pockets II and III are very similar to one another as they lie along the noncrystallographic two-fold axis, and thus identical residues (Val20A, Ile33B and Leu29A for pocket II and vice versa for pocket III) from both chains of the D/D domains are involved in the hydrophobic interaction. Both pockets have an optimal fit for an isoleucine but a few other residues fit snugly as well. Pocket IV is not as tightly packed as pocket I, even though they have very similar residues. This allows for some leniency on the residues present in this pocket, especially for the second residue (the one nearest to the N-terminus of the amphipathic helix). As mentioned previously, PKA-RIα has, relatively, many polar sidechains just outside the hydrophobic cleft (Figure 6). These allow dAKAP2 to create salt bridges with PKA-RIα to increase the stability of the interaction. For example, Glu632, Asp645 and Gln649 form hydrogen bonds with Lys30B, Gln26A and Lys30A, respectively.

Conclusions and Future Perspectives

Most AKAPs identified thus far are RII-specific. The various structural studies show that this is due to there being a smaller and less restrictive binding interface compared to PKA-RI. PKA-RII has four pockets which require only six residues to satisfy the binding requirements whereas PKA-RI has four pockets which require eight residues. In addition, there is a larger polar surface surrounding the binding cleft of PKA-RI than PKA-RII. These structural factors finally decide which PKA isoform the AKAPs can localize to a specific spot inside the cell to create a spatiotemporal signaling cascade.

Most AKAPs have different PKA binding domains and thus have different affinities for the various PKA-R isoforms. As a result of this mutations for various AKAPs have been created which allow them to be PKA-RI and/or PKA-RII specific. Recently however, the reverse experiment was performed by creating AKAP selective PKA-R isoforms, engineered through structure-based phage selection. Various residues in the D/D were mutated to optimize AKAP specificity. This novel set of tools could allow the study of specific localized pools of PKA-R, for instance at the plasma membrane or the ER(46).

Every year novel AKAPs are discovered(47-53). This is not surprising as there are many different pathways with which PKA is involved and these are not all at the same location. By narrowing down the binding requirements via these structural studies one may create a search engine based on various factors to possibly discover the remaining PKA-RI/RII AKAPs. Tools such as these have been created but could still be improved via this data(54). In addition, for AKAPs discovered thus far but not having a defined PKA binding domain this tool could be used as well to identify the binding region. Of course, additional experiments such as binding affinity assays, confocal imaging or competitive studies have to be performed to confirm the site.

Functionally, the PKA/AKAP interaction is not the only important feature of cAMP-signaling. AKAPs bind more than just PKA. Also phosphodiesterases, phosphatases, ion channels, receptors, adenylyl cyclases, other kinases and many more proteins are described. To fully appreciate the molecular details of localized cAMP signaling the field also needs to invest in techniques complementary to X-Ray crystallography and NMR. One of such tools could be native mass spectrometry. For instance, via native mass spectrometry the complex consisting of dimeric AKAP79 (with each a PKA-RII dimer attached), four Ca(2+)/calmodulin(CaM)-dependent protein phosphastase heterodimers and two CaM molecules was revealed(55). This led to the current understanding of how AKAP79 concentrates CaM at the plasma membrane.

Various diseases and ailments are associated with PKA in various tissues and locations throughout the cell(56-63). Also, re-distribution of PKA towards a different pool of AKAPs has been observed in for instance heart failure(64). Now having a much greater knowledge concerning the structural interaction specific inhibitors could be created to possibly disturb or enhance the interaction between a single AKAP and its localized pool of PKA. This could ultimately lead to better, more specific drugs which more accurately disrupt a single maladaptive pool of cAMP/PKA.

So far only the PKA-RIα and PKA-RIIα interactions with AKAPs have been exposed. It would be interesting to see what the factors implicating PKA-RIβ and PKA-RIIβ binding with AKAPs are. The recently solved structures of PKA-RIβ and PKA-RIIβ show them being vastly different from their α isoform counterparts(65, 66). This could potentially lead to a much different interaction. Therefore, in order to see and understand the binding of the β regulatory subunits with their respective AKAPs, their interaction structures would be of much value.

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Chapter 3

A Small Novel A-Kinase Anchoring Protein (AKAP) That Localizes Specifically Protein Kinase A-Regulatory Subunit I (PKA-RI) to the Plasma Membrane

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Summary

A-kinase anchoring proteins (AKAPs) provide spatio-temporal specificity for the omnipotent cAMP-dependent protein kinase (PKA) via high affinity interactions with PKA regulatory subunits (PKA-RI, RII). Many PKA-RII-AKAP complexes are heavily tethered to cellular substructures, whereas PKA-RI-AKAP complexes have remained largely undiscovered. Here, using a cAMP-affinity based chemical proteomics strategy in human heart and platelets, we uncovered a novel, ubiquitously expressed AKAP, termed small membrane (sm) AKAP due to its specific localization at the plasma membrane via potential myristoylation/palmitoylation anchors. In vitro binding studies revealed specificity of smAKAP for PKA-RI (Kd=7nM) over PKA-RII (Kd=53nM) subunits, co-expression of smAKAP with the four PKA R-subunits revealed an even more exclusive specificity of smAKAP for PKA-RIα/ß in the cellular context. Applying the singlet-oxygen generating electron microscopy probe miniSOG, indicated that smAKAP is tethered to the plasma membrane and is particularly dense at cell:cell junctions and within filopodia. Our preliminary functional characterization of smAKAP provides evidence that, like PKA-RII, PKA-RI can be tightly tethered by a novel repertoire of AKAPs, providing a new perspective on spatio-temporal control of cAMP-signaling.

Introduction

In the cellular context signaling regulated by the small second messenger, cAMP, involves a plethora of signaling proteins. A key player is cAMP-dependent protein kinase (PKA). In the resting state the catalytic subunits (PKA-C) are tightly bound and inhibited by regulatory subunits (PKA-R). This inactive holoenzyme is organized as a hetero-tetramer ($(PKA\text{-}R)^2(PKA\text{-}C)^2$) and becomes activated by cAMP binding (1). The dimerization and docking (D/D) domain of the PKA-R isoforms allows binding to the so-called A-kinase anchoring proteins (AKAPs), the key-regulator scaffolds of cAMP's intracellular specificity(2-4). The hallmark signature motif of the AKAPs is a small sequence of 17-20 amino acids that forms an amphipathic helix that associates tightly with the PKA-R D/D domain(5). Besides targeting PKA activity to a specific location within the cell, most AKAPs function as scaffolds for a variety of other signaling proteins such as phosphatases, phosphodiesterases, and other kinases. These macromolecular protein complexes create efficient signaling hubs that allow for a localized cAMP regulatory mechanism(4). For example, AKAP18δ localizes to the heart sarcoplasmic reticulum and creates a signaling complex comprised of AKAP18δ, phospholamban, PKA and sarcoplasmic reticulum Ca2+-ATPase to regulate intracellular Ca2+ levels upon β-adrenergic stimulation(6).

In 1984 Lohmann et al.(7) reported on the first AKAP, namely the microtubule-associated protein 2 (MAP2), when purifying PKA-RIIα. Since then over 70 different AKAPs originating from around 30 different genes have been identified(3, 4, 8). Initially, PKA-R overlay assays(7) and yeast two-hybrid (Y2H) screens were used for the discovery of new AKAPs. For instance, Rab32, a member of the Ras superfamily of small G-proteins, was shown by Y2H to interact with PKA-RII and co-localizes to mitochondria(8). More recently, mass spectrometry based chemical proteomics experiments with immobilized cAMP have proven their value in capturing, identifying and characterizing novel AKAPs(3, 9). This specific PKA-AKAP enrichment approach led, for instance, to the discovery of novel AKAPs such as SPHKAP and the PALM2-AKAP2 fusion protein(10, 11).

In mammalian systems there are four genetically distinct and functionally non-redundant isoforms of PKA regulatory subunits: RIα, RIβ, RIIα and RIIβ, which all contain a (D/D) domain capable of AKAP binding. Subtle differences, especially between the RI and RII isoforms, seem to induce selectivity in binding to a subset of AKAPs. Most reported AKAPs have a preferred specificity for PKA-RII, while three AKAPs: D-AKAP1(12), D-AKAP2(13) and the recently discovered Opa1(14), are dual-specific AKAPs. We identified sphingosine kinase type 1-interaction protein (SPHKAP) as the first PKA-RI specific AKAP(10, 15). Detailed structural analysis of the RI and RII D/D domain in complex with the earlier mentioned amphipathic helix A-kinase binding (AKB) domain of the dual-specific D-AKAP2 revealed that interaction with PKA-RI requires a larger interaction surface which leads to a larger set of unique constraints to bind PKA- RI(5, 16). These restrictions also seem to drive the specificity of the very small subset of the AKAPs known to interact tightly with PKA-RI. Array-based synthetic peptide screening led to detailed knowledge on the importance of particular amino acid combinations at key locations in the AKB domain that contribute to RI over RII specificity(17, 18).

Here we report on the identification and preliminary functional characterization of a novel, small (i.e. 11 kDa) PKA-RI specific A-kinase anchoring protein, which we refer to as the small-membrane AKAP (smAKAP). smAKAP is tethered to the plasma membrane most likely through a dual acylation of its N-terminal Met-Gly-Cys- motif (myristoylation and palmitoylation, respectively). Through biochemical experiments in vitro and in vivo, we show that smAKAP has the capacity to target specifically PKA-RI isoforms to the plasma membrane; similar to both AKAP250 (gravin) and AKAP18α, which perform this task for PKA-RIIα(19, 20). Using a novel singlet oxygen generating electron microscopy probe (miniSOG)(21) we confirm smAKAP's localization to plasma membranes, its enrichment at cell:cell junctions and its association with filopodia.

Materials and Methods

cAMP pull downs

Human left ventricular heart tissue and human platelets were collected from individuals without any diagnosed cardiovascular disease. All samples were obtained with the appropriate consent according to the Helsinki convention. The lysate preparation and cAMP pull downs were performed in earlier work in human heart(22) and platelets(23).

RT-PCR

Total RNAs of spleen, liver, kidney, brain, lung, uterus, stomach, intestine, ovaries, skeletal muscle, heart and colon were isolated from a female mouse using TRIzol reagent (Invitrogen). Subsequently the RNAs were treated by DNAse I followed by addition of Oligo dT12-VN (Promega) and Superscript II (Invitrogen). Finally the PCR reaction was completed using Taq polymerase (Invitrogen) and the appropriate primers (Eurogentec) for mouse smAKAP and mouse GAPDH.

Creating plasmids

The human smAKAP gene was isolated from mouse heart cDNA and by applying the enzyme free cloning method, the amplified cDNA was cloned into pLICHIS vector(24). Human smAKAP was subcloned from pLICHIS into the BamHI-HindIII site of a mCherry vector(25), into the BamHI-HindIII site of the miniSOG vector(21) and into the EcoRI-BlpI site of the mGFP vector(26). The single site mutations (G2A, C3A) and double site mutation (G2A/C3A) of the human smAKAP sequence in the mGFP vector were made by QuikChange mutagenesis (Agilent Technologies). PKA-RIα tagged with mCherry and RI specific D-AKAP2 were described previously(27, 28). The mKO2 tagged PKA regulatory (R) subunits were generated by fusing respective R subunits with mKO2 to the C-terminus with PCR and inserted between EcoRI and NotI sites in pcDNA3 (Invitrogen, San Diego). There is a SalI site as linker in RIα, RIβ and RIIα constructs and a BamHI site as linker in the RIIβ construct.

Binding affinity

Full length PKA-RIα/β and PKA-RIIα/β dimers were purified as described previously(29,

30). The 5-TAMRA N-terminus tagged TVILEYAHRLSQDILCDALQQWAC peptide was synthesized at the peptide facility of the Netherlands Cancer Institute (Amsterdam, Netherlands). Flat bottom black 96-well plates (Thermo) were used for the fluorescence polarization readings of each regulatory subunit dimer with the 5-TAMRA tagged peptide, which was excited at 535 nm (5- to 10 nm bandpass) and emission monitored at 580 nm (5- to 10 nm bandpass), by the Tecan Genios Pro 96/384 Multifunction Microplate Reader (Tecan). The binding experiments were each performed four times after which they were put into the non-linear regression model of one site saturated binding in GraphPad Prism 5.0.

Cell culture

HeLa and HEK293 cells were acquired from the American Type Culture Collection. Prior and post to the transfections, the cells were sustained in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker) containing 10% heat-inactivated fetal bovine serum (Omega Scientific Inc., San Diego, CA, USA) and 2 mM Glutamax (Invitrogen) in 35mm glass bottom dishes (MatTek). The dishes for HEK293 cells were coated with poly-d-lysine. Cells were grown to 75% confluence and transfected using PolyFect according to the manufacturer's protocol for the specific cell type (QIAGEN).

Fluorescence Imaging

Cells were fixed by ice-cold 4% formaldehyde in PBS and subsequently washed twice with PBS. The confocal images were acquired with a Leica TCS SPE II confocal system with a 63x water objective lens.

Electron Microscopy

smAKAP-miniSOG was co-transfected with either RIα-mCherry or RIα-mKO2 to help identify transfected cells. 14 hours post-transfection, cells were fixed in 2.0% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M sodium cacodylate buffer (pH 7.4) for 5 minutes at room temperature, and then moved to ice for a duration of 45 minutes for optimal cell ultrastructural preservation. The cells were washed 5 times in ice-cold 0.1 M sodium cacodylate buffer (pH 7.4), each taking two minutes, in order to remove excess aldehydes. Cells were treated for 15 min in blocking buffer (50 mM glycine, 10 mM KCN, and 10 mM aminotriazole) to reduce nonspecific background reaction of diaminobenzidine (DAB). Images of cells were acquired using a Leica TCS SPE II confocal system using both 488-nm and 568-nm laser excitation and a 63x water objective immersion lens. Cells expressing high level of smAKAP-miniSOG were photooxidized. Afterwards, cells were washed with 0.1M sodium cacodylate buffer, post-fixed with 1% osmium tetroxide (Electron Microscopy Sciences) in 0.1M sodium cacodylate buffer, dehydrated in an ethanol series, embedded in Durcupan AMC Fluka epoxy resin (Sigma), sectioned at 70-80nm by a Leica ultracut UCT ultramicrotome, post stained in 2% aqueous uranyl acetate (Electron Microscopy Sciences) for 10 minutes and imaged with a JEOL JEM1200EX transmission electron microscope at either 60kV or 80kV according to(21). The stereo images were acquired at 10° intervals.

Statistics and Quantification

The two fluorescent profiles were compared by ImageJ 1.43 by plotting a line through the

visible cell and then creating a plot profile. The profiles were compared and an R2 correlation coefficient was computed using MS Excel.

Results

Discovery of smAKAP by chemical proteomics and bioinformatics

Following a chemical proteomics based strategy described earlier(9, 31) an unknown protein, with the connotation MGC13057, belonging to the UPF0733 family (Q9BSF0, C2orf88), was observed as selective binder in cAMP-based affinity pull-down experiments(22, 23), both in human heart tissue and human platelets. LC-MS/MS analyses allowed the mapping of 92% of the sequence of MGC13057 affinity-captured from both these tissues, except the ultimate N-terminal residues, (Figure 1A). Applying and aligning our previously established Hidden Markov model (9) with the sequence of MGC13057 suggested that the YAHRLSQDILCDAL (AA61-74) sequence could be a potential A-kinase anchoring motif making MGC13057 a putative novel AKAP.

BLAST analysis and alignment of the retrieved homologues of MGC13057 revealed a good conservation across mammals, but also to other vertebrate species such as bird (Taeniopygia guttata) and fish (Danio rerio). Although in the latter species the degree of identical amino acids diminished, some regions, including the putative AKB motif and the N-terminus, still showed strong similarity/identity (Figure 1B). A helical wheel alignment revealed the presence of an amphipathic helix, a hallmark of AKAPs, with one side of the helix consisting primarily of hydrophobic residues and the other side of charged/hydrophilic residues, further suggesting that this uncharacterized protein may be a novel AKAP (Figure 1C).

MGC13057's expression across various mouse tissues was tested by means of mRNA reverse transcription PCR. The mRNA expression level appeared to be ubiquitous although it was much less in spleen and liver (Figure 1D). As expected, mRNA levels were relatively high in murine heart, since we originally detected MGC13057 in human heart tissue.

MGC13057 displays PKA-RI selectivity

We first further investigated the sequence of the well- conserved proposed AKB domain of MGC13057 to possibly define its PKA-R subunit specificity. The AKB-domain of MGC13057 was therefore aligned with AKB-domains of PKA-RII specific AKAPs, dual-specific AKAPs and RI-specific AKAPs (Figure 2A). Similarity between MGC13057's AKB domain and those of PKA-RII was poor (Figure 2A), however, the alignments with PKA-RI binding AKAPs proved very strong. For instance, in MGC13057 there are four helical turns with hydrophobic domains. In the first domain the PKA-RI specific alanine is conserved, as well as, the bulky tyrosine residue (phenylalanine in SPHKAP(10)) that fits well in the deeper grooved D/D domains of the PKA-RI isoforms, but less in the PKA-RII isoforms(16). Similarly, in MGC13057 there are two large polar residues present after the final hydrophobic domain which merely arrest PKA-RII binding, and are also found in D AKAP1, D-AKAP2, AKAP-CE and partially in SPHKAP and RIAD(17). This suggests that MGC13057 is, like SPHKAP, potentially a PKA-RI specific AKAP. To verify the isoform specificity, in vitro binding assays

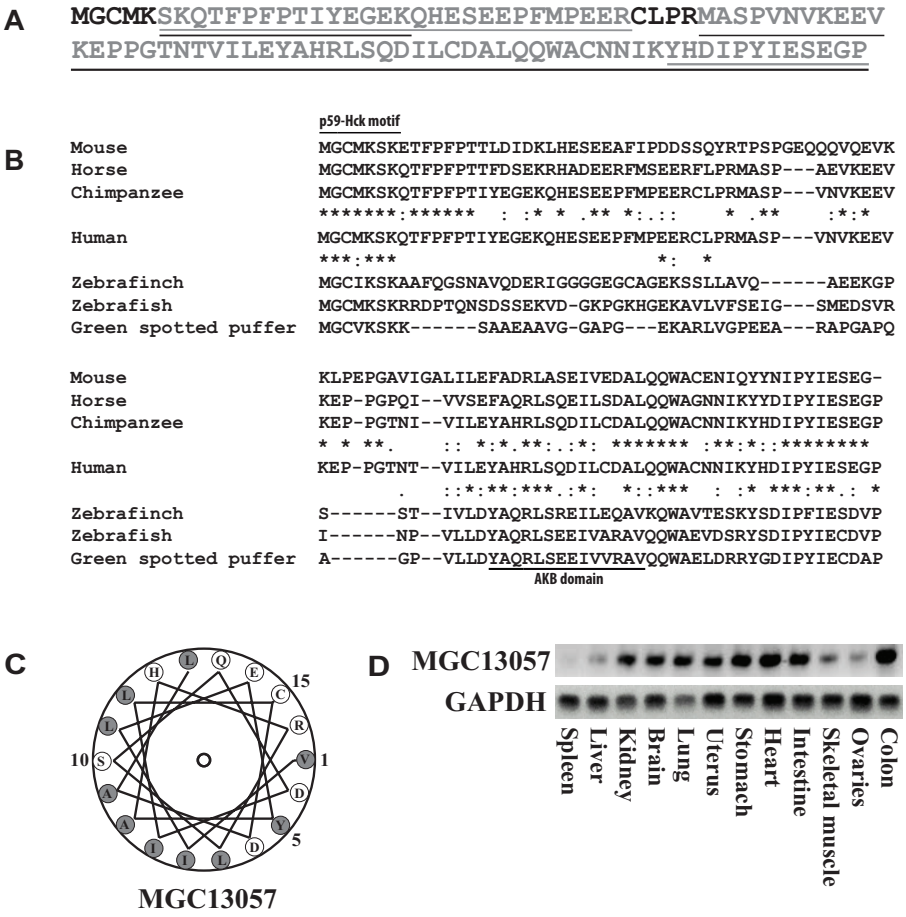

Figure 1 *Discovery of MGC13057as a putative AKAP.*

(A) A chemical proteomics analysis by means of cAMP bound resin was performed upon human heart (left ventricle) and platelets leading to the detection of the hypothetical protein MGC13057. Sequence coverage (gray text) of this small protein was nearly complete with peptides identified in heart (gray lines) and platelets (black lines). (B) Sequence alignment of human MGC13057 with various orthologues in other species (from top to bottom: mouse (Q9CPS8), Horse (XP_001502030.1), Chimpanzee (XP_003309413.1), human (Q9BSF0), zebrafinch (XM_002191648.1), zebrafish (P0C8S0) and green spotted puffer (Q4RTJ5)). Identity (*) and similarity (:) in several regions, including the AKB domain and p59Hck motif are annotated. (C) Helical wheel alignment of the putative AKB domain reveals an amphipathic helix with a hydrophobic surface on one side (shaded). (D) mRNA expression of the MGC13057 gene in several mouse tissues by RT-PCR.

were performed with fluorescence anisotropy. Binding between all four full-length PKA-R subunits and a 24 amino acid long synthetic peptide TVILEYAHRLSQDILCDALQQWAC that mimics the AKB-domain of MGC13057 (AA56-79) were probed. For read-out of binding, the peptide was labeled with a 5-carboxytetramethylrhodamine (5-TAMRA) tag. This in vitro assay clearly indicated an almost 10-fold preference in binding affinity between the PKA-RI isoforms (KD ~7nM)) and the PKA-RII proteins (KD ~ 53 nM) (Figure 2B), verifying in vitro MGC13057's preference for PKA-RI. These are the highest affinities reported so far for PKA-RI subunits binding to AKAPs (Figure 2C).

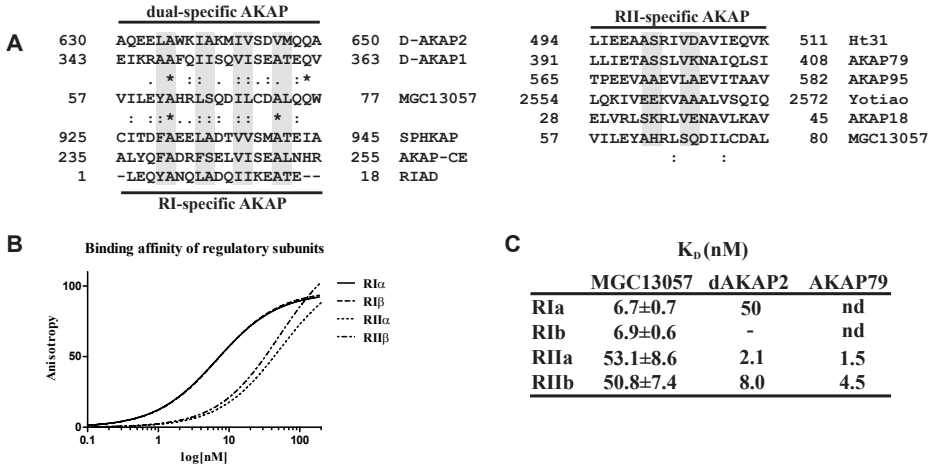

Figure 2 *MGC13057 is a PKA-RI specific AKAP.*

(A) Alignment of the MGC13057 AKB-domain (57-77) with the AKB domains of dual- (left, top) and PKA-RI (left, bottom) specific AKAPs. The same was done with the AKB domains of several established PKA-RII specific AKAPs (right). Identity (*) and similarity (:) are annotated. (B) Fluorescence anisotropy measurements to determine the binding affinity of the MGC13057 AKB-domain peptide, tagged with 5-TAMRA (excitation at 535 nm and emission at 580 nm) with the full length regulatory subunit dimers: PKA-RIα (solid line), PKA-RIß (dash), PKA-RIIα (alternating dash) and PKA-RIIß (short dash). (C) Depicted are the KD values and standard deviations (n=4).

Localization and specificity in vivo

As the previous experiments indicated that MGC13057 is a putative PKA-RI specific AKAP, we set out to confirm this in a cellular context. The human MGC13057 gene was subcloned and fused to the green fluorescent protein GFP. Both HEK293 and HeLa cells were transiently transfected with this construct. Fluorescence microscopy showed strong localization of MGC13057 at the plasma membrane and its particular association with filopodia (Figure 3A). Based on its size and membrane localization we subsequently refer to it as small-plasma membrane AKAP (smAKAP). Using confocal imaging and scanning through the cell we confirmed the localization to the plasma membrane and its strong presence at cell:cell junctions when smAKAP was expressed in Hela cells (Figure 3B).

Next, fluorescent imaging co-localization studies of smAKAP with the four PKA-R isoforms were performed to further investigate smAKAP's specificity, and PKA-R's localization in the cellular context. For these studies we engineered, in addition to the smAKAP-GFP construct, the four R-subunit-mKO2 constructs. When we carried out a dual transfection of smAKAP-GFP and RIα-mKO2 in Hela cells, smAKAP and PKA-RIα strongly co-localize at the plasma membrane, specifically to filopodia, suggesting they tightly interact in the cellular context (Figure 3C). Strong co-localization at the plasma membrane was also observed when PKA-RIα-mKO2 was co-transfected with smAKAP-GFP (Figure 3D). In contrast, when we co-expressed smAKAP with either PKA-RIIα-mKO2 or PKA-RIIβ-mKO2, we observed no co-localization with smAKAP-GFP at the plasma membrane (Figure 3E/F).

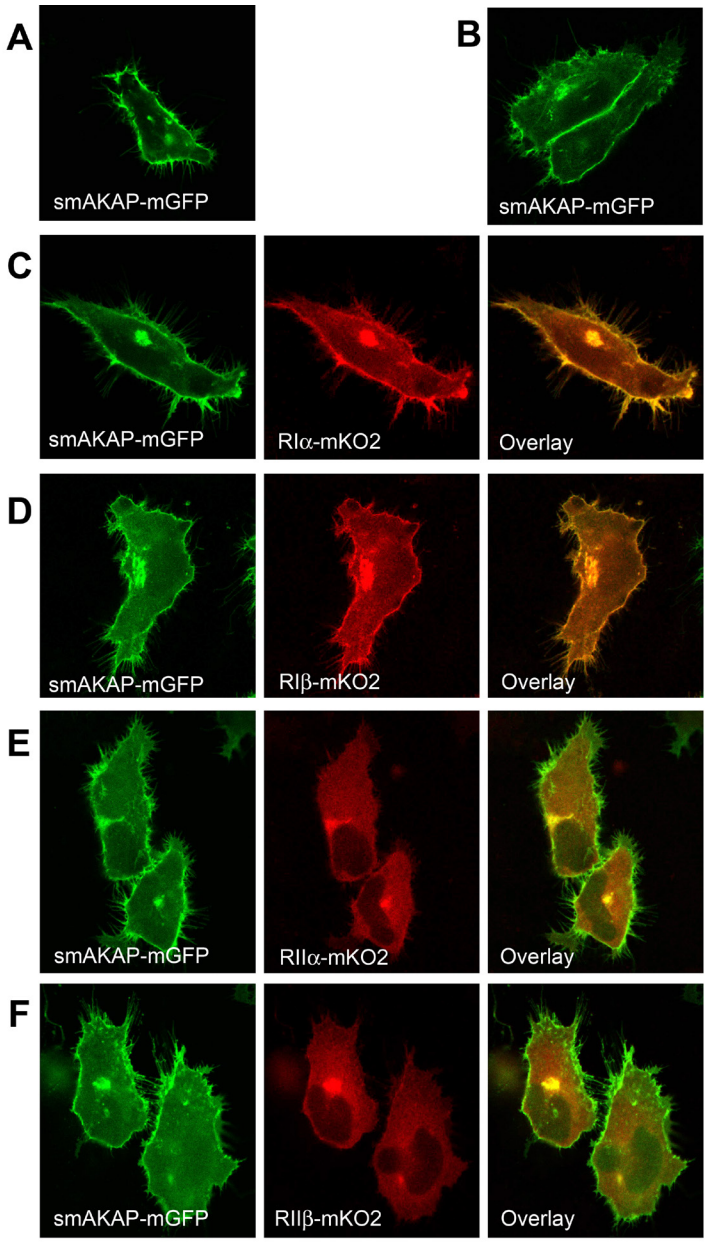

Figure 3 *smAKAP assertively relocates PKA-RI to the plasma membrane.*

(A) Transfection of HeLa with smAKAP-mGFP shows smAKAP present at the plasma membrane and filopodia. (B) A stronger presence of smAKAP at cell:cell junction is displayed by smAKAP-mGFP. (C) PKA-RIα-mKO2 and smAKAP-mGFP strongly co-localise. (D) smAKAP-mGFP and PKA-RIβ-mKO2 also co-localise. (E) In contrast PKA-RIIα-mKO2 and smAKAP-mGFP display no co-localisation. (F) Likewise smAKAP-mGFP and PKA-RIIβ-mKO2 do not co-localise. The protein expression levels are presented in Figure 5.

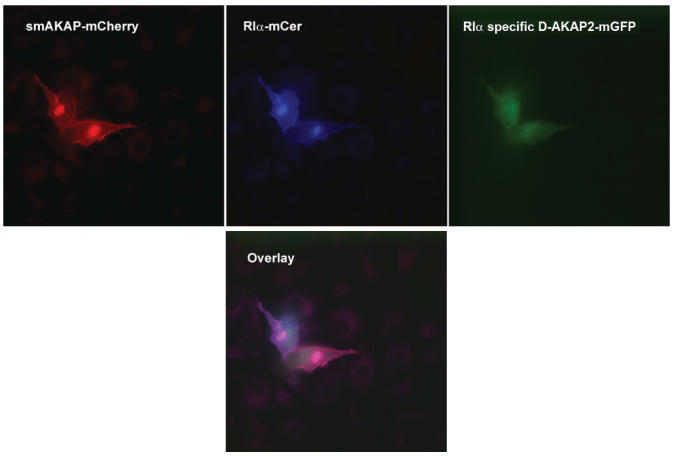

Figure 4 *HEK293 is triply transfected with smAKAP (mCherry, left), PKA-RIα (mCer, middle) and RI specific D-AKAP2 (GFP, right) to show how smAKAP outcompetes RI specific D-AKAP2 for PKA-RIα binding (Overlay, bottom). See also figure 5E and F.*

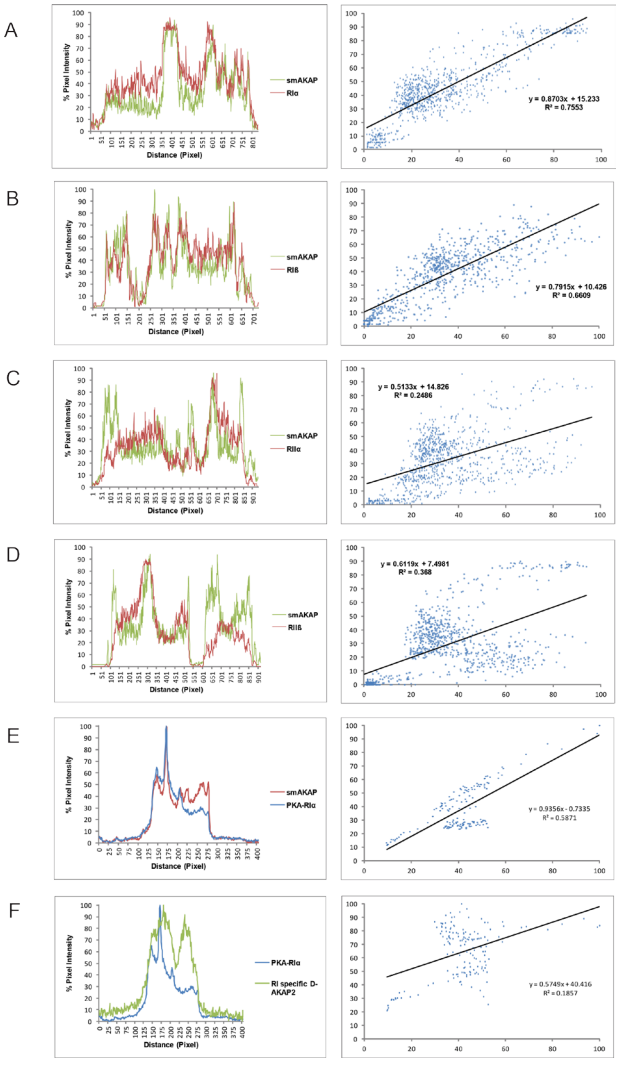

Figure 5 *Line plot profile and correlation analysis of colocalization of smAKAP with (A) RIα (See also Figure 3C) (B) RIβ (See also Figure 3D) (C) RIIα (See also Figure 3E) (D) RIIβ (See also Figure 3F) and (E) RIα (See also Figure 4), whereas (F) is of RI specific D-AKAP2 with RIα (See also Figure 4). The Plot profiles (left) display the difference in fluorescence intensity along a line taken through the cell. Scatter plots (right) show the pixel distribution with which the R^2 was calculated.*

While smAKAP-GFP was enriched to the plasma membrane, PKA-RIIα and PKA-RIIβ were localized to the cytoplasm.

Despite the presence of the competing PKA-RI tethering D-AKAP2, which localizes to the cytosol, PKA-RIα is not released from smAKAP at its location at the plasma membrane (Figure 4). These data clearly indicate that smAKAP specifically binds to PKA-RI in cells, and demonstrates that these isoforms are not always cytosolic as initially hypothesized(32, 33). This is further supported by the large differences in r^2 between the fluorescence intensity profiles of each co-localisation (Figure 5).

Post-translational modifications in the N-terminus of smAKAP mediate membrane interaction

Having established that smAKAP localizes to the plasma membrane we next explored the requirements for this localization. In addition to the AKB domain, another well-conserved region of smAKAP (Figure 1B) is at the N-terminus, which contains both a Gly (G2) and a Cys (C3) residue. This "two-signal mode" for protein acylation and membrane anchoring is highlighted by proteins belonging to specific members of the Src family of kinases, most of which are modified at their N-termini with both myristate and palmitate(34). Interestingly, the 7 amino acid N-terminal stretch of smAKAP is identical to that of the oncogenic tyrosine kinase p59-Hck, which is known to be localized at the cell membrane (and at caveolae), and membrane localization of Hck requires both palmitoylation at Cys-3 and myristoylation at Gly-2 (Figure 6A)(35). The guanine nucleotide-binding protein G(s) subunit alpha isoform, which activates adenylyl cyclase (a transmembrane protein) and is thus part of the PKA signaling pathway, can also be both myristoylated and palmitoylated, in turn allowing it to be near smAKAP in regulating PKA's activity(36). Myristoylation

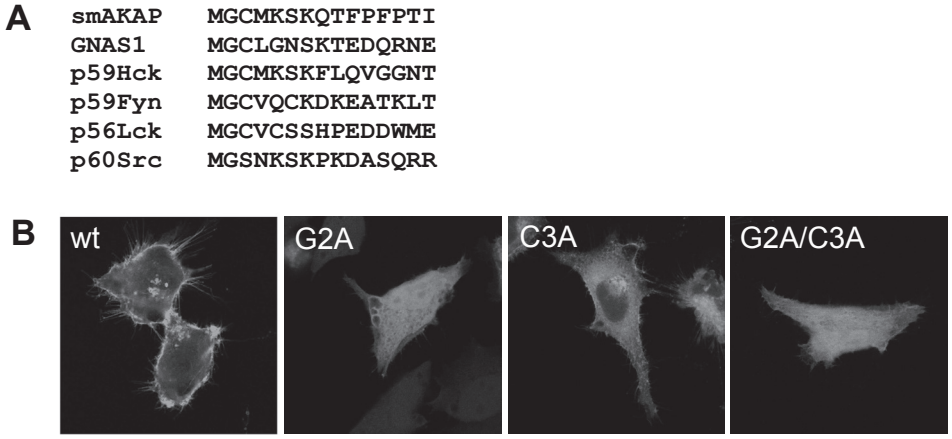

Figure 6 *Modification by lipids of the N-terminus of smAKAP is crucial for localization.*

(A) Sequence alignment of the first seven N-terminal amino acids of smAKAP with various proteins, known to carry myristoylated and/or palmitoylated glycines/cysteines. (B) Transfection of mutated forms of smAKAP-GFP, G2A (left), blocking myristoylation, results in loss of plasma membrane localization. To a lesser degree, the single mutation, C3A (middle), which blocks palmitoylation of smAKAP-mGFP, similarly hampers membrane localization (middle). The double mutation, G2A/C3A, fully negates binding of smAKAP-mGFP to the mem-

usually occurs co-translationally, while palmitoylation is a posttranslational modification (PTM)(37, 38). The N-terminus of smAKAP thus provides a well-known motif that has the ability to undergo palmitoylation and myristoylation, likely mediating a lipid-induced localization of smAKAP to the plasma membrane(36, 39). Unfortunately, we were unable to detect peptides of the N-terminus of smAKAP in our proteomics experiments, but with hindsight this may be partly caused by its extensive lipidation. To establish the importance of these acylation sites for membrane localization, we mutated smAKAP at G2 and C3 with two single alanine mutations (G2A, C3A) and a double mutation (G2A, C3A) in the full-length human smAKAP-GFP construct. Figure 6B clearly shows that plasma membrane localization is abolished when either site is mutated, and thus as well with the double mutation, indicating that both these residues are essential. This is a strong indirect indication of acylation through both palmitoylation and myristoylation and that they are both essential and likely driving smAKAP's localization to the plasma membrane, similarly as described for p59-Hck(35).

Electron microscopy imaging further defines the localization of smAKAP

To more precisely map the localization of smAKAP we used electron microscopy and the recently introduced mini Singlet Oxygen Generator (miniSOG)(21). MiniSOG is a genetically modified flavoprotein, which produces singlet oxygen upon excitation with green light (Figure 7A). Diaminobenzidine (DAB) will then be oxidized to form an insoluble osmium-philic polymer which gives contrast to the ultrastructure near the tagged fusion protein. The smAKAP cDNA was fused to miniSOG and transiently transfected into HEK293 or HeLa cells for photo-oxidation. RIα-mCherry was co-transfected to help deduce the location of smAKAP-miniSOG via red fluorescence. Via this enhanced imaging method a much higher resolution was obtained allowing us to observe that smAKAP was localized at the plasma membrane (Figure 7C,D,E). In these images one can also very clearly see how the smAKAP is enhanced at cell:cell contact sites (Figure 7,D,E,F), and furthermore the localization of smAKAP to filopodia (Figure 7G,H). In addition to its localization at the plasma membrane, we could observe some localization to internalized vesicles as expected by both the suspected myristoylation and palmitoylation(35). It is shown how a protrusion of the membrane, containing smAKAP, is formed on the plasma membrane junction between two cells, utilizing both plasma membranes. Potentially, this is then released into the adjacent cell, possibly indicating cell-to-cell signaling (Figure 7F).

Discussion

In cellular biology the ability to organize enzymes into multi-protein complexes allows for a high degree of fidelity, efficiency and spatial precision in signaling responses. In cAMP regulated signaling, localization of signaling complexes around AKAP scaffolds is a key element in controlling and regulating intracellular communication. Here, using chemical proteomics, we discovered a novel AKAP, termed smAKAP, which specifically and tightly targets PKA-RI to the inside of the plasma membrane. The tethering of PKA-RI to the plasma membrane by smAKAP contradicts many previous reports that regard PKA-RI to

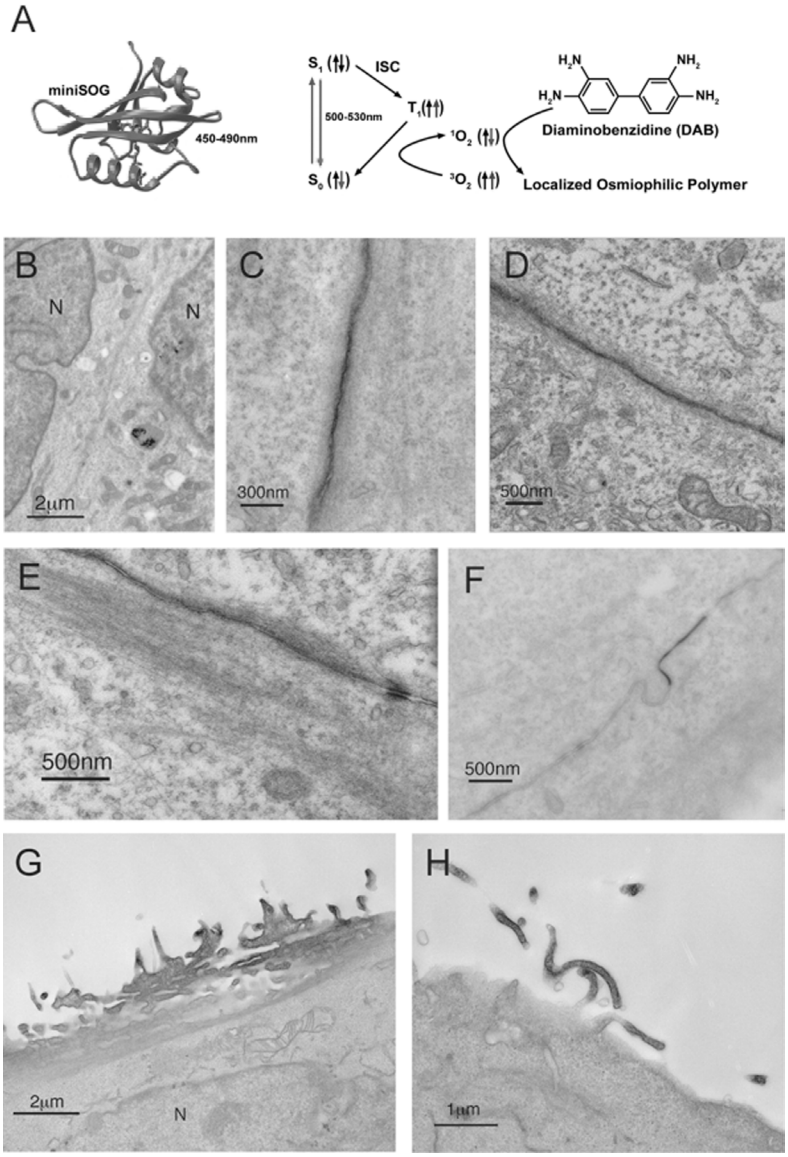

Figure 7 *Electron microscopy imaging reveals localization of smAKAP.*

(A) MiniSOG is a flavoprotein whose excitation is ~500-530nm and leads to the production of singlet oxygen. Upon the addition of DAB to a dish, the DAB located around the excited miniSOG will polymerize and create an osmiophilic polymer. Adapted from 21 (B) The control, which was not exposed to oxygen, had no DAB polymerisation at the plasma membrane as seen between two cells. N stands for nuclei. (C) Electron microscopy of the miniSOG-tagged smAKAP reveals plasma membrane localization in HEK293 cells after photo-oxidation (black areas) and smAKAP is on the intracellular side of the membrane. (D)(E) In certain regions, actin fibers are visible on the intracellular side of the membrane and smAKAP concentrated at a junction between two cells. (F) One area of the plasma membrane of adjoining cells is highly stained with DAB with a bulge occurring. (G) smAKAP in an isolated HeLa cell is also located on the plasma membrane and especially enriched in filopodia. (H) A higher magnification image of filopodia showing the enrichment of the smAKAP

be 'freely' diffused in the cytosol(32, 33), although this general view has been questioned more recently(10, 12-14). For instance, a study involving FRET-based reporters of cAMP suggested not only specific PKA-RII but also PKA-RI anchoring sites to be present in cardiomyocytes(40). PKA-RI tethering was initially suggested by the discovery of AKAP-CE in Caenorhabditis elegans, which bound PKA-RI(41). Moreover, in human erythrocytes it was established, via isolating various organelles followed by immunoblotting, that PKA-RI is tightly bound to the plasma membrane(42-44). Although RI-subunits were typically found to be more diffuse in the cytoplasm than RII-subunits, they could also be recruited in a more dynamic way as evidenced by the migration of RI to the cap site in activated lymphocytes(45) and following treatment of cardiac myocytes with H2O2(46). Furthermore, it was shown that overexpressed RIα and C-subunits were still associated with AKAP220 even though they were diffuse in the cytoplasm; however, targeting of the RIα subunit to multivesicular bodies only occurred upon activation of the complex(47).

The recently identified dual-specific AKAP, Opa1, binds PKA-RIα strongly with a KD = 12.5 ± 2.8 nM, and this is the first dual-specific AKAP to bind PKA-RI with higher affinity than PKA-RII(14). For comparison, the first PKA-RIα specific AKAP, SPHKAP, had a KD = 73 ± 9 nM for RIα(15) and the dual specific AKAP D-AKAP2 had a KD of 48 nM for RIα(27). Opa1was the strongest binding PKA-RI AKAP before we reported here our smAKAP with its apparent KD = 6.7 ± 0.7 nM (based on in vitro AKB peptide binding, Figure 2B/C).

Recently, it was noted that AKAPs bind to lipid rafts via PTM's such as palmitoylation(48) and bind to multivesicular bodies(47) allowing the signaling scaffolds to be moved throughout the cell. In line with these findings, the EM imaging in Figure 5 hints at how a double plasma membrane vesicle can form at the cell:cell contact sites and then migrate into the adjacent cell. This possibly suggests a mechanism for how cytosolic content, including the smAKAP complex, can be carried from one cell to the other, but further investigations need to follow in order to deduce smAKAP's physiological functions.

It is also noteworthy that the smAKAP is localized to the filopodia and overexpression of smAKAP appeared to increase the number of filopodia. RIα is thought to be associated with cell migration mediated by the α4 integrin, and this localization to the filopodium would be consistent with this function(49).

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Chapter 4

PKA-induced phosphorylation inhibits its association to A-kinase anchoring proteins

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Summary

The A-kinase anchoring protein (AKAP) smAKAP has a unique feature; it solely interacts with the type I regulatory subunit dimer of cAMP-dependent kinase (PKA-RI). Here, we determined the crystal structure of smAKAP's A-kinase Binding domain (smAKAP-AKB) in complex with the dimerization/docking domain of PKA-RIα (PKA-RIαD/D). This provided a unique interaction surface with a large number of contact residues to drive smAKAP's high specificity. Complementary hydrogen/deuterium exchange studies with full length proteins showed the interaction is limited to the AKB-D/D interface. In the core of the AKB domain, we identified a conserved PKA phosphorylation site (Ser66), which, when phosphorylated ablated binding of PKA-RI. The inhibitory mechanism of smAKAP's Ser66-phosphorylation stems from a distinct helix disruption, not a steric effect and is also present in other AKAPs. These findings represent a novel mechanism which could have major implications for the regulation of localized cAMP-signaling events.

Introduction

Many important cellular functions are regulated by signaling through parallel cAMP-dependent pathways(2). Amongst other targets, intracellular cAMP activates cAMP-dependent protein kinase (PKA)(3). PKA is a holo-enzyme consisting of a homo-dimerized regulatory subunit (PKA-R) with a catalytic subunit (PKA-C) bound to each PKA-R. Four genetically distinct PKA-R isoforms are expressed in mammalian systems, designated as PKA-RIα, PKA-RIβ, PKA-RIIα and PKA-RIIβ. Upon binding of cAMP to PKA-R, PKA-C is released and becomes active to subsequently phosphorylate Ser/Thr residues on protein substrates(4). Due to PKA's importance and involvement in many parallel pathways, it needs additional levels of regulation in space and time. It achieves this through interaction with the protein scaffolding family of A-kinase anchoring proteins (AKAPs), that localize PKA, often together with many other signaling proteins (phosphatases, phosphodiesterases, other kinases, etc.) to a specific location in the cell(5, 6). At present, more than 30 mammalian AKAPs have been reported and experimentally verified(5, 6). Thus far, mainly AKAPs binding to the PKA-RII isoforms have been identified, as well as a handful of dual specific AKAPs, binding to both PKA-RI and PKA-RII (dAKAP1(7), dAKAP2(8) and Opa1(9)).

Initially, PKA-RI was proposed to be cytosolic and not anchored, however recently, we reported the chemical proteomics based discovery of two novel AKAPs with exclusive specificity for PKA-RI, small membrane AKAP (smAKAP)(1) and sphingosine kinase interacting protein (SKIP, SPHKAP)(10, 11). smAKAP has an unprecedented affinity for PKA-RIα (Kd = 6.7 nM) and PKA-RIβ (Kd = 6.9 nM) and localizes only these two isoforms to the plasma membrane using myristoylation and palmitoylation at Gly2 and Cys3, respectively. Its small size of only 11 kDa makes it the perfect model system to investigate further the specific determinants of PKA-RI's interaction with its unique set of AKAPs.

Structural characterization showed that AKAPs bind to the N-terminal dimerization and docking (D/D) domain of the PKA-R dimer via their A-kinase binding (AKB) domain(12). This AKB-domain is an amphipathic helix of 3-4 turns long and its hydrophobic edge interacts with the hydrophobic face of the D/D domain. What drives PKA-R/AKAP specificity is only partially understood, although it is clear that both sides of the interaction have adopted specific structural determinants. The D/D domain structures of both PKA-RIα and PKA-RIIα demonstrated a tightly packed X-type bundle consisting of four helices(13, 14). The monomers are positioned anti-parallel to one another and in the core there is a large hydrophobic plateau to accommodate AKAP binding. The N-terminal helix (α0) shows isoform specific differences. Specifically, in RIα it contains cysteines which lead to two identical inter-chain disulfide bridges between Cys16 and Cys37. This connection reinforces the monomer-monomer interaction along the boundary and the anti-parallel alignment(15). Also, the RIα D/D module presents a deeper cleft which allows the presence of larger hydrophobic amino acids in the AKB-domain(13). This was later confirmed by peptide screening(16-18) and the presence of a phenylalanine residue in the AKB peptide of SPHKAP(10) (Figure 1A).

More recently, crystal structures revealing the interactions of PKA-RI and PKA-RII with the

dual specific dAKAP2 (gene name AKAP10) provided a first glimpse into isoform specific interactions(19, 20). Firstly, the PKA-RII-dAKAP2 interaction is indeed characterized by a smaller interaction surface than the PKA-RI-dAKAP2 interaction. The former utilizes only two hydrophobic pockets of the AKB domain to interact, while in the latter, four hydrophobic contact sites were observed. The larger interaction surface is largely due to the disulfide bridges mentioned above, which create a more stable domain at the end of the D/D helices(13). Since the PKA-RI specific AKAPs have only recently been discovered(1, 10), no detailed information on their structural determinants has been elucidated yet.

PKA is targeted close to its substrates at specific locations in the cell via AKAPs. PKA recognizes its substrates via a rather promiscuous phosphorylation motif: [R/K]-[R/K]-X-[S/T][noP](21, 22). PKA has been found to phosphorylate AKAPs, although only to a limited extent, suggesting that a regulatory mechanism controls AKAP complex composition, activity or perhaps localization. A few illustrative examples include PKA phosphorylation of AKAP-Lbc on Ser1565 to allow 14-3-3β/ξ and binding at this site to inhibit the Rho-GEF activity (23). Similarly, AKAP79 has a number of PKA phosphorylation sites, which allow binding to phosphatidylinositol 4,5-bisphosphate(24).

Here, we report that smAKAP contains a putative PKA phosphorylation site within its AKB-domain, which drastically influences the PKA-RI-smAKAP interaction. To investigate the structural basis for this observation further, we first solved the crystal structure of the PKA-RIαD/D:smAKAP-AKB complex. This not only provided novel insights into what makes smAKAP specific towards PKA-RIα/β, but also led us to define the role of smAKAP phosphorylation in the AKB domain. Using a set of complementary cell imaging, biophysical, bioinformatics and structural biological techniques we uncovered and structurally characterized a novel auto-inhibitory mechanism of PKA-RI anchoring. This further extends the repertoire of nature's tools to regulate localized cAMP signaling.

Materials and Methods

Protein purification

Bovine full-length PKA-RIα(25) (P00514) and PKA-RIαD/D (AA 12-61)(20) were purified as previously described. His-smAKAP (and mutant) were expressed in E. coli (BL21 (DE3)) (20). At OD600 the cell culture was induced with isopropyl β-D-thiogalactopyranoside and grown for another 6 h at 37 °C prior to harvesting at 6000 rpm. The cells were resuspended in lysis buffer (50 mM KH_2PO_4, 300 mM NaCl, 10 mM imidazole, 5% glycerol, 5 mM β-mercaptoethanol, pH 8.0) after which they were lysed using a Microfluidizer (Microfluidics, USA) at 18,000 p.s.i. The cells were then spun down for 75 min at 4 °C in a Beckman JA20 rotor. The supernatant was then run on a Profinia (Bio-Rad, USA) according to the native IMAC protocol. The eluent was dialysed in 20 mM tris, 100 mM NaCl, 5 mM DTT, pH 8.3). The resulting protein was purified on an S75 column and concentrated to 30 mg/mL. The smAKAP-AKB peptide was dissolved into 0.5 M tris at pH 8.5 to a concentration of 5 mg/mL. The complex formed, consisting of PKA-RIαD/D and smAKAP-AKB peptide, had a final concentration of, respectively, 10 mg/mL and 2.52 mg/mL, and was used for crystallization.

Crystallography

Crystals of PKA-RIαD/D:smAKAP-AKB complex appeared approximately 2 months after being set with an Oryx8 protein crystallization robot at room temperature at a 2:3 ratio of protein solution:crystallizing (crystallizing solution: 0.1 M Citric acid pH 3.5, 28% w/v Polyethylene glycol 8,000). Data were collected on the synchrotron beamline 8.2.2 of the Advanced Light Source, Lawrence Berkeley National Labs (Berkeley, CA). The data were processed and scaled via HKL2000(26). For phasing the PKA-RIα D/D:dAKAP2-AKB structure (3IM4) was used as molecular replacement with CCP4 using Phaser(20, 27). This model was further refined in COOT and Refmac 5.2(28, 29). The refinement statistics are shown in Table 1.

Table 1 Collection and refinement statistics of the crystallographic data.

	PKA-RIαD/D:smAKAP-AKB
Data collection	
Space group	$P2_12_12_1$
Cell dimensions (Å)	
a	37.5
b	55.7
c	57.3
No. of molecule per asymmetrical unit	1
Resolution (Å)	2.0
R_{merge} (%)	7.6 (46)
Completeness (%)	98.0 (100.0)
I/sigma	26.9 (4.0)
No. reflections	8480
Refinement	
Resolution (Å)	40.0-2.0
R_{work} / R_{free} (%)	21.0/24.1
R.m.s. deviations	
Bond lengths (Å)	0.024
Bond angles (°)	1.9
Ramachandran angles (%)	
most favored	90.9
disallowed	none

*Values in parentheses are for the highest-resolution shell: (2.00-2.05 Å)

Structure analysis

The final crystallography model was evaluated using MolProbity(30).

Hydrogen-deuterium exchange mass spectrometry (HDX-MS)

A 30-fold dilution with either 100% H_2O, pH 7 for the non-deuterated experiments or deuterium oxide (Sigma Aldrich, Germany), pD 7, for deuterated experiments was carried out for the following complexes: unbound smAKAP (60 pmol), smAKAP-excess PKA-RIα (60 pmol of smAKAP and 75 pmol of PKA-RIα), unbound PKA-RIα (60 pmol) and PKA-RIα-excess smAKAP (60 pmol of PKA-RIα and 75 pmol of smAKAP). Diluted samples were incubated at room temperature for time intervals of 0 min for the non-deuterated experiments and 10 sec, 1 min, 10 min, 60 min and 240 min for the deuterated experiments. The deuteration reaction was quenched by pH reduction to 2.5 with a 1:1 dilution using ice cold 4 M guanidine hydrochloride adjusted to pH 1.85. Quenched samples were immediately injected into a 50 µL injection loop on a nano-ACQUITY UPLC system with HDX technology (Waters, USA). Online digestion was performed using an in-house built immobilised pepsin column for 2 min in 0.05% formic acid in H_2O, (flow rate of 25 µL/min), held at a temperature of 15°C. Peptides were trapped and desalted online using an ACQUITY UPLC BEH C18 1.7 µm VanGuard Pre-column (Waters, USA) at 0°C, with subsequent elution onto an ACQUITY UPLC BEH C18 1.7 µm, 1 mm x 100 mm column (Waters) held at 0°C. Peptide separation was achieved using a 7 min linear acetonitrile gradient (5%-85%) in 0.1% formic acid (flow rate of 40 µL/min). The eluent was directed into a Xevo G2 instrument (Waters, USA) with electrospray ionisation and lock-mass correction (using Glu-fibrinogen peptide). Mass spectra were acquired in MSE mode over the m/z range 50-2000. Two blank injections were performed between each sample injection to prevent sample carry over. Peptides were identified prior to deuteration using ProteinLynx Global Server 2.5 software (Waters, USA).

Calculation of exchange data

Deuterium uptake was calculated and compared to the non-deuterated control samples using DynamX 1.0.0 software (Waters, USA). Experiments were carried out in triplicate at each time point. Absolute deuterium incorporation at a given time point was determined by comparison with t = 0 in the non-deuterated sample. The deuterium incorporation at a given time point corresponded to the centroid value across the backbone amide population. Results were averaged across repeat analyses and a standard deviation derived. To examine the differences in a comparable way, the percentage difference uptake (Da) between the excess and unbound sets was calculated per peptide using equation 1.

$$\%\ \text{D uptake per peptide} = \frac{(\text{D uptake} - \text{unbound}\ (\text{Da})) - (\text{D uptake} - \text{excess}\ (\text{Da}))}{(\text{number of amino acids} - \text{N—terminus} - \text{number of prolines})} \quad (1)$$

Phosphorylation analyses

Human His-tagged smAKAP (wt and S66D) (10 µM)(1) was taken up in a reaction buffer containing $MgCl^2$ (10 mM), ATP (200 µM), [^{32}P]ATP (500-1000cmp ϱmol-1) and MOPS (50mM) (pH 7.5). Addition of recombinant PKA-Cα (20 nM)(31) and/or PKG Iα (20 nM)(32). 10 µl of reaction volume was quenched with 90 µl of 30% Acetic acid at the following time points: 0, 5, 10 and 15 min. The phosphorylation assays were also analyzed by LC-MS/MS to identify the exact phosphorylation site. An in-solution digestion of His-smAKAP using trypsin was

performed as described previously(33). The sample was desalted with an HBL Oasis system (Waters, USA). The desalted sample was dried down and reconstituted in 10% formic acid. A vented-column setup was used for analyzing protein digests through an Agilent 1200-Series LC system coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Germany) with conditions as described elsewhere(34, 35). Additionally, from seven different AKAPs (AKAP1 - RRRAAFQIISQVISEATEQ, AKAP4 - RRFYVNRLSSLVIQMAHKE, AKAP5 - RRLIETASSLVKNAIQLSI, AKAP9 - RRKVAAALVSQIQLEAVQE, AKAP10 - RRELAWKIAKMIVSDVMQQ, AKAP18 – RRAELVRLSKRLVENAVLK and smAKAP - RRILEYAHRLSQDILCDAL) the AKB domains were synthesized (NKI, Netherlands) including a double arginine label at the N-terminus for efficient capture on phosphocellulose. These were incubated with six kinases according to protocol: PKB (NEB, USA), CKB1 (NEB, USA), MEK1 (SignalChem, Canada), MEK2 (SignalChem, Canada), PKA-Cα(31) and PKG 1α(32). The reactions were spotted onto a phosphocellulose filter disk (Sigma-Aldrich, Netherlands). After four washes with 0.5% phosphoric acid the filter disks were rinsed with acetone, dried, and then analyzed on the 32P channel in liquid scintillant.

Cell culture

HeLa and HEK293 cells were cultured and transfected with the plasmids (smAKAP-wt-GFP, PKA-RIα-mKO2, PKA-RIα-mKO2, PKA-C-HA and smAKAP-S66E-mGFP) as described previously(1).

Fluorescence imaging

Cells were washed once with ice-cold PBS, fixed in 4% ice-cold formaldehyde in PBS and afterwards washed twice with PBS. Upon addition of Calyculin A and 8-CPT-cAMP live imaging was employed. The confocal images were obtained with a Zeiss LSM700 confocal system with a 63x oil objective lens.

Mutagenesis

The single site mutations (S66D and S66E) were introduced in the previously described smAKAP-GFP plasmid(1) and pLICHIS His6-smAKAP plasmid via the QuikChange mutagenesis kit (Agilent Technologies, USA).

Circular Dichroism and Fluorescence Anisotropy

Four peptides (1) IVILEYAHRLSQDILCDALQQWAC, (2) IVILEYAHRLpSQDILCDALQQWAC, (3) IVILEYAHRLDQDILCDALQQWAC and (4) IVILEYAHRLEQDILCDALQQWAC (mutations in bold) were synthesized and purified (NKI, Netherlands). These were dissolved in a 0.1M Tris buffer at pH 8.5. CD measurements were carried out on a Jasco J-810 spectropolarimeter and analysed via the Jasco Spectra Manager. For fluorescence anisotropy measurements on the interaction between PKA-RIα and smAKAP, peptides 1 and 2 were N-terminally tagged with 5-TAMRA as described previously(1). Measurements were also performed as described previously(1).

Molecular dynamics

Molecular dynamics was performed with the program YASARA version 8.12.26 in an AM-

BER03 force field(36). The simulations were run as described previously(36, 37) with the exception of the following. In an aqueous continuous phase the smAKAP-AKB peptide from structure solved in this article was placed in a 50 Å x 50 Å x 50 Å box. Simulations were run for 25 ns, and coordinates were saved every 7.5 ps. The results were then analyzed using analysis programs written in our laboratory. The AKB-Phospho peptide was constructed using the FoldX package.

Statistical methods

Statistical analyses used the Michaelis-Menten model, as implemented in Prism (version 5.0a).

Figures

Structure images were generated by using Pymol Version 1.5.0 (http://www.pymol.org/) and YASARA 8.12.26. Figures were created by using Adobe Photoshop and Illustrator.

Accession material online

Coordinates and diffraction intensities for the PKA-RIαD/D:smAKAP-AKB complex, Table 1 and Table 2 can be accessed here: http://www.2shared.com/file/L0tP_ngF/Online_material.html

Results

Structural Analysis of the PKA-RIα-smAKAP Interaction

To understand the selectivity of smAKAP towards PKA-RI better, we set out to determine the crystal structure of the complex consisting of the D/D-domain of PKA-RIα and the AKB-domain of smAKAP. As expected, the overall structure deduced was similar to previously reported PKA-R D/D:AKB structures (13, 17, 19, 20, 38). The complex crystallized with one RIα-D/D dimer (the monomer whose N-terminus is next to the N-terminus of smAKAP is named 'A' (AA12-58) whereas the other monomer is named 'B' (AA12-61)) bound to one smAKAP AKB-domain (AA 56-79), which is named 'C'. The majority of residues in the smAKAP peptide had clear electron density, however, $Trp77^{C}$ and $Cys79^{C}$ did not, therefore the sidechains of $Trp77^{C}$ and $Cys79^{C}$ have been omitted from the model. Similarly, most of the residues of both PKA-RIαD/D monomers are accounted for, except $Ser12^{B}$, $Leu13^{B}$, $Lys57^{A}$ and $Gly58^{A}$, of which the sidechains have been omitted, and $Glu59^{A}$, $Ala60^{A}$ and $Lys61^{A}$ which have been omitted entirely.

The structure of the RIαD/D dimer consists of an anti-parallel, four-helix bundle. The smAKAP-AKB peptide lies diagonally across the hydrophobic interaction surface of the PKA-RIαD/D domain (Figure 1B). The root mean square deviation (RMSD) between the PKA-RIαD/D of the dAKAP2(20) structure and the smAKAP complex presented here is 0.39 Å (Cα carbons), indicating that binding of AKB peptides with different PKA-R specificity has little influence on the conformation of PKA-RI. The RMSD between the AKB domains is larger, 0.58 Å (Cα carbons), as expected due to the presence of several differences

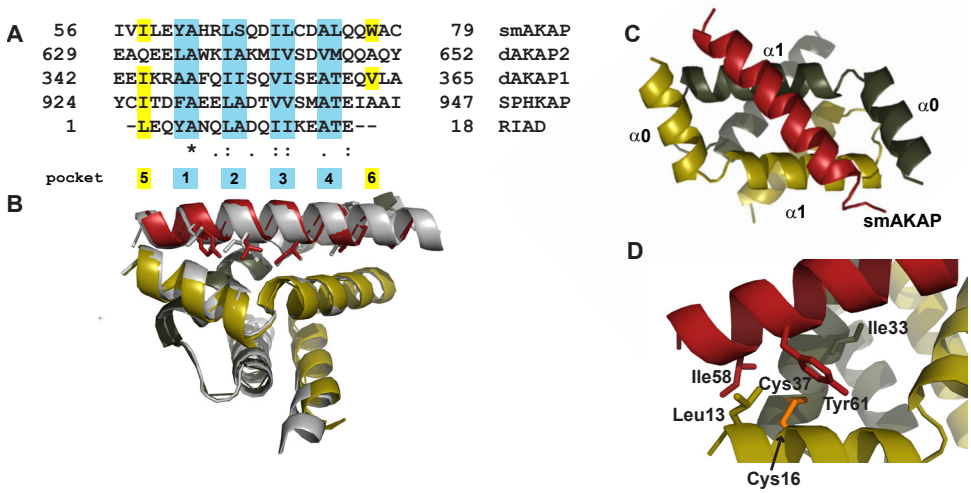

Figure 1 *Crystal structure of the smAKAP-AKB/PKA-RIαD/D complex.*

(A) A sequence alignment of four AKAPs whose AKB domains each bind to PKA-RI with the established four binding pockets highlighted in blue. (B) A side view displaying the interaction surface between smAKAP-AKB (red) and PKA-RIαD/D (gold (A)/green (B)) with an overlay of dAKAP2:PKARIαD/D (silver). (C) A top view of smAKAP overlaying PKA-RIαD/D. (D) The N-terminus of the AKB domain (red) establishes the fifth pocket. Tyr61^{C} and Ile58^{C} (highlighted in yellow in the sequence alignment of (A)) bury the disulfide bond Cys16^{A}-Cys37^{B} into a hydrophobic pocket. Leu13^{A} of α0-helix (gold) and Ile33^{B} of α1-helix (green) also close up the hydrophobic pocket.

between the AKB domains of dual-specific and PKA-RI specific AKAPs. There are four binding pockets in the D/D domain which each allow two hydrophobic residues of the amphipathic helix to dock (Figure 1A). The smAKAP-AKB domain has the hydrophobic amino acids fitting snugly in each binding pocket. What makes the smAKAP interaction different is that at each end of the domain there are additional hydrophobic interactions which assist in anchoring to the RIαD/D domain by burying the disulfide bonds into hydrophobic pockets. This creates a possible fifth and sixth pocket. In the fifth pocket, the disulfide bridge between Cys16^{A} and Cys36^{B} is surrounded by Ile33^{B} and Val34^{B} of helix αI, Leu13^{A} of helix α0 and Ile58^{C} and Tyr61^{C} (of the amphipathic helix), creating an enhanced hydrophobic pocket (Figure 1C). In addition, there are hydrogen bonds between side-chains of both proteins, Gln76^{C} and Lys30^{A} in helix α1 and Asp72^{C} and Gln26^{A} in helix α1, which also help to stabilize the interaction due to many polar residues of PKA-RIα protruding from the edges of the interaction surface. All these factors lead to a very strong and specific interaction of smAKAP with PKA-RIα. This is further illustrated by the conservation of Ile58^{C} throughout species and in SPHKAP (Ile926) (10), RIAD (Leu1) (18) and dAKAP1 (Ile344) (7) but not in dAKAP2 (Gln631) (8) (Figure 1A). Tyr61^{C} is also conserved in RIAD, whereas in SPHKAP another bulky hydrophobic amino acid resides at this site (Phe929). The bulky amino acid at this position has been proposed earlier as a PKA-RI specifier, as it would only fit in the deeper groove of the PKA-RI D/D domains(16, 20). It is likely that at the other end of the AKB domain, where the second disulfide bridge resides (Cys16^{B} and Cys36^{A}), a putative sixth pocket is formed in a similar way with Leu74^{C} and Trp77^{C}. Although the sidechain of 77^{C} is not well resolved in the structure one could assume a function similar to the

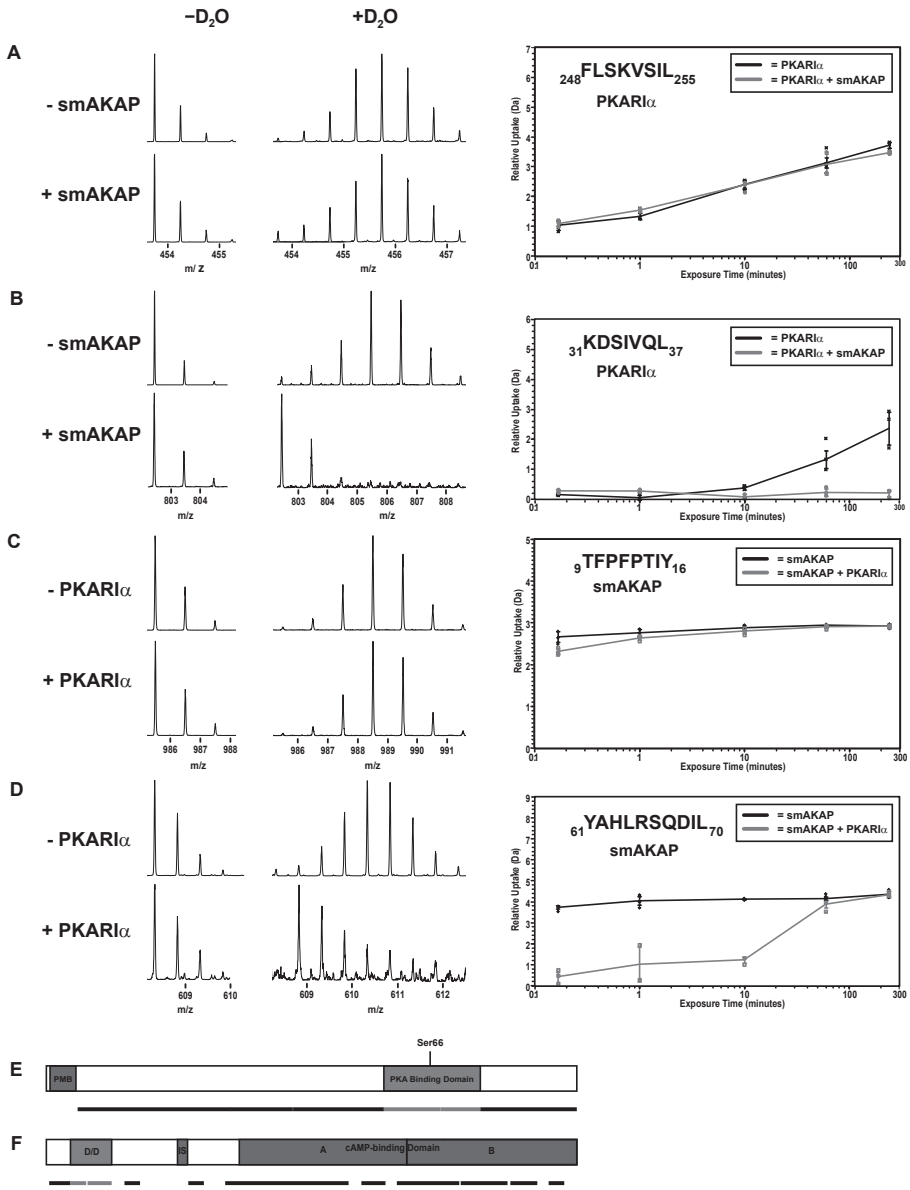

Figure 2 *H/D exchange profiling of the interaction surface of full length smAKAP and PKA-RIα.*

(A) No difference in deuterium uptake was observed between PKARIα (black) and PKARIα+smAKAP (gray) for AA248-255 (B) whereas for AA31-37 there was an increasing difference in deuterium uptake starting after 10 minutes. (C) For AA9-16 of smAKAP there was no difference in deuterium incorporation between smAKAP (black) and smAKAP+PKARIα (gray) (D) whereas a large initial difference in uptake was noted for AA61-70. The known domains of smAKAP (E) and PKARIα (F) are shown. In red are the domains which showed interaction between smAKAP and PKARIα via HDX. Below are representative peptides that best reflect the sequence coverage obtained (gray – differential deuterium incorporation and black – no difference deuterium incorporation).

Ile58^{C} and Tyr61^{C} couple at the other end of the helix due to the internal symmetry present. However, the putative sixth pocket is much less defined and neither Leu74^{C} or Trp77^{C} seem conserved in the other PKA-RI specific AKB domains of SPHKAP and RIAD (Figure 1A) (10, 18).

Probing the interaction surface of the full-length PKA-RIα and His6-smAKAP using H/D exchange mass Native mass spectrometry Native mass spectrometry

A small domain upstream of the classical AKB domain, designated as RISR (RI specifier region), was reported to assist in PKA-RI specificity in dual specific AKAPs(39). Although smAKAP does not contain a putative RISR region, we investigated the smAKAP:PKA-RIα interaction surface for putative contact sites outside the AKB domain using hydrogen/deuterium exchange (HDX) mass spectrometry with the full length proteins. smAKAP, in the presence and absence of an excess of PKA-RIα, was incubated in deuterium oxide for various time points between 10 seconds and 4 hours. The reverse experiments, using an excess of smAKAP, were also conducted. As expected, we observed a distinct difference in deuterium incorporation in different regions. For instance, peptide AA248-255 displayed no difference in deuterium uptake (Figure 2A), whereas peptides in the N-terminal region such as AA19-37 revealed a substantial reduction in deuterium uptake (Figure 2B and Online material Table 1). To a minor extent, the peptides between AA38 and AA67 also revealed differential deuterium uptake (Online material Table 1). Although we were able to cover large parts of the PKA RIα sequence (77%), no other region displayed uptake differences. Similarly, focusing on smAKAP, we observed that only the peptides covering the region AA61-78, which is the AKB-domain (Figure 1A), displayed a substantial decrease in deuterium uptake, whereas all other peptides, covering nearly the whole sequence of smAKAP (96%) displayed no substantial difference in deuterium uptake in the absence of PKA-RI (Figure 2C, D and Supplementary Table 2). These results clearly indicate that only the AKB domain of smAKAP (Figure 2E), and no region up- or down-stream, is in stable contact with the D/D domain of PKA-RIα (Figure 2F).

The AKB-domain of smAKAP is phosphorylated by PKA

BLAST analysis and sequence alignment of smAKAP and its homologues revealed several well conserved serines and threonines(1). One of them, Ser66, lies in the middle of the AKB domain and, displays the phosphorylation motif H-R-L-S (Figure 3A), a PKA substrate site proposed from peptide screens(22, 40). This PKA motif in smAKAP is conserved from zebrafish to human with the positively charged residue (arginine or histidine) occupying either the -2 or -3 positions with respect to the serine (Figure 3A). Even though serine to alanine mutations are very common in evolution, Ser66 in smAKAP remains stable across species. Interestingly, Ser66 seems to reside on the hydrophobic side of the amphipathic AKB domain helix (Figure 3B), which could possibly induce steric and electrostatic hindrance for the hydrophobic based binding of smAKAP with PKA-RI.

An *in vitro* phosphorylation assay with purified His6-smAKAP and the kinases PKA (catalytic subunit) and its closest homologue PKG Iα revealed that PKA phosphorylated smAKAP whereas PKG Iα did only marginally (Figure 3C). Phosphorylation of

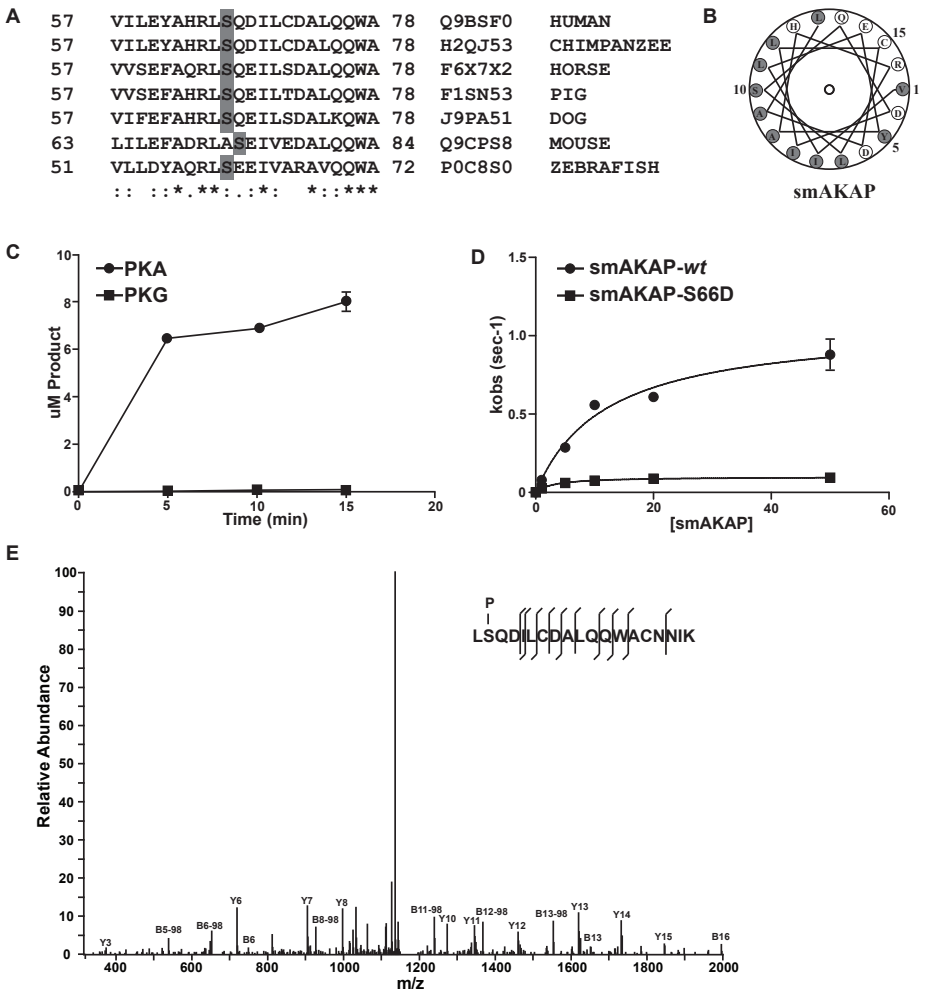

Figure 3 *PKA phosphorylates smAKAP at Ser66 in the A-kinase Anchoring Domain.*

(A) Sequence alignment of human smAKAP (AA 57-78) with various orthologues in other vertebrate species. Identity (*), similarity (:) and the serines highlighted in gray. (B) Helical wheel alignment of the smAKAP's AKB domain reveals an amphipathic helix with a hydrophobic surface on one side (gray) which contains Ser66. (C) ^{32}P *phosphorylation assay using PKA-Cα and PKG Iα as kinase and recombinant His6-smAKAP as substrate. (D)* ^{32}P *phosphorylation assay using PKA-Cα on recombinant smAKAP-wt and smAKAP-S66D. (E) Annotated MS/MS spectrum of the peptide LpSQDILCDALQQWACNNIK with a phosphorylated serine at position 2, corresponding to Ser66 in smAKAP.*

smAKAP-wt had a V_{max} of 1.068±0.081 μM/sec and a K_m of 11.91±2.42 μM. The filter binding assay was repeated with Ser66 mutated to an aspartic acid (smAKAP-S66D), which showed no detectable levels of phosphorylation (Figure 3D). To confirm further that Ser66 is indeed the substrate site of PKA, a digest of in vitro phosphorylated smAKAP was analyzed by LC-MS/MS analysis. The tandem MS-spectrum of the only observed phosphopeptide Lp-SQDILCDALQQWACNNIK (Mascot score 89) unambiguously identifies Ser66 as the phosphorylation target site of PKA (Figure 3E).

Serine66 phosphorylation effectively abrogates binding of PKA-RI

We showed previously using a fluorescence polarization assay that smAKAP has a strong affinity (K_d ≈ 7 nM) for PKA-RIα and RIβ(1). Executing this assay with the AKB peptide carrying a phosphorylated serine (IVILEYAHRLpSQDILCDALQQWAC), we noted a dramatic decrease in the binding affinity (K_d > 500 nM (Table 2)). HeLa cells transfected with smAKAP-wt-GFP and PKA-RIα-mCherry(1) or PKA-RIα-mKO2 (Figure 4A), show strong co-localization along the plasma membrane. In order to deduce the impact of phosphorylated smAKAP in the cellular context, phosphomimetic mutants were created: smAKAP-S66E¬-GFP and smAKAP-S66D-GFP. Notably, when the HeLa cells were transfected with these mutants along with PKA-RIβ-mKO2 (Figure 4B), smAKAP still localized at the plasma membrane, while both PKA-RI isoforms now predominantly resided in the cytoplasm, evidence that phosphorylation of Ser66 inhibits PKA-RI binding to smAKAP. In order to deduce if the phosphorylation of Ser66 by PKA occurs in a cellular context, HeLa cells were subjected to a triple transfection (smAKAP-wt-GFP, PKA-RIβ-mKO2 and PKA-C-HA). After addition of 8-CPT-cAMP and Calyculin A, disruption of the association between smAKAP-wt-GFP and PKA-RIβ-mKO2 was observed by live cell microscopy (Figure 4C). Upon phosphorylation of smAKAP, most of the PKA-RIβ cannot bind to smAKAP anymore and relocates to the cytosol.

Table 2 *Fluorescence anisotropy measurements to determine the binding affinity (n=3, K_d ± standard deviation in nM) of the phosphorylated smAKAP AKB-domain peptide, tagged with 5-TAMRA (excitation at 535 nm and emission at 580 nm) with the full length regulatory subunit dimers: PKA-RIα and PKA-RIβ. The smAKAP wt, dAKAP2 and AKAP79 results were previously published(1).*

	smAKAP *wt*	smAKAP-P	dAKAP2	AKAP79
RIα	6.7±0.7	>500	50	nd
RIβ	6.9±0.6	>500	-	nd

As the interaction between smAKAP and PKA-RIα was determined by both crystallography and hydrogen/deuterium exchange studies, we were able to explore further the detailed molecular mechanism of the binding abrogation induced by Ser66-phosphorylation. Initial-

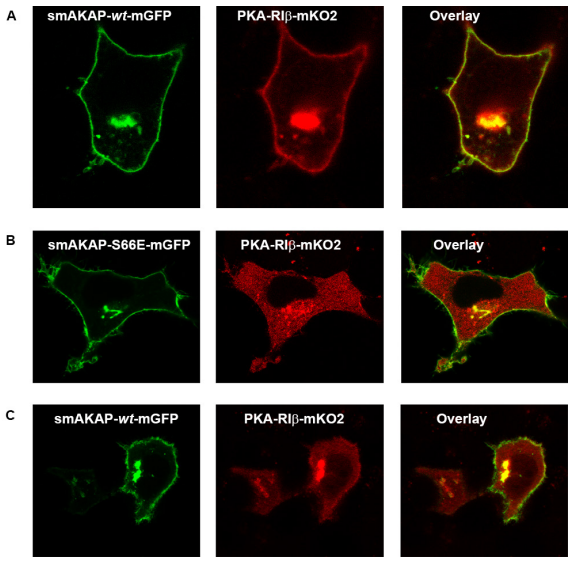

Figure 4 *Phosphorylated smAKAP no longer localizes PKA-RI at the plasma membrane.*

(A) Transfection of HeLa cells with smAKAP-wt-mGFP and PKA-RIβ-mKO2 show co-localization at the plasma membrane. (B) In contrast, in the HeLa cells transfected with the phosphomimetic smAKAP-S66E-mGFP and PKA-RIβ-mKO2 no co-localization occurs. (C) After adding 8-CPT-cAMP and Calyculin A, smAKAP-wt-mGFP is partially phosphorylated upon Ser66 by PKA-C, thus releasing PKA-RIβ-mKO2 into the cytosol.

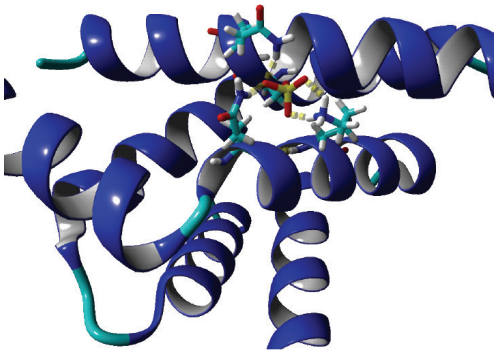

Figure 5 *Via HADDOCK we modeled smAKAP-AKB-Phospho:PKARIαD/D.*

Unexpectedly, this resulted in a much stronger interaction. As shown there is a larger interaction surface due to the phosphoserine sticking out of the hydrophobic domain which in turn allows hydrogen bonding with Gln26 and Lys 30 of PKARIα and Gln67 of smAKAP.

ly, we hypothesized that electronegativity and steric clashing could inhibit binding of the phosphorylated AKB domain peptide (AKB-Phospho) to the hydrophobic PKA-RIαD/D. However, molecular modeling using HADDOCK(41, 42), of smAKAP AKB-wt, AKB-S66D, AKB-S66E and AKB-pSer66 with PKA-RIαD/D suggested that the phosphorylated wild type and the phosphomimetic mutants could bind at least as well to the D/D domain of PKA-RIα as the non-phosphorylated equivalent. These data indicated that a phosphorylated Ser66 can still fit within the hydrophobic groove and is able to extend its side-chain with the electronegative moiety out of the hydrophobic groove and create hydrogen bond interactions with $Lys30^{B}$ and $Gln26^{B}$ of PKA-RIα (Figure 5). Clearly, this is only a model, as experimentally we observed complete disruption of binding upon phosphorylation, prompting us to investigate other putative modes of binding disruption.

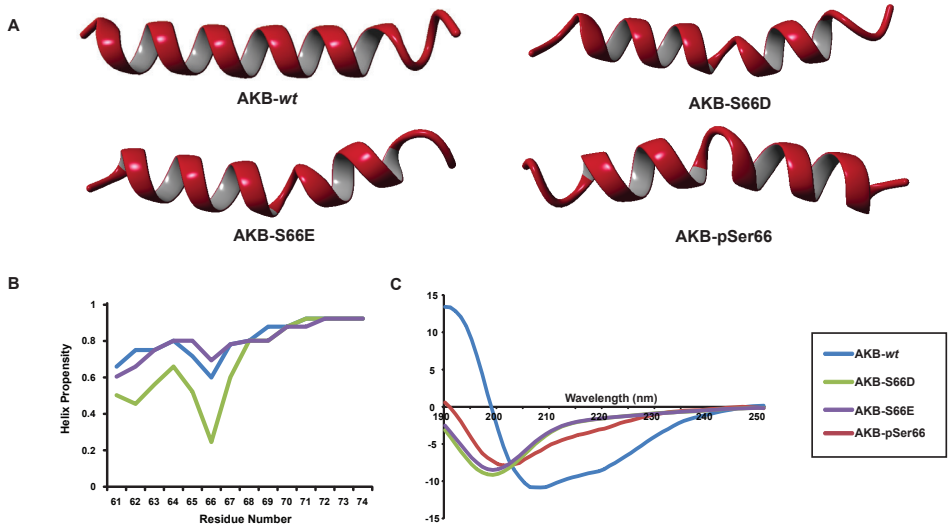

Figure 6 *Loss of helical structure due to Ser66 phosphorylation abolishes PKA-R-smAKAP interaction.*

(A) 20 ns molecular dynamics simulations were run of the AKB-wt peptide in water. The peptide started to lose its helix from the N and C termini. In contrast, the AKB-S66D, AKB-S66E and AKB-pSer66 peptides already lost their secondary structure in the middle near the serine after 1ns. (B) Helix propensity prediction of AKB-wt, AKB-S66D and AKB-S66E were measured via NetSurfP 1.1. Upon the mutation of serine to aspartic acid there is a severe drop in helix propensity. NetSurfP 1.1 is unable to use phosphorylated serines. (C) CD measurements revealed a helix propensity of ~45% for the AKB-wt peptide. The AKB-S66D, AKB-S66E and AKB-pSer66 peptides showed no secondary structure.

Molecular mechanism of binding disruption

In molecular dynamic (MD) simulations on the AKB helices of AKB-wt, AKB-S66D, AKB-S66E and AKB-pSer66, all peptides partially or even fully lost their secondary structure in 20 ns simulations. The AKB-wt helix started to lose its helicity relatively slowly at both termini of the peptide after ~1 ns. In contrast, the other three peptides quickly lost helicity next to the mutated serine after ~1 ns (Figure 6A). This suggests that the phosphorylation causes a severe distortion in the helix, which could possibly lead to the entire amphipathic helix being lost. Using NetSurfP 1.1, the helical propensity was determined of the AKB-wt, AKB-S66D and AKB-S66E peptides (Figure 6B). Mutation to an aspartic acid caused a severe drop in helical propensity which led us to investigate this further using circular dichroism (CD). The four peptides used in the MD simulations (AKB-wt, AKB-S66D, AKB-S66E and AKB-pSer66) were synthesized and measured by CD. As suggested by the MD simulations, only the AKB-wt peptide displayed a strong α-helix propensity (~45%) whereas the phosphoserine and phospho-mimetic mutants showed no secondary structure (Figure 6C). This confirms the findings of the MD simulations and suggests that the formation of an amphipathic helix, required for PKA binding, is seriously hampered when Ser66 of smAKAP is phosphorylated.

AKB domains of various other AKAPs are phosphorylated

In order to see if other AKAPs can also potentially use this mechanism, we screened AKB domains in the literature for the presence of serine, threonine and tyrosine residues. This is the case for the AKB domains of AKAP1, AKAP4, AKAP5, AKAP9, AKAP10, AKAP18 and smAKAP. The relevant AKB peptides were synthesized and incubated with the kinases PKB, CKB1, PKA, MEK1, MEK2 and PKG. Interestingly, not only smAKAP was strongly phosphorylated by PKA, but AKAP4 and AKAP18 (Figure 7). AKAP10, which contains a putative MEK1/MEK2 motif, could be phosphorylated by PKB, MEK1 and MEK2, whereas CKB1 phosphorylated AKAP5 and AKAP1. Solely the AKB domain of AKAP9 did not show any phosphorylation by this set of kinases.

Discussion

Thus far, molecular understanding of PKA-RI:AKAP interactions has been limited to studying its interaction with the dual-specific dAKAP2(20) and the creation of PKA-RI specific peptide libraries(18). Here we took advantage of the recent discovery of two PKA-RI specific AKAPs, SPHKAP(10) and smAKAP(1), to extend our understanding of these specific interactions.

Comparison of dAKAP2 binding to either PKA-RI and PKA-RII revealed a remarkable difference in interaction surface(19, 20). The PKA-RIα:dAKAP2 interaction makes contact through four hydrophobic pockets on dAKAP2's amphipathic helix (Figure 1A), whereas PKA-RII utilizes only two hydrophobic binding pockets, resulting in a much larger interaction surface for PKA-RIα:dAKAP2(20). In the structure of the PKA-RIαD/D:smAKAP-AKB complex solved here, we clearly see similar contacts via four binding pockets (Figure 1A).

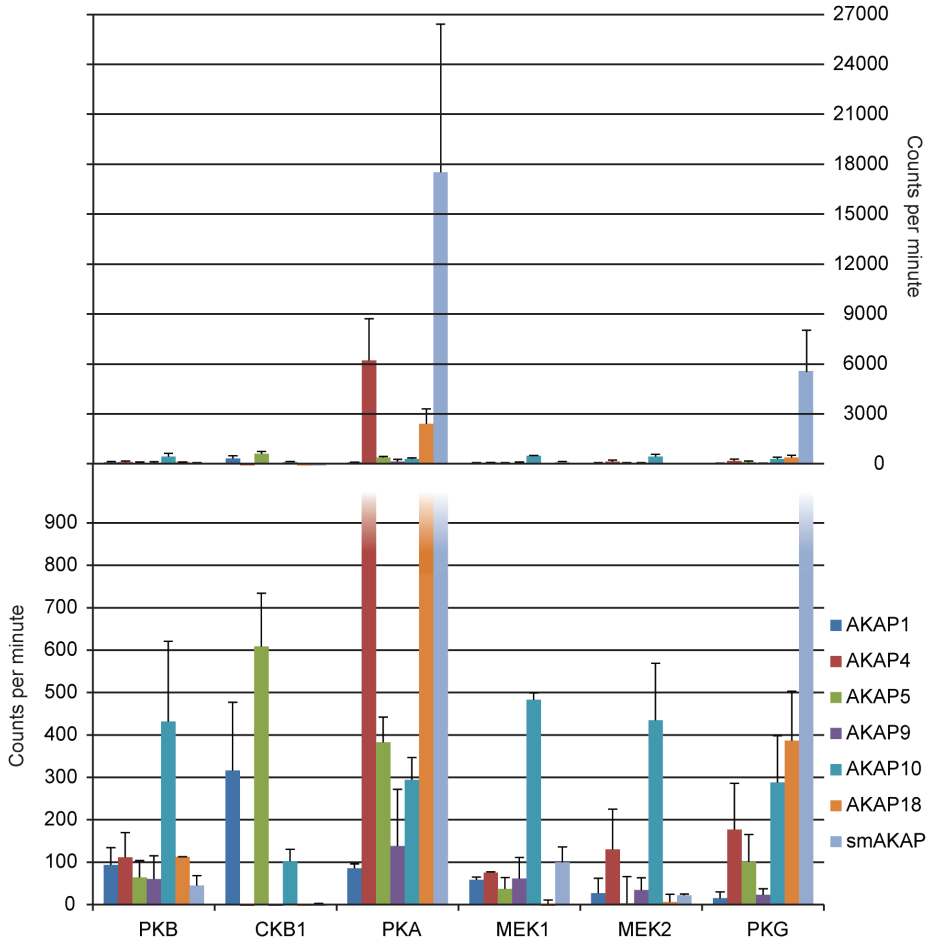

Figure 7 *Multiple AKAPs can be phosphorylated at their AKB domain.*

The AKB domain peptides of AKAP1, AKAP4, AKAP5, AKAP9, AKAP10, AKAP18 and smAKAP, which all contain a Ser/Thr/Tyr residue, were incubated in vitro with six kinases (PKB, CKB1, PKA, MEK1, MEK2 and PKG) for 30 min at 30 ºC (n = 2). The phosphorylation was monitored via ^{32}P incorporation using a liquid scintillant counter. Except the AKB domain of AKAP9, all other AKBs became phosphorylated, either by PKA (especially smAKAP, AKAP18 and AKAP4) and/or other kinases.

Another interesting difference between the D/D-domains of PKA-RIα and RIIα is the presence of two identical disulfide bridges between Cys16 of one PKA-RIα monomer and Cys37 of the second PKA-RIα monomer(13, 38). These reside at the edges of the AKAP interaction surface of the PKA-RIα-D/D-domain(13). It was suggested that these disulfide bridges could contribute to the interaction with dAKAP2, although in the crystal structure of Sarma et al.(20), no direct interaction with any of dAKAP2's residues was observed. Interestingly, smAKAP did show these interactions as $Ile58^{C}$ and $Tyr61^{C}$ at the N-terminal end (Figure 1C) and $Leu74^{C}$ (possibly in conjunction with Trp77) seal both disulfide bridges into the hydrophobic pockets. An additional interaction between $Ile58^{C}$ and $Leu13^{A}$ of PKA-RIα (helix α0) also assists. In dAKAP2, the locations of Gln631 and Met647 are identical to those of

smAKAP's Ile58^{C} and Leu74^{C} respectively, however these are not able to accomplish the same task with respect to binding(20).

These additional hydrophobic interactions could account for the much stronger binding affinity that PKA-RIα displays for smAKAP compared to dAKAP2(1, 16). Three other PKA-RI binding sequences have an isoleucine or leucine at the equivalent Ile58 position of smAKAP (Figure 1A): dAKAP1 (dual specific, Ile344) SPHKAP(10) (RI-specific, Ile926) and RIAD(18) (synthetic, Leu1). dAKAP2, does not have a hydrophobic residue at the Ile58 equivalent position but a glutamine (Gln631, Figure 1A). A peptide substitution array performed on the AKB domain of dAKAP2 showed that phenylalanine, leucine, isoleucine or valine substitution of Gln631 increased the PKA-RIα binding affinity of dAKAP2 6-fold(16). These findings support the importance of creating an additional, fifth, hydrophobic pocket around Ile58C and the Cys16A:Cys37B disulfide bridge to drive high affinity PKA-RIα binding. Our structure also suggests that Tyr61C, located in pocket 1, is part of this extended hydrophobic interaction. This residue is likely not only important for affinity, but also for PKA-RI specificity. For instance, if the first amino acid in pocket 1 of dAKAP2 (Leu634) is mutated to an aromatic residue the PKA-RII binding affinity decreases approximately 40-fold. As well, when RIAD was generated(18) it was observed that PKA-RI tolerates a bulky (aromatic) amino acid at that position (Tyr4 of RIAD), while PKA-RII did not, in line with the observation of Banky et al. that PKA-RIα contains a much deeper hydrophobic groove in the D/D-domain to accommodate this large side chain(13). This was confirmed by the establishment of SPHKAP as the first PKA-RI specific AKAP (Phe929)(10).

In further support of the proposed sixth pocket at the C-terminal end of smAKAPs AKB domain, Burns-Hamuro *et al.*(16) also created a dAKAP2 AKB domain peptide in which both Gln631 and Met647 (equivalent to Ile58 and Leu74 of smAKAP) were substituted for phenylalanines. This resulted in an affinity increase for PKA-RIα of 24-fold, instead of 6-fold when substituting only Gln631 for phenylalanine. PKA-RII affinity was not affected, indicating that these interactions do not drive specificity, but rather affinity. The conservation of a hydrophobic amino acid at Trp77 (smAKAP, Figure 1A) in the other PKA-RI binding AKAPs suggests this could further assist binding in this region, however due to disorder of Trp77 in our crystal structure verification of this hypothesis awaits.

AKAPs accommodate spatial/temporal signaling of PKA. As such, AKAPs anchor PKA close to its substrates in a specific cellular compartment allowing it to respond instantly to local rises in cAMP-levels. Phosphorylation of substrates may continue until halted via several feed-back loops. The best described mechanism proceeds through nearby phosphodiesterases (PDEs) which degrade cAMP to AMP, thereby terminating PKA activity at the second messenger level(43). Often, specific PDEs and PKA are sequestered by the same AKAP(6). An example of such a negative feedback mechanism is membrane bound PDE3A which is activated by PKA phosphorylation(43). Dropping concentrations lead to dissociation of cAMP from PKA-R and subsequent re-formation of the inactive PKA holoenzyme on the AKAP. Dephosphorylation of PDE3 by a nearby, or even AKAP-anchored, phosphatase then resets the system to respond to rises in cAMP again.

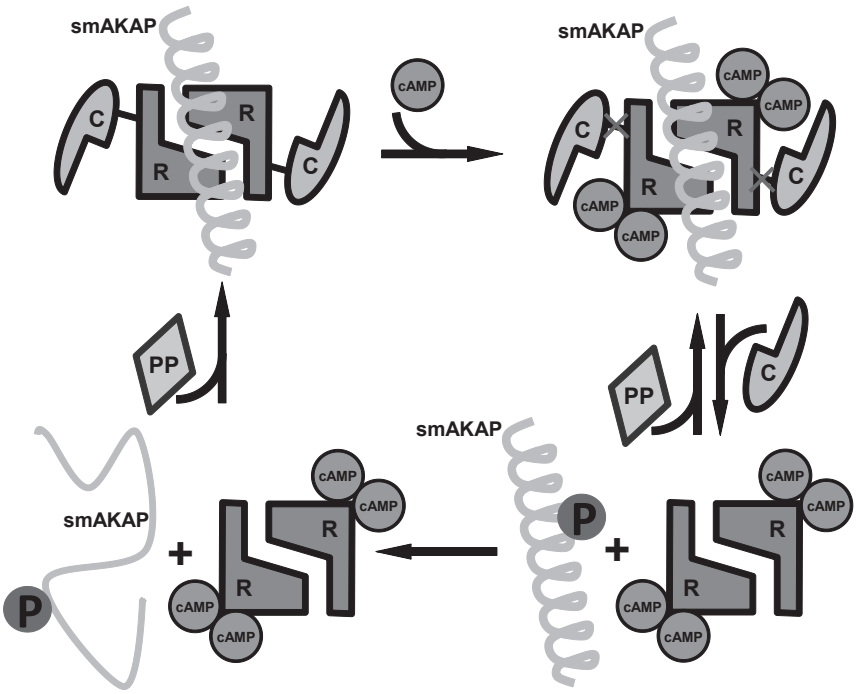

Figure 8 *Proposed model for how PKA Ser66 phosphorylation disrupts smAKAP binding.*

The PKA holoenzyme is tethered to smAKAP (signified by the amphipathic helical AKB domain as the remainder of the protein is unstructured). To each of the PKA-R in the dimer, a PKA-C is non-covalently bound. Upon binding of two cAMP to each PKA-R, the catalytic subunits are released. Upon release, PKA-C is able to phosphorylate Ser66 of smAKAP. Due to the phosphoserine present in the amphipathic helix, the secondary structure is lost and the energy barrier too high to form an α-helix again. A protein phosphatase could potentially dephosphorylate Ser66, in turn lowering the energy barrier to form an α-helix and allowing smAKAP to bind to PKA-RIα again.

Direct disruption of PKA:AKAP interaction could be another potential mechanism to control localized cAMP signaling, however, thus far this has only been shown pharmacologically. For instance, the Ht31 peptide(44) and more recently the isoform specific peptides super-AKAP-IS(17) (PKA-RII) and RIAD(18) (PKA-RI) have been proven to be useful tools in disrupting the PKA:AKAP interaction. FMP-API-1, a small molecule, was also shown to inhibit the binding of PKA to AKAPs, through an allosteric effect away from the D/D domain(45). Here we show an alternative negative feedback loop in which PKA inhibits its own anchoring to smAKAP by phosphorylating Ser66 in the AKB domain. Based on these findings we propose the following model (Figure 8): At low cAMP concentrations, smAKAP is bound to the PKA holoenzyme. Once the cAMP concentration increases, it will bind to PKA-RI, causing PKA-C to be released. The released PKA-C, which is still in close vicinity of smAKAP, will phosphorylate smAKAP when the regulatory subunit dimer dissociates under equilibrium conditions. This phosphorylation results in the amphipathic helix AKB domain of smAKAP to (partially) unfold. As it unfolds, PKA-RI gains more freedom to operate as the dimer cannot re-bind to smAKAP. If the phosphorylation is removed, by a nearby phosphatase, the random coil AKB domain can re-form its α-helix, allowing PKA-RI to bind to smAKAP again. That this may be a bona fide mechanism used by other

AKAPs as well is illustrated by the presence of Ser, Thr and Tyr residues in at least 6 other human AKB domains (Figure 9), of which several (AKAP1/AKAP4) are already annotated as sites of phosphorylation in online phosphoproteomics databases. Not all of these bear the typical PKA phosphorylation motif in the AKB domain, although AKAP4 and AKAP18 do. It was shown that the RLS motif also caused a strong phosphorylation event on aquaporin-0 by PKA which is anchored on AKAP2, leading to preserve fluid circulation within the lens(46). Additionally, we have shown that it does not necessarily have to be PKA which could assist in inhibiting the interaction between PKA and AKAPs as various other kinases seem to phosphorylate different AKAPs (Figure 7). This could indicate that the mechanism revealed here is a more common inhibitory mechanism for PKA:AKAP interactions.

What is the physiological role of PKA-RI release from smAKAP? This is a question we cannot answer at this time, although it is tempting to speculate that release of the PKA-RI subunit induces a (temporary) signal termination at the smAKAP location. However one could also imagine that PKA-RI release may assist in more efficient capturing of the catalytic subunit when cAMP concentrations are dropping. This should be particularly important in the light of the very strong interaction between smAKAP and PKA-RI. Or PKA phosphorylation of smAKAP could play a role in the dynamics of degradation of this particular PKA signaling node.

In conclusion, the observation that PKA phosphorylates smAKAP within the AKB domain to inhibit its own anchoring led us to solve the crystal structure of PKA-RIα interacting with smAKAP. This is the first structure of PKA-RI with one of its specific AKAPs, of which only two have been identified (smAKAP and SPHKAP). The structure revealed several interesting points: (i) the interaction between PKA-RIα and smAKAP utilizes six hydrophobic contact sites, and extends over a much larger portion of the amphipathic helix, whereas PKA-RII interactions typically use less hydrophobic pockets; (ii), phosphorylation of smAKAP's AKB domain by PKA causes destabilization of the amphipathic helix which reduces smAKAP's affinity for PKA-RIα at least 1000-fold. This makes it tempting to speculate that PKA-RIα interacting with its specific AKAPs occurs at much higher affinities, that may actually require the observed phosphorylation event to break the interaction under physiological conditions. Additionally, other AKAPs have been shown to be phosphorylated as well in their AKB domains which suggests the phosphorylation being a key disruptor of PKA anchoring to an AKAP.

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Chapter 5

A Systematic Evaluation of Old and Identification of Novel PKA-AKAP Interaction Motifs

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Summary

Protein kinase A (PKA), or cAMP-dependent protein kinase, is localized to specific locations in the cell via A-kinase anchoring proteins (AKAPs). The regulatory subunits of PKA (PKA-RIα, RIβ, RIIα and RIIβ) each form a homo-dimer and the dimerization domain interacts with a small helical region, designated as the amphipathic helix, present in each of the over 40 so far described AKAPs. This allows for tight anchoring of PKA and efficient communication with other signaling proteins in a spatial and temporal manner. The hydrophobic interaction surfaces of the PKA-R dimer and several AKAP helices have been described with meticulous structural detail. Despite this knowledge, not every proposed AKAP has its PKA-RIα and/or PKA-RIIα binding motif specified. Here we created an efficient bioinformatic tool, termed THAHIT, to accurately map the PKA binding motif of all reported AKAPs and for each predict its specificity towards PKA-RIα and/or PKA-RIIα, based on all available structural parameters. In addition, we define several novel helical domains on thus far poorly characterized AKAPs. Using this tool additional anchoring sites on existing AKAPs could be proposed. To verify the validity of these newly predicted anchoring sites and their putative specificities we used computational modeling approaches (HADDOCK docking studies) and affinity studies (fluorescence anisotropy and cellular co-localization studies). Next we used THAHIT to identify new AKAPs in cAMP-based chemical proteomics discovery datasets, retrieving a new 330 kDa AKAP, which we further characterized as a PKA-RIIα binder. Altogether THAHIT provided a comprehensive overview of known and new AKAP-PKA interaction domains enabling to define their specificity.

Introduction

Many cellular processes, such as cell proliferation(2) and remodeling of the actin cytoskeleton(3), are regulated via spatially and temporally segregated cAMP signaling pathways. A key component in these pathways is the cAMP-dependent protein kinase also known as protein kinase A (PKA)(4). PKA is a heterotetrameric kinase consisting of a regulatory subunit (PKA-R) dimer with each subunit binding to a catalytic (PKA-C) subunit(5). There are four, genetically distinct, PKA-R isoforms: PKA-RIα/β and PKA-RIIα/β and three PKA-C isoforms; α, β and γ. Early on, cAMP and PKA were thought to be freely diffusible throughout the cell. However, in 1984 the first A-kinase anchoring protein (AKAP), MAP2(6), was reported and since then many more followed to currently comprise a family of well over 40 unique AKAP genes(7, 8). Many of these also include alternative splicing variants, expanding the family to well over 70 different protein species. AKAPs bind to PKA with high affinity and sequester it to a specific location inside the cell, close to a localized pool of cAMP and a specific set of substrates. Along with PKA, the AKAPs often tether many other signaling proteins, such as adenylate cyclases, phosphodiesterases, phosphatases and other kinases. In this way, a very efficient signaling hub is created that is regulated in both space and time to allow segregation of the functionally distinct cAMP regulated pathways(8).

MAP2, was discovered using a method called the RII-overlay(6). This methodology was less successful using PKA-RI and therefore all 'early' AKAPs were reported to have PKA-RII specificity. Later, in the 1990s using a two-hybrid screen, the first dual-specific AKAPs, dAKAP1 and dAKAP2, were revealed, which bind both PKA-RI and PKA-RII isoforms(9). The capturing of AKAPs via PKA-R binding to immobilized cAMP, the cAMP pull-down, is nowadays also frequently used(10, 11). The AKAPs are then identified via western blotting and/or mass spectrometry. This methodology led to the recent discovery of the first PKA-RI specific AKAPs, SPHKAP and smAKAP(12-14). Other methodologies used include the T7-phage display to discover Chromodomain Helicase Binding Protein 8 and bioinformatics with follow-up studies to discover Glycogen synthase kinase 3β interaction protein (GSKIP) as AKAPs(15, 16).

Carr et al. described the AKAP domain responsible for the high affinity interaction between PKA and AKAPs for the first time(17). The domain consists of a 3-4 turn amphipathic helix which uses its hydrophobic edge to dock to the dimerization/docking (D/D) domain of PKA(18). Each AKAP has one or more amphipathic alpha helices which bind to the regulatory subunit dimer. Several structural studies have elucidated meticulous molecular detail on the interaction between PKA-RIα and PKA-RIIα with different AKAP helices by means of, for instance, crystal/NMR structures, mutation analyses and peptide arrays(19-22). The D/D-domain of PKA-R consists of an X type four-helix bundle with a hydrophobic docking surface for the hydrophobic side of the AKAPs kinase binding domain(18). There is a larger interaction surface present on PKA-RIαD/D than on PKA-RIIαD/D, which causes more restrictions on binding for PKA-RI interacting AKAPs(19).

The most common means of initially attempting to locate the PKA binding domain is via determining the presence of an amphipathic helix using bioinformatic tools. Afterwards there are several options, with pros and cons to each, such as peptide arrays(22), amino

acid substitutions (with proline as a helix disruptor being the common choice), deletional mapping combined with Western blotting, co-localization studies or Co-IPs and binding affinity studies between the predicted domain and PKA-R. By means of these various studies many of the PKA-RIIα and PKA-RIα binding domains have been confirmed. However, not every report of a novel AKAP in the literature has presented the interaction region with equal detail.

Based upon a confident dataset of previously confirmed amphipathic helices we developed here a software tool, THAHIT (THe AKAP/amphipathic Helix Identification Tool), to identify PKA binding domains based upon various stringent structural factors. Besides localizing the amphipathic helix in the protein sequence, it is also able to predict the specificity of the helix towards PKA-RIIα or PKA-RIα. This led us to predict stringent motifs for both PKA-RIα and PKA-RIIα directed amphipathic AKAP helices to reveal additional binding determinants up- and down-stream of the actual helix. We also observed that many identified AKAPs may contain, yet undiscovered, additional PKA binding sites. Additionally, various chemical proteomics datasets which contained possible novel AKAPs were scanned using THAHIT resulting in the discovery of a novel AKAP we termed *vl*AKAP, for very large AKAP. We validated all observed novel sites using a complimentary set of techniques including computer assisted docking studies, fluorescence anisotropy binding affinity studies, evolutionary conservation analyses and co-localization in the cellular context using confocal microscopy. THAHIT proves to be a versatile software tool to predict and specify existing, but more importantly, also novel AKAP-PKA anchoring domains and here we present the first comprehensive overview of all PKA-AKAP interaction domains and their putative specificity.

Materials and Methods

Development of THAHIT

The software tool was written in Python and makes use of the package Biopython 1.62.

Cell culture

HEK293 cells were sustained in Dulbecco's modified Eagle's medium (Lonza, Switzerland) containing 10% heat-inactivated fetal bovine serum (Lonza, Switzerland) and Glutamine (Lonza, Switzerland) in 35 mm glass bottom dishes (MatTek, USA). The dishes for imaging were coated with poly-D-lysine. Cells were grown to 75% confluence and transfected with the plasmids (WAVE2-GFP, WAVE2ΔC-GFP and PKA-RIIα-mCherry) using PolyFect according to the manufacturer's protocol for the specific cell type (QIAGEN, Germany).

Fluorescence imaging

Cells were washed once with ice-cold PBS, fixed in 4% ice-cold formaldehyde in PBS and afterwards washed twice with PBS. The confocal images were obtained with a Zeiss LSM700 confocal system with a 63x oil objective lens.

Statistics and Quantification

The two fluorescent profiles were compared by ImageJ 1.43 by plotting a line through the visible cell and then creating a plot profile. The profiles were compared, and an R^2 correlation coefficient was computed using MS Excel.

RT-PCR

Total RNAs of brain, skeletal muscle, ventricle, stomach, spleen, liver, kidney, lung, ovary and testicle were isolated from a female rat using TRIzol reagent (Invitrogen). Afterwards the RNAs were treated by DNaseI followed by addition of oligo(dT)12-VN (Promega) and Superscript II (Invitrogen). Finally, the PCR was completed using Taq polymerase (Invitrogen) and the appropriate primers (Eurogentec) for rat vlAKAP and rat GAPDH.

Protein and Peptide Synthesis

Seven peptides: (WAVE1 AA20-43) RGIKNELECVTNISLANIIRQLSS (vlAKAP AA1299-1322) CLLEDKARELVNEIIYVAQEKLRN (AKAP7γ AA294-317) AELVRLSKRLVENAV-LKAVQQYLE (Ezrin AA84-107) FYPEDVAEELIQDITQKLFFLQVK (AKAP10 AA629-652) EAQEELAWKIAKMIVSDIMQQAQY (RIAD) TVLEQYANQLADQIIKEATE and (super-AKAP-IS) QIEYVAKQIVDYAIHQA were synthesized and purified to ~95% (NKI, Netherlands). The peptides were N-terminally tagged with 5-TAMRA. Full-length PKA-RIα and PKA-RIIα dimers were purified as described previously(23).

Fluorescence Anisotropy

Measurements were carried out with the PHERAstar microplate reader (BMG LABTECH GmbH, Germany) using the FP module with excitation at 540 nm and emission at 590 nm. Flat bottom black 96-well plates (Thermo) were used. Each of the binding experiments was carried out four times after which they were put into the non-linear regression model of one-site saturated binding in GraphPad Prism 5.0.

Results

Foundations of the AKAP/amphipathic Helix Identification Tool THAHIT

To build the THAHIT, first a comprehensive list of all known human PKA-RIα and/or PKA-RIIα specific AKAPs was gathered(6, 9, 13-16, 24-57). Afterwards we accumulated the available data for all known PKA-RIα and/or PKA-RIIα binding regions of these AKAPs. We selected amphipathic helices based on stringent validation criteria using information in the literature to create a high confident amphipathic helix collection. Solely sequences confirmed by multiple biochemical assays (deletion, mutation, proline substitution etc.) were used for THAHIT. These sequences were manually aligned based on knowledge of crystal/NMR structures, peptide array and binding affinity data (Figure 1A/B). The alignments are color-coded and the binding motifs are clearly visible by the alternating yellow/red color representing the hydrophobic and hydrophilic amino acids. We first aligned all confirmed PKA-RII sequences to evaluate the appropriate parameters for the identification of PKA-

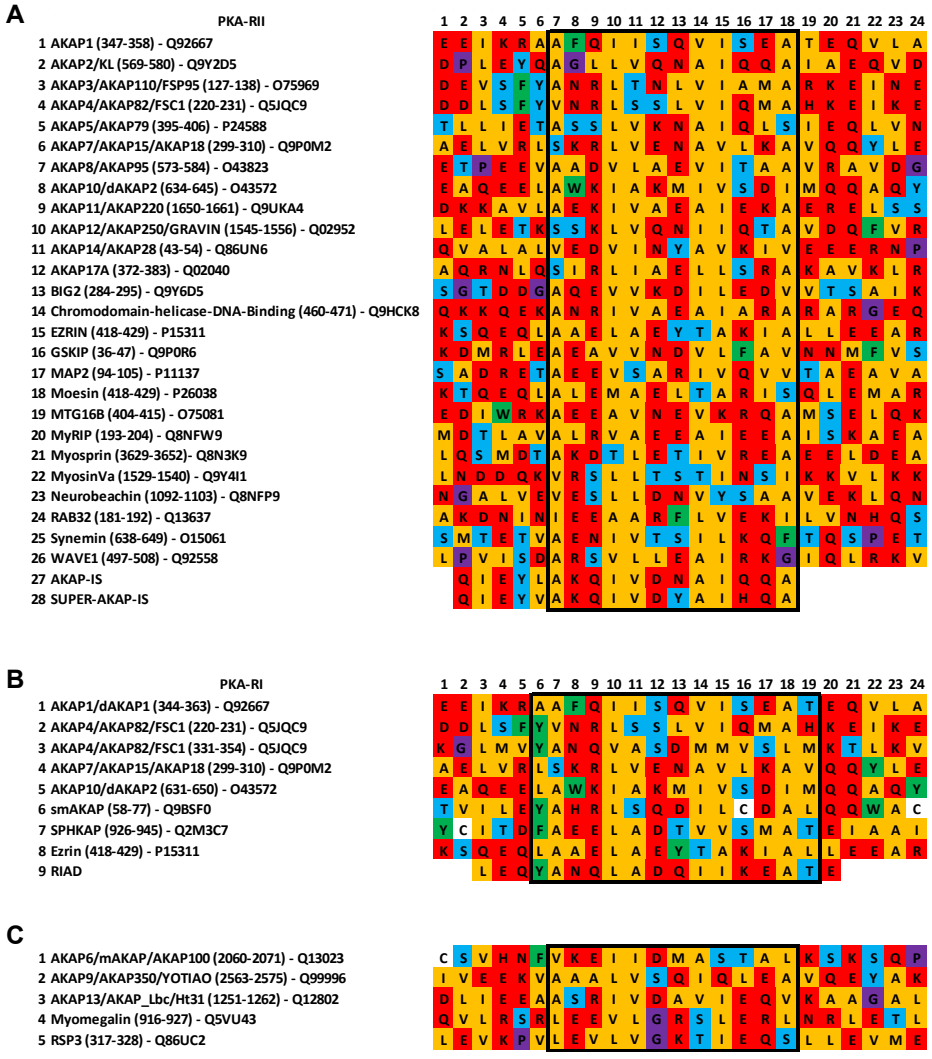

Figure 1 *Alignments of PKA-R Binding Domain Helices.*
Aligned in (A) are all known PKA-RIIα binding domains with substantial evidence for their genuineness. The residues are coded as follows: orange – aliphatic, red – polar, blue – S/T, green – bulky and purple – G/P. Aligned in (B) are all known PKA-RIα binding domains (exhibiting substantial evidence for their genuineness) with similar coding as described above. (C) THAHIT enables also the identification of binding domains in AKAPs that had not yet substantial evidence for their (non-)proposed PKA-R binding domain.

RII binding helices by THAHIT (Figure 1A). Previous AKAP motif search tools paid mostly attention to the hydrophobic side of the helix(16), but since more and more structural evidence also suggests a role for some of the hydrophilic residues in the interaction(22), here we incorporated sequence requirements for both sides of the amphipathic helix. The hydrophilic surface is important in that it is never a buried surface. Thus it is always in contact with water and therefore prefers polar residues. First of all the hydrophobic domain requires the following 12-residue motif: [A/V/L/I/S]-X-X-[A/V/L/I]-[A/V/L/I/S/T]-X-X-[A/V/

L/I/S/T]-[A/V/L/I]-X-X-[A/V/L/I/S]. Next the hydrophilic domain (X) requires a minimum of three X to consist of H/R/K/D/E/N/Q (residues colored red in sequence alignments) or 2 plus at least one S/T. For additional stringency, for the 12-residue motif a minimum helix propensity score of 85% was demanded(58). No proline residue is permitted within this entire motif or at least three residues downstream and at least one residue upstream of the motif as this heavily disturbs the helix propensity. The last parameter we adopted was a pI value range from 3.0 - 6.5, which was previously used in a bioinformatics motif search for novel AKAPs(16).

The PKA-RIα helix alignments to titrate out specific THAHIT parameters resulted in a longer 14-residue motif (Figure 1B), consisting of the following: [A/L/F/Y]-[A/L/I]-X-X-[L/V/I]-[A/V/I/S]-X-X-[A/V/I/T/M]-[A/V/L/I/M]-X-X-[A/L/I]-[V/L/T/M/H] where at least three X consist of H/R/K/D/E/N/Q or 2 plus at least one S/T/Y. The same proline, helix propensity and pI parameters are inferred. All the above-mentioned parameters were programmed into THAHIT to allow the evaluation of collections of sequences for the presence of putative amphipathic helices and to specify their preference for PKA-RI and/or PKA-RII. THAHIT was tested on various datasets and proved useful in many different ways as outlined in detail below.

Identification and verification of amphipathic helices on reported AKAPs

THAHIT was first used for the evaluation of all known human PKA-RIα and PKA-RIIα binding AKAPs reported in the literature, which were collected in a single FASTA-file. THAHIT subdivided the hits and aligned the PKA-RIα and PKA-RIIα binding helices. As expected, THAHIT identified and specified all amphipathic helices used as input to build the tool correctly. In addition, we could predict the exact binding sequences of AKAPs for which the biochemical validation did not meet the criteria stated above (Figure 1C). For instance, the exact amphipathic helices of myomegalin(46), myosinVa(47) and WAVE2(57) were for the first time pinpointed. Also, on several AKAPs reported in the literature without a specified helix, we were unable to define the likely helix motif (e.g. for pericentrin, α4-integrin and MyosinVIIa), suggesting these proteins may possibly interact with PKA via an alternative mechanism. All results were, manually scrutinized against the published data. We now aligned the amphipathic helix sequences of all reported AKAPs from literature and the ones identified/confirmed via THAHIT using pLogo based on binding preference. A highly conserved motif was created for both the PKA-RIα and PKA-RIIα binding domains (Figure 2A/B). The PKA-RIIα motif is much more defined, likely as there is a larger set of known PKA-RIIα binding AKAPs (Figure 2A). In the PKA-RII motif the central double hydrophobic residues stand out (position 10, 11 and 14, 15). A double hydrophobic residue seems less important at the flanking regions where alanine residues at position 7 and 18 seem to suffice. Besides these detailed motif elements we discovered a clear consensus of polar residues three residues upstream of Ala7 (position 4). Notably, 22 out of a total of 33 AKAP sequences have a polar residue here, and at the same distance downstream of Ala18 (position 21, 22/31). Amongst these, there seems to be a strong preference for glutamic acid (11/22 in both position 4 and 21). This was thus far not acknowledged; therefore we set out to investigate the nature of this additional hydrophilic region. We used molecular dock-

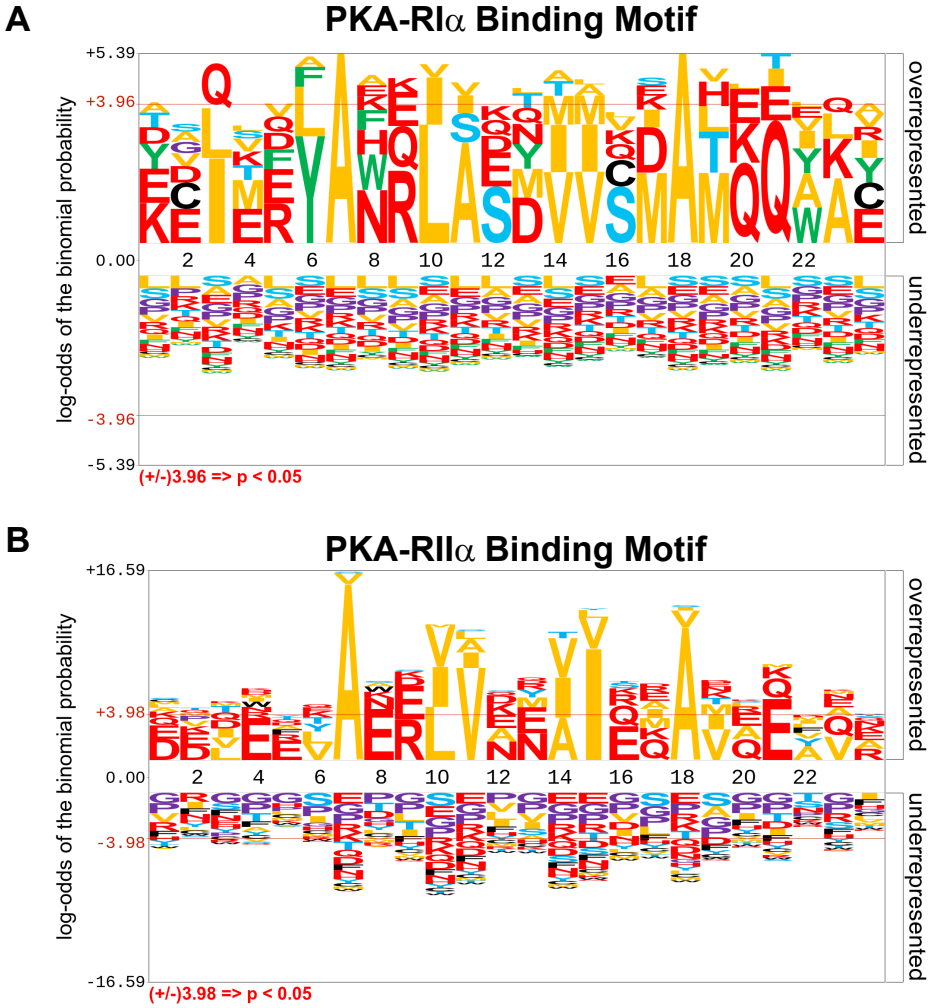

Figure 2 *Using all helix sequences depicted in Figure 1 a higher definition amphipathic helix motif was created using pLogo.*
Motifs of the PKA-RIIα and PKA-RIα binding amphipathic helices are depicted in (A) and (B) respectively.

ing studies with the HADDOCK software(59, 60) to elucidate the potential interactions of these polar residues. HADDOCK gives a score to a model based upon electrostatic energy, Van der Waals energy, desolvation energy and restraints violation energies. The crystal structures of PKA-RIIα's D/D-domain and the amphipathic helix of dAKAP2 revealed that Glu631 can form a hydrogen bridge with Arg22 of PKA-RIIα (Figure 3), supporting its conserved importance in the PKA-AKAP interface.

For several AKAPs, such as AKAP4(61), SPHKAP(14) and AKAP11(34), multiple PKA binding domains have been identified and these were all also found using THAHIT. We hypothesized that other AKAPs may also contain additional, currently overlooked, binding sites. In our analysis, THAHIT recognized in total 16 novel additional PKA-RIα and/or

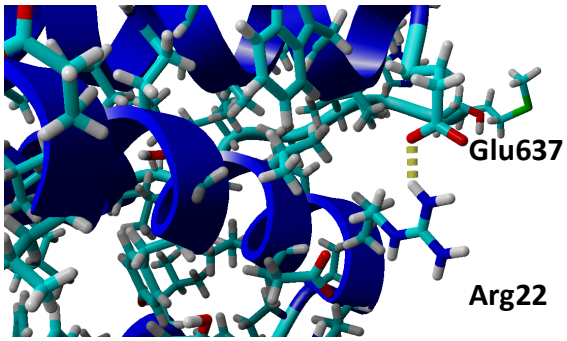

Figure 3 *A HADDOCK based docking structure of d-AKAP2 and PKA-RIIα.*

Shown is the interaction between the previously not annotated conserved polar residue at position 4 (Glu637) of the helix (see Figure 2) and Arg22 of PKA-RIIα which interact and thus play a role in the PKA-AKAP affinity.

Table 1 *A list of THAHIT's predicted additional PKA-RIα and PKA-RIIα binding domains for known* AKAPs.

	Region	PKA-RIα	PKA-RIIα
AKAP2	586-582	QAGLLVQNAIQQAI	
AKAP6	1624-1635		VGELSKRTLDLL
AKAP9	572-586	VAADVLAEVITAAV	
AKAP11	619-630		VSEALSNALKDL
AKAP11	689-700		AKDLSESVIQEA
AKAP12	1366-1377		SEEVSKQLLQTV
AKAP14	38-52	VALALVEDVINYAV	
Ezrin	88-102	VAELLIQDITQKLF	
MyRIP	192-206	VALRVAEEAIEEAI	
MyRIP	641-652		LCNISTEVLKVI
Neurobeachin	480-491		VKAIVTHSIHSA
Neurobeachin	843-854		ILKVVATLLKNS
Neurobeachin	2805-2816		VHTITGDLLRAL
Synemin	637-650	VAENIVTSILKQFT	
WAVE1	25-36		LECVTNISLANI
WAVE2	25-36		LECVTNITLANV
WAVE3	26-37		LECVTNSTLAAI

PKA-RIIα binding sites on 12 different AKAPs (Table 1). For instance, THAHIT suggested an additional PKA-RIα/PKA-RIIα binding domain at the N-terminus (AA84-107) of Ezrin (another dual-specific anchoring domain was already annotated via Co-IPs at the C-terminus AA412-435(42)). Also, an additional PKA-RII binding domain at the N-terminus (AA20-43) of WAVE1 and the homologues gene products WAVE2 (AA20-43) and WAVE3 (AA19-42) was observed. To confirm the validity of these new predicted PKA binding domains, biochemical validation experiments were performed as described in more detail below.

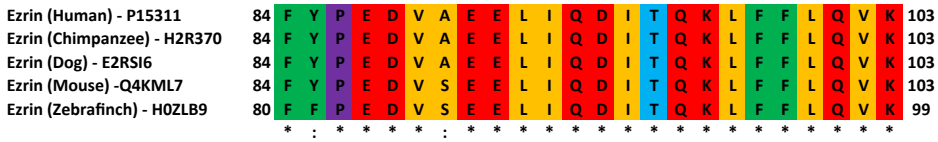

Figure 4 *Conservation of the putative PKA-RIα/PKA-RIIα binding domain of Ezrin (AA88-102) extracted by THAHIT. Using ClustalW the domain was aligned and showed 100% conservation in a wide range of species; human, chimpanzee, dog, mouse and zebrafinch.*

Ezrin

The novel PKA binding domain of Ezrin (AA84-107) as extracted from the sequence by THAHIT is 100% conserved from human to zebrafinch, according to BLASTp analyses (Figure 4). To further investigate this putative binding site we first used HADDOCK modeling using a set of crystal structures available for the PKA-RIα and PKA-RIIα D/D domain binding to several different AKAP amphipathic helix peptides(59, 60). By comparing the HADDOCK scores of different (putative) AKAP helices docking onto the two PKA-R structures we aimed to evaluate goodness of fit for the domains THAHIT predicted. We used several known models as internal calibrants. The affinities and PKA-R specificities of these are known and chosen such that they present various affinities towards PKA-RIα and RIIα: superAKAP-IS (PKA-RIIα specific), smAKAP (PKA-RIα specific) and AKAP10 (dual-specific) and AKAP7 (AA294-317, strong RII, modest RI binding). Scrambled peptides were used as negative controls. The final HADDOCK structures were qualitatively inspected based upon positioning and helicity. We found that the novel Ezrin (AA84-107) domain docked better onto PKA-RIα than AKAP7 and the scrambled peptide, but had a poorer HADDOCK score than RI-specific smAKAP and dual-specific AKAP10 (Table 2), suggesting it to also be a dual-specific site. Therefore we also performed binding predictions with the PKA-RIIα structure (Table 2). We observed a remarkable strong score for AKAP7, which was even better than the engineered RII specific sequence of superAKAP-IS. The new domain in Ezrin scored poorer than AKAP7 and AKAP10 but still had a better score than the scrambled peptide. To confirm these in silico results, we measured the actual affinities using fluorescence anisotropy binding studies with full-length recombinant PKA-RIα, PKA-RIIα and synthetic peptides of the different amphipathic helices tagged with a 5-TAMRA. The novel amphipathic helix of Ezrin had a K_d = 78 nM for PKA-RIα compared to AKAP7 having a K_d = 119 nM whereas AKAP10, smAKAP and RIAD presented with K_d = 9.4 nM, K_d = 4.3 nM and K_d = 2.0 nM, respectively (Table 3). This novel site also interacts with PKA-RIIα, reiterating it is a dual-specific site (K_d = 32 nM). This was a lower affinity than exhibited by the peptides originating from superAKAP-IS, AKAP7 and AKAP10 but still three orders better than the scrambled peptide (Table 3). This confirms THAHIT's prediction of THAHIT that Ezrin has

Table 2 *HADDOCK scores of various PKA-R binding domain peptides docking onto PKA-RIα and PKA-RIIα. The lower the score the stronger the interaction (a dash means not acquired).*

	PKA-RIα	PKA-RIIα
smAKAP(AA56-79)	-139.8 +/- 3.5	-
superAKAP-IS	-	-83.7 +/- 14.4
AKAP7γ (AA294-317)	-110.1 +/- 2.0	-91.3 +/- 3.0
AKAP10 (AA629-652)	-126.1 +/- 1.7	-82.8 +/- 1.1
Ezrin (AA84-107)	-120.7 +/- 8.0	-72.3 +/- 2.4
WAVE1/WAVE2 (AA20-43)	-	-85.4 +/- 4.5
***v*/AKAP (AA1299-1322)**	-	-82.5 +/- 1.7
Scrambled	-81.3 +/- 3.4	-54.7 +/- 4.8

Table 3 *Assessing PKA-AKAP interaction by AKAP mimicking peptides and fluorescence binding assays.*

The same set of PKA-R binding domain peptides from table 2 were synthesized with a 5-TAMRA tag attached. Using fluorescence anisotropy the binding affinities were measured between these peptides and PKA-RIα/PKA-RIIα. A dash means not acquired.

	PKA-RIα (nM)	PKA-RIIα (nM)
smAKAP(AA56-79)	4.3	-
superAKAP-IS	-	0.6
AKAP7γ (AA294-317)	119	0.6
AKAP10 (AA629-652)	9.4	4.3
Ezrin (AA84-107)	78	32
WAVE1/WAVE2 (AA20-43)	-	20
***v*/AKAP (AA1299-1322)**	>1000	0.7
Scrambled	>1000	>1000

not only a PKA-RIα/PKA-RIIα binding domain at the C-terminus but also an additional one at the N-terminus. Additionally it confirms that THAHIT has the ability to properly predict PKA-RIα binding motifs.

WAVE1/WAVE2

Interestingly, WAVE1 is a validated AKAP for which a PKA-RIIα binding domain has been annotated via deletion mapping and Co-IPs at the C-terminus (AA491-514)(56). WAVE1, WAVE2 and WAVE3 seem very homologous in this C-terminal region (Figure 5A), but a crucial difference is present within the amphipathic helix, where a crucial hydrophobic amino acid (V500) in WAVE1 is substituted by an aspartic acid (D439), which hampers PKA binding according to THAHIT. The original WAVE1 study provided experimental evidence (RII overlay and Co-IP with PKA-C) corroborating that the C-terminus of WAVE2 and WAVE3 do not have PKA-RII binding capabilities and therefore these two proteins were not considered as AKAPs. However, THAHIT also predicts a novel RII binding region at the N-terminus of WAVE1, 2 and 3 (Table 1, Figure 5B). THAHIT's suggested PKA-RIIα binding domains of WAVE1 and /WAVE2 are well conserved from human to zebrafinch, according to BLASTp analyses (Figure 5C). Using WAVE2 as a representative, we performed HADDOCK and fluorescence anisotropy studies. PKA-RIIα HADDOCK binding studies of the WAVE1/WAVE2 N-terminal putative amphipathic helix were again compared to the control set used for Ezrin. The WAVE1/WAVE2 peptide had a score slightly poorer than superAKAP-IS and a bit better than AKAP10 but a much better result than the scrambled peptide (Table 2). The actual affinities we acquired by fluorescence anisotropy reflected the modeling data perfectly; AKAP7 showed a binding affinity, K_d = 0.6 nM, as strong as superAKAP-IS (K_d = 0.6 nM) for PKA-RIIα. WAVE1/WAVE2 had a weaker binding affinity (K_d = 20 nM) than AKAP10 (K_d = 4.3 nM) (Table 3). As mentioned, WAVE2 was initially not thought to have a PKA-RIIα binding domain and therefore not to be an AKAP(56). To further strengthen WAVE2 is indeed an AKAP via this N-terminal region, a co-localization study using a full length WAVE2-GFP construct and a WAVE2 construct lacking the C-terminal amphipathic helix (WAVE2ΔC-GFP) were co-transfected with PKA-RIIα-mCherry. Confocal microscopy showed that the WAVE2ΔC-GFP which only contains the N-terminal amphipathic helix also acts as an AKAP by co-localizing with PKA-RIIα in situ (Figure 5D, E).

THAHIT Identifies a Novel AKAP; vlAKAP

To further assess the functionality of THAHIT we analyzed several previously published chemical proteomics datasets of cAMP pull-downs to illustrate the potential of the software tool to identify novel AKAPs(12, 62, 63). When screening the data used in the identification of smAKAP(12) in human platelets and heart tissue, THAHIT readily identified smAKAP with the appropriate prediction for PKA-RIα binding. When screening the identifications from other chemical proteomics experiments performed in rat and mouse heart(62, 63), in which we identified SPHKAP for the first time, we encountered an unknown protein

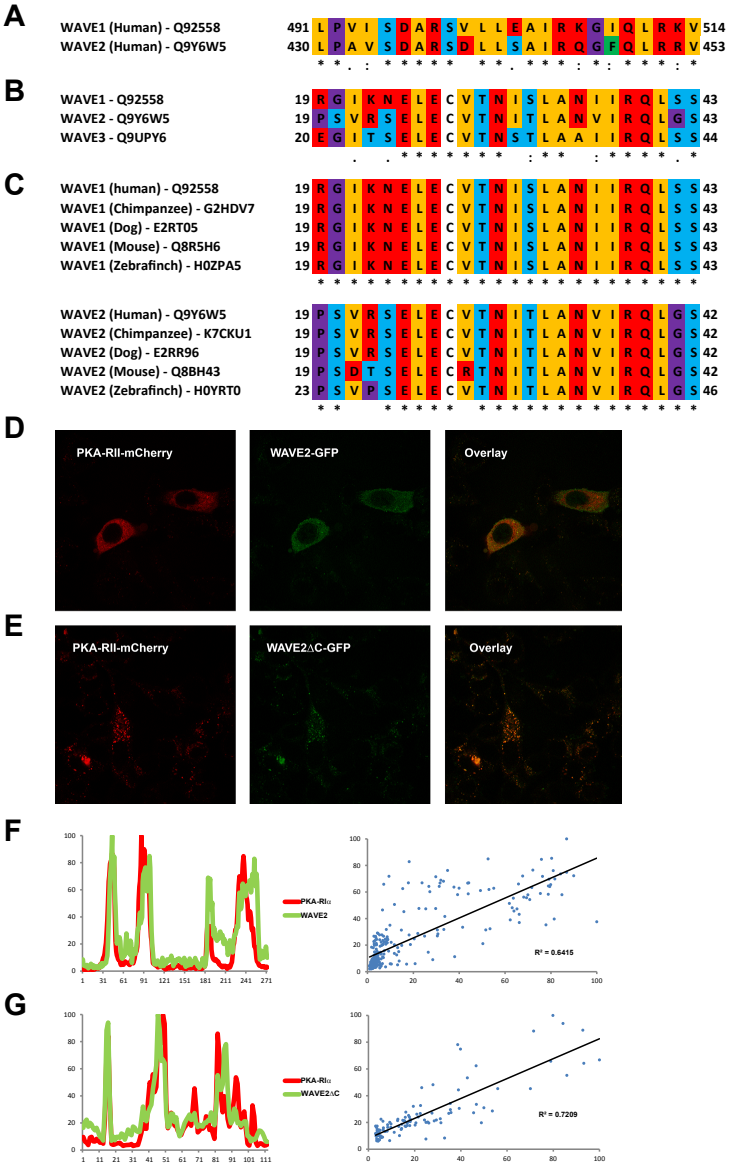

Figure 5 *Characterizing the WAVE PKA-RIIα N-terminus binding domain.*

(A) A sequence alignment of the known PKA-RIIα C-terminus binding domain of WAVE1 with its isoform WAVE2. (B) A sequence alignment of THAHIT's proposed N-terminal PKA-RIIα binding domains of WAVE1, WAVE2 and WAVE3. (C) Sequence conservation of the putative amphipathic N-terminal amphipathic helices of WAVE1 (AA25-36) and WAVE2 (AA25-36) using ClustalW over a wide range of species; human, chimpanzee, dog, mouse and zebrafinch. (D) PKA-RIIα-mCherry and full-length WAVE2-GFP are both expressed in HEK293 cells. Confocal imaging shows the N-terminus of WAVE2 co-localizing with PKA-RIIα. (E) Additionally, as suggested by THAHIT, HEK293 cells expressing PKA-RIIα-mCherry and WAVE2ΔC-GFP show both proteins co-localizing as well. For both (D) and (E) a line plot profile and correlation analysis of co-localization was performed ((F) and (G), respectively). The plot profiles display the difference in fluorescence intensity along a line taken through the cell. Scatter plots (far right) show the pixel distribution with which the R^2 was calculated.

(IPI00370756, Human accession - XP_005247174.1) with a molecular weight of 330 kDa. It contained a possible PKA-RIIα interacting helix between AA1299-1322. We termed this protein *vl*AKAP, for very large AKAP. Like with the other AKAPs, the helix is very well conserved amongst species (Figure 6A). To confirm that this full-length protein exists and not just the first 1365 residues as suggested in UniProt (B4DLE8), we matched 14 peptide spectra (of the chemical proteomics datasets) spread along the entire rat protein (Figure 6B). These peptides were all identified in the same gel band in the higher molecular weight region around the 260 kDa marker. To investigate whether *vl*AKAP has more conserved domains, we performed a BLAST analysis. First we checked for putative conserved domains in both the human and rat sequences. These were only found at the very C-terminus (Figure 6B, C). Between AA2043-2092, AA2138-2176, AA2188-2232, AA2233-2275, AA2281-2322, AA2323-2365, AA2376-2412, AA2413-2455 and AA2502-2543 there are beta/gamma crystallin domains for the human whereas the rat showed only five of such domains at the N-terminus. Further downstream, at the very C-terminus between AA2575-2677, there is a ricin B-type lectin domain. The former domains were first only recognized as the major constituents of the vertebrate eye lens(64). The ricin domain is described as a carbohydrate

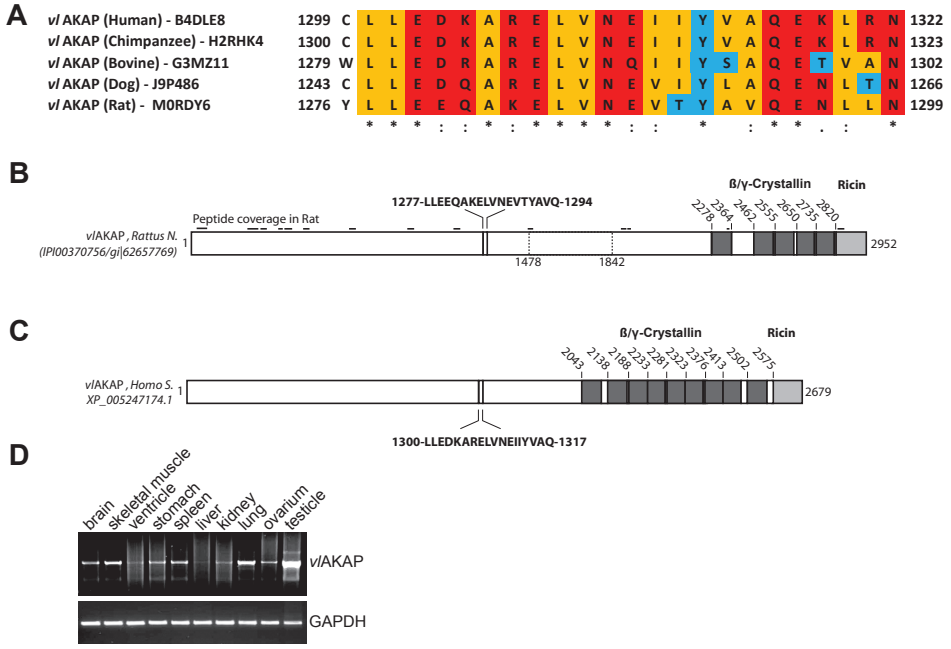

Figure 6 *Characterizing the novel AKAP, vlAKAP, discovered via assessing chemical proteomic datasets using THAHIT.*

(A) An aligment of human/chimpanzee/bovine/dog/rat vlAKAP PKA-RIIα binding domain sequences. (B) Rat vlAKAP, pulled down from rat ventricular tissue in a previous study1 with the putative PKA-RII binding amphipathic helix depicted (AA1277-1294). vlAKAP contains several ß/γ crystallin domains (dark grey) at the C-terminus, as well as a ricin domain depicted in light grey. At the top, the 14 identified unique peptides are depicted at their proper location in the sequence, corroborating with the size of vlAKAP. (C) Human vlAKAP with the putative amphipathic helix depicted contains (D) Semi-quantitive RT-PCR analysis of vlAKAP in several tissues and organs of adult rats, based on amplification of a C terminal 1335 base pair fragment. Representative results of three independent experiments are shown. GAPDH was used as control.

binding domain. The expression of *vl*AKAP was tested across various rat tissues by means of mRNA RT-PCR. It was ubiquitously expressed with the largest expression in testicles, lungs and skeletal muscle (Figure 6D). Again, we applied HADDOCK and fluorescence anisotropy studies to confirm this domain further (AA1299-1322). The HADDOCK results suggested a very strong binding affinity (Score = -82.5 +/- 1.7) comparing very well with

other PKA-RII binding AKAPs (Table 2). The in vitro binding affinity studies further confirm this site with a K_d = 0.7 nM for binding to PKA-RIIα, close to the value of the strongest RII binding AKAPs superAKAP-IS and AKAP7 (Table 3). Thus via THAHIT's screening of the chemical proteomics datasets and additional verification using conservation analysis, docking and in vitro binding studies we identified a novel PKA-RIIα binding AKAP: *vl*AKAP.

Discussion

Here, based on available protein-interaction data we developed a bioinformatics tool termed "THe AKAP/amphipathic Helix Identification Tool" (THAHIT), to characterize the wide repertoire of proteins that interact with the regulatory subunits of protein kinase A. We show that THAHIT can identify on protein sequences PKA-RIα and PKA-RIIα binding domains, as illustrated by the discovery of new functional helices on WAVE1/WAVE2 and Ezrin. In 2000 when WAVE1 was first identified as an AKAP via Co-IPs, it was shown that WAVE2 (and WAVE3 as well) did not co-precipitate with PKA and that only the C-terminus of WAVE1 pulled down PKA-RIIα(56). However, recently another study was published which performed similar Co-IPs and did show co-precipitation of WAVE2 and PKA-RIIα(57). This study did indeed find the N-terminus of WAVE2 binding PKA as well but they did not further specify the responsible helix. Interestingly, a possible explanation for the differing Co-IP results is the following: there is a crystal structure of the Actin Regulatory WAVE Complex (PDB: 3P8C) which includes the N-terminal PKA-RIIα binding domain suggested here(65). In the crystal structure the PKA-binding amphipathic helix is bound to the Abl interactor 2. These data suggest that there are varying cellular conditions which prohibit or allow WAVE1 and WAVE2 to pull down PKA due to overlapping binding domains, thus displaying one of the cons of Co-IPs: false negatives. However, based upon these findings we confirm WAVE1 and WAVE2 to have a PKA-RIIα binding domain at the N-terminus (AA26-37 and AA25-36, respectively). We suggest WAVE3 to contain a PKA-RIIα binding domain as well, although its amphipathic helix is not as good of a fit based on HADDOCK as WAVE1 and WAVE2 (Figure 5A, Table 2).

THAHIT did not assign a fitting amphipathic helix to every AKAP. This was expected for AKAPs α4 integrin(39) and pericentrin(40). In the discovery manuscripts, pericentrin is described to require a domain stretching 100 residues to bind to PKA-RIIα, whereas α4 integrin binds directly to PKA-C and pulls down PKA-RIα via the catalytic subunit(39, 40). One may question if these AKAPs can be considered canonical, or should maybe be considered as subclasses, as it seems that the PKA-R binding motif is much conserved throughout other AKAPs. THAHIT was also unable to detect a suitable interaction helix on MyosinVIIa.

This AKAP was discovered via Co-IPs and its putative PKA binding domain was narrowed down to the last 209 AA of the FERM domain(48), but even after manual inspection, we could not identify a suitable amphipathic helix motif in this domain. This suggests that MyosinVIIa may not be a direct interactor of PKA-RIIα and therefore not an AKAP. Further studies should shed light on this. One possible explanation may be MyRIP, a documented AKAP(50) with a PKA-RIIα binding domain as also found via THAHIT. MyRIP is reported to bind MyosinVIIa via the FERM domain and may therefore provide the link between PKA-RIIα and MyosinVIIa to explain the co-immunoprecipitation results(66).

These examples illustrate the necessity to narrowly define the PKA binding domain. Co-localization studies or IPs provide a good initial attempt to find out if the proteins co-localize in the cellular context, but these do not distinguish between direct or indirect interactions. To evaluate this, one should perform, for instance, binding affinity studies using fluorescence anisotropy(12), surface plasmon resonance(38, 67) or isothermal calorimetry (ideally using full length PKA-R). This should be performed with well-recognized PKA-R binding domains as controls. Another method to confirm the binding domain is using a peptide array (here there is no secondary interaction possible), as done with MyRIP for instance(50), although the 3-dimensional structure of the AKAP is likely compromised. Ideally, a complementary set of methods is used to provide sufficient evidence for the PKA-R/AKAP interaction under study.

To conclude, THAHIT helped us create a greater understanding and clearer picture of the PKA binding motifs of AKAPs and we could imply additional hydrophilic interactions as novel important binding parameters. Here we propose a list of new PKA-RIα and PKA-RIIα binding domains for existing AKAPs suggesting that many AKAPs anchor more than one PKA enzyme. The exact nature of this curious observation is of high interest. THAHIT also allowed us to discover a novel AKAP: *vl*AKAP, while at the same time it also led to questioning some previously published AKAPs and their domains as this was not unambiguously defined for each AKAP. This should instigate one to precisely determine the interaction surface between the PKA-R and AKAP in question via the suggested methods.

Acknowledgements

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Chapter 5

Chapter 6

Summary and Outlook

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Samenvatting en Vooruitzicht

Summary

In **chapter one** there is a general introduction regarding the communication between cells in our body which consists of a very structured and complicated chemical network. To streamline this network, there is a group of proteins called A-kinase anchoring proteins (AKAPs) which function as signalling hubs for the action of the omnipotent cAMP-dependent protein kinase (PKA). AKAPs sequester themselves, along with PKA and a variable collection of other signalling proteins, such as kinases, phosphatases and phosphodiesterases to various locations in a cell. This allows spatial and temporal regulation of cAMP-signaling.

Since 1984, when the first AKAP (MAP2) was discovered, more than forty AKAPs have been identified. Much research has been done concerning this group of proteins, from structural studies to the effect of genetic variations in human AKAP genes. Here we focus on the discovery of novel AKAPs and characterization of their structure/function relationships.

PKA is a heterotetrameric protein consisting of a regulatory subunit (PKA-R) dimer and a catalytic subunit (PKA-C) attached to each PKA-R. Upon binding of the messenger cAMP to PKA-R, the catalytic subunit is released which can then phosphorylate its targets. PKA-R exists in four genetically distinct isoforms: Iα, Iβ, IIα and IIβ.

Numerous studies have been published in the last three decades regarding the structural interactions between AKAPs and PKA-R. In **chapter two** we have carefully tracked the progression of this particular aspect of cAMP-signaling. The PKA-R dimer interface is formed via a domain at its N-terminus named the docking and dimerization domain (D/D). From NMR and crystal structure data we know it forms an X-fold shape of helices, also termed a four-helix bundle, with its surface featuring a hydrophobic groove. The PKA-RIα isoform has a larger surface than PKA-RIIα due to the extension of the D/D domain with two disulfide bridges at each end. This keeps it very rigid in comparison to PKA-RIIα which displays a more loose N-terminus with more flexibility. Most likely due to the more stringent binding restrictions found for PKA-RI so far there are currently many more PKA-RII binding AKAPs known. Dual-specific AKAPs have also been described. These bind either PKA-R, albeit with different affinities. At the other end of the interaction, each AKAP contains at least one amphipathic helix (one side of the helix is hydrophobic whereas the other is hydrophilic) to dock onto the PKA-R dimer interface. Specific residues in this helix determine the specificity.

Novel AKAPs are discovered via various methods. One of the recently best established and most effective is chemical proteomics. Using cAMP-functionalized beads, PKA-R can be enriched along with secondary interactors such as AKAPs. These PKA-AKAP enriched fractions are then characterized via mass spectrometry. In **chapter three** we apply this method to heart tissue and freshly isolated platelets in an effort to find novel, unknown AKAPs. An until then theoretical protein (Q9BSF0) was pulled down and we confirm this to be a novel AKAP. We named it small membrane AKAP (smAKAP) as it is one of the smallest AKAPs (95 AA) and locates itself primarily to peripheral membranes. Myristoylation and

palmitoylation at Gly2 and Cys3, respectively, are shown to be responsible for this localization. smAKAP is ubiquitously expressed and its PKA-R binding domain is well conserved. Interestingly, this domain proved to be entirely PKA-RI specific, only the second of its kind.

In **chapter four**, we utilize smAKAPs unique PKA-RI specificity to extend the knowledge reviewed in chapter two with a novel crystal structure of smAKAPs amphipathic helix bound to the D/D domain of PKA-RIα. We identify several additional residues which enhance the interaction of PKA-RI specific amphipathic helices. For example, the two disulfide bridges present on the edges of the PKA-RIα dimeric interface are in very close proximity of smAKAP's hydrophobic residues just outside either end of the previously established PKA-RIα binding motif. This creates an additional hydrophobic niche for the specific, high affinity interaction with the PKA-RIαD/D domain.

Interestingly, we found a PKA phosphorylation motif around Ser66, exactly in the middle of smAKAP's PKA binding domain. Via various experiments we confirm that PKA-C phosphorylates Ser66. To investigate the function of this "auto"-phosphorylation, we performed HADDOCK docking studies with our new crystal structure. These suggested pSer66 to stick out of the hydrophobic groove and induce hydrogen bridges with polar residues of PKA-RIα, thereby creating an even stronger binding AKAP. Interestingly, in vitro and in vivo binding studies proved the opposite, i.e. Ser66 phosphorylation by PKA blocks the interaction, and also the localization. We initially hypothesized that phosphorylation of Ser66 creates a large polar residue on the hydrophobic surface of the amphipathic helix of smAKAP and thereby blocks binding to PKA. Further structural confirmation revealed a helix disrupting mechanism to be responsible. These data suggest a novel regulatory mechanism in local cAMP signaling in which PKA inhibits it's own binding, and thus localization, via phosphorylation smAKAP on the amphipathic helix.

The conservation of PKA-binding amphipathic helices between different AKAP families is relatively poor and this has hampered us to unambiguously identify all existing AKAPs by sequence homology. The increased structural understanding of PKA-RIα's and PKA-RIIα's with their own repertoire of AKAPs (**chapters two and four**) inspired us to develop a bioinformatic tool to more accurately recognize PKA binding motifs in AKAPs. The tool shown in **chapter five** was built based on knowledge of currently known and well established PKA-RIα and PKA-RIIα binding motifs. Previously, motifs were searched solely on the hydrophobic residues and pI of the binding domain. Here we have evaluated and incorporated additional paramaters including restraints for the hydrophilic domain, helix propensity score and a pI score. In a first test, this new software tool, THe Akap/amphipathic Helix Identification Tool (THAHIT), has identified every PKA-R binding domain known in the current literature. Not only that, but it has also predicted additional binding motifs in known AKAPs. It is already known that several AKAPs, such as SKIP, have multiple PKA binding domains so why should other known AKAPs not have them either? Confirmatory experiments on a couple of THAHIT's predictions, a PKA-RIα/PKA-RIIα binding domain on the N-terminus of Ezrin and a PKA-RIIα binding domain on the N-terminus of WAVE1 and WAVE2, were performed to see how accurate THAHIT is. Initially it was shown that these sites are very well conserved. Next, HADDOCK docking studies, fluorescence anisot-

ropy binding affinity studies and co-localization studies using confocal microscopy showed that these newly identified AKAP-domains bind very well, proving that THAHIT is very effective.

When applied to existing chemical proteomics data, THAHIT identified a putative novel AKAP: very large AKAP (*vl*AKAP). The same confirmatory techniques were applied to this AKAP to further confirm it being a real AKAP. Interestingly *vl*AKAP is one of the strongest PKA-RIIα binding AKAPs known today.

Outlook

Many different signaling mechanisms control the various corresponding pathways in cells and thus, ultimately, our body. One of these is the self-inhibiting phosphorylation mechanism which we proposed in **chapter four**. The docking of PKA onto smAKAP is blocked by PKA phosphorylating the binding site on smAKAP. This is the immediate effect of the mechanism but what influence does this have on the entire pathway it is involved in? In addition, many AKAPs contain serines, threonines or tyrosines in their PKA binding helices, suggesting this to be a more wide-spread mechanism, likely also mediated by other kinases than PKA. It would be interesting to target various other AKAPs (such as AKAP4 and AKAP11), for which large-scale proteomics experiments have uncovered confirmed phosphorylatable residues in their PKA binding domain. Here we have witnessed the phosphorylation event and studied its initial function. Via further biophysical, biochemical and cell biological experiments via for instance surface plasmon resonance, phosphomimetics and mutagenesis, the exact function and dynamics of the mechanism can be monitored in much more detail.

Not all AKAPs have been discovered yet. In this thesis two novel AKAPs have been revealed via chemical proteomics, bioinformatics and various other techniques. Most of the recent novel AKAPs have been found by means of pull-downs. However, a major issue with this technique is that some AKAPs may not be covered. For instance due to their strong attachment to more insoluble membrane-rich regions within the cell, or they occur at such a low expression level that it is currently impossible to detect them, or their interaction with PKA may be more transient. One of the top ten hits we found using THAHIT was the PKA binding motif of *vl*AKAP (**chapter five**). Incidentally we retrieved it from rat tissue via chemical proteomics as well. This does suggest that quite likely some of these other top hits could be an AKAP as well. Using the proposed methods in **chapter five** these hits should be screened in order to verify if these are indeed AKAPs. Additionally, this will lead to more insight on the PKA binding motif and can therefore improve the method for predicting the site as well.

In all, we discovered two novel AKAPs: smAKAP and *vl*AKAP. Not only that, but we created a list of proteins which are very promising PKA-RIα and/or PKA-RIIα binding AKAPs. This was done using the tool THAHIT which is based upon structural data garnered from previous structural studies and the crystal structure of smAKAP bound to PKA-RIα which we solved. Finally, we elucidated a novel inhibitory mechanism which prevents PKA from

docking onto certain AKAPs due to a phosphorylation in the PKA binding domain.

Samenvatting

In **hoofdstuk een** is er een korte samenvatting over de communicatie die tussen cellen in ons lichaam wordt mogelijk gemaakt door zeer gestructureerde en gecompliceerde chemische netwerken. Om dit netwerk gestroomlijnd te krijgen, is er een groep "anker"-eiwitten, genaamd A-kinase anchoring proteins (AKAPs), die functioneren als dataknooppunten voor de actie van het eiwit cAMP-dependent protein kinase (PKA). AKAPs positioneren zichzelf, samen met PKA en een variabele collectie signaal eiwitten, als kinases, phosphatases en phosphodiesterases naar diverse plekken in de cel. Deze ankereiwitten zorgen ervoor dat signaaleiwitten op hun locatie een tijdelijke verbinding met hen aangaan.

Sinds in 1984 de eerste AKAP (MAP2) werd ontdekt, zijn er meer dan veertig AKAPs geïdentificeerd. Sindsdien is veel onderzoek gedaan naar deze groep eiwitten, van structurele studies tot het effect van genetische variaties in de humane AKAP genen. In deze studies hebben wij ons geconcentreerd op de ontdekking van nieuwe AKAPs, de beschrijving van hun structuur en functionele relaties.

PKA is een heterotetramerisch eiwit dat bestaat uit een regulerende subeenheid (PKA-R) (dimeer) en een katalytische subeenheid (PKA-C). Als de boodschapper cAMP zich bindt aan PKA-R komt de katalytische subeenheid PKA-C vrij zodat die zijn doelen kan fosforyleren. PKA-R heeft vier genetisch verschillende isoformen: Iα, Iβ, IIα en IIβ.

De afgelopen drie decennia zijn talrijke studies gepubliceerd over de structurele interacties tussen AKAPs en PKA-R. In **hoofdstuk twee** is de progressie hiervan beschreven. De PKA-R dimeer koppeling wordt gevormd door een domein aan de N-terminus genaamd het docking and dimerization domein (D/D). Gebaseerd op NMR-en kristalstructuur data weten we dat het een X-fold model (bestaande uit α-helices) is met een oppervlak waarin zich een zogenoemde hydrophobische spleet bevindt. De PKA-RIα isoform heeft een groter oppervlak dan PKA-RIIα vanwege de verlenging van het D/D domein via de disulfidebruggen aan elk uiteinde. Dit zorgt ervoor dat de PKA-RIα heel erg stug is in vergelijking met PKA-RIIα dat een veel flexibelere N-terminus heeft. Tot nu toe zijn veel meer AKAPs bekend die PKA-RII binden dan er AKAPs zijn die verbindingen aangaan met PKA-RI. Dat wordt zeer waarschijnlijk veroorzaakt door de striktere voorwaarden voor het vormen van verbindingen met PKA-RI.

Dual-specific AKAPs, die zowel PKA-RI als PKA-RII kunnen binden, zij het met verschillende voorkeuren, zijn ook beschreven. Als we kijken naar de andere kant van de koppeling, is het zo dat elke AKAP tenminste een amphipathische helix bevat (een zijde van de helix is hydrophobisch terwijl de andere zijde hydrophilisch is) die op de PKA-R dimeer interface aansluit. Aan de hydrophobische zijde kunnen zich verschillende aminozuren bevinden die daarmee de specificiteit van de AKAPs bepalen.

Nieuwe AKAPs worden ontdekt via verschillende methoden. Tot de meest recente behoort chemical proteomics dat zeer effectief is. Door middel van cAMP-gefunctionaliseerde bolletjes kan PKA-R worden verrijkt samen met secundaire interactoren als AKAPs. Deze PKA-AKAP verrijkte fracties worden dan gekarakteriseerd door middel van massaspectro-

metrie. In **hoofdstuk drie** werd deze methode toegepast op hartweefsel en vers geisoleerde bloedplaatjes in een poging nieuwe AKAPs te ontdekken. Een tot dusver theoretisch eiwit (Q9BSF0) was op die manier verrijkt dat door bevestiging door andere experimenten inderdaad een nieuwe AKAP bleek te zijn. We noemden het small membrane AKAP (smAKAP) omdat het een van de kleinste AKAPs is (95 aminozuren lang) en zich vooral aan het membraan hecht. smAKAP is een in elk weefsel aanwezig eiwit waarvan het PKA-R binding domein zeer goed geconserveerd is. Het is bewezen dat dit domein geheel PKA-RI specific is, alleen maar de tweede tot nu toe.

In **hoofdstuk vier** werd smAKAP's unieke PKA-RI specificiteit gebruikt om de kennis die we in **hoofdstuk twee** bespraken te vergroten via een nieuwe kristalstructuur van smAKAP's amphipathische helix gebonden aan het D/D domein van PKA-RIα. We identificeren enkele aanvullende aminozuren die de interactie van PKA-RI specifieke amphipathische helices verhogen. Bijvoorbeeld, de twee disulfide bruggen die zich aan de uiteinden van de PKA-RIα dimerische oppervlakte bevinden zijn vlakbij smAKAP's hydrophobische aminozuren die zich ietsjes buiten elk uiteinde van het eerder beschreven PKA-RI binding motief bevinden. Dit creëert een extra hydrophobische nis die zorgt voor de specifieke, hoge affiniteitinteractie met het PKA-RIαD/D domein.

Nu bevindt zich ook een PKA-fosforylatiemotief rond het aminozuur serine dat zich exact in het midden van smAKAP's PKA binding domein bevindt. Via verschillende experimenten bevestigen we dat PKA-C die serine fosforyleert. Om de functie van deze 'auto'-fosforylatie na te gaan hebben we met de nieuwe kristalstructuur en de bio-informatische tool HADDOCK onderzoek gedaan naar binding tussen PKA-RIα en smAKAP. Deze studies suggereerden dat de gefosforyliseerde serine uit de hydrofobische spleet steekt en waterstofbruggen vormt met de polaire aminozuren van PKA-RIα. De veronderstelling was dat dit een nog sterkere binding zou bevorderen. Maar, in vitro en in vivo bindingstudies toonden het exact tegenovergestelde aan; dat de fosforylatie van deze serine via PKA de interactie blokkeert en dus ook het lokaliseren van PKA. Aanvankelijk veronderstelden we dat de fosforylatie van deze serine een groot polair aminozuur aan de hydrofobische zijde van de amphipathische helix van smAKAP vormde en daardoor de binding van PKA blokkeerde. Nader structureel onderzoek toonde aan dat een gefosforyleerde serine de vorming van een α-helix verstoort. De data suggereerden dat een nieuw regulerend mechanisme in lokale cAMP signalering zorgt voor het zelfblokkeren van het binden van PKA aan de amphipathische helix door middel van de fosforylering van smAKAP via PKA.

De conservering van PKA-bindende amphipathische helixen tussen verschillende AKAP families is relatief slecht en dit belemmerde ons om ondubbelzinnig alle bestaande AKAPs te identificeren door middel van sequentiehomologie. Het toegenomen structurele begrip van PKA-RIα's en PKA-RIIα's met hun repertoire aan AKAPs (**hoofdstukken twee en vier**) inspireerde ons om een bio-informatische tool te ontwikkelen die zeer nauwkeurig het PKA binding motief kan herkennen. Deze tool in **hoofdstuk vijf** is gebaseerd op de momenteel bekende en gevestigde PKA-RIα- en PKA-RIIα-binding-motieven. Eerder werden motieven alleen maar gevonden via de hydrofobische aminozuren en het iso-elektrisch punt van het bindings domein. Hieraan hebben we meerdere parameters toegevoegd, zoals de heliciteit

en de hydrofilische aminozuren. In een eerste test heeft deze nieuwe bio-informatische tool, 'The Akap/amphipathic Helix Identification Tool' (THAHIT), alle PKA-R-binding- domeinen geïdentificeerd die tot nu toe bekend zijn in de literatuur. Daarnaast heeft het ook aanvullende binding-domeinen in bekende AKAPs geïdentificeerd. Het is al bekend dat verschillende AKAPs (bijvoorbeeld SKIP) meerdere PKA-binding-domeinen hebben. Wij stelden ons de vraag waarom er niet nog meer AKAPs met meerdere van dat soort bindingen zouden zijn. Bevestigende experimenten op enkele van THAHIT's voorspellingen (een PKA-RIα/PKA-RIIα- binding-domein aan de N-terminus van Ezrin en een PKA-RIIα-binding-domein aan de N-terminus van WAVE1 en WAVE2) werden uitgevoerd om te zien hoe nauwkeurig THAHIT is. Eerst werd gekeken naar de conservering van deze domeinen. Vervolgens werden HADDOCK-binding-studies, fluorescence anisotropy bindingaffiniteit-studies en co-localisatiestudies door middel van confocale microscopie uitgevoerd. Deze toonden aan dat deze nieuw geïdentificeerde AKAP domeinen heel goed binden en dus bevestigen dat THAHIT zeer effectief is.

Toen THAHIT werd toegepast op bestaande chemical proteomics-data heeft het een potentiële nieuwe AKAP geïdentificeerd: very large AKAP (*vl*AKAP). Dezelfde bevestigende technieken werden toegepast op deze AKAP om te bevestigen dat het een echte AKAP betrof. Het bleek dat *vl*AKAP behoort tot de sterkst bindende PKA-RIIα-AKAPs die tot nu toe bekend zijn.

Vooruitzicht

Veel verschillende signaleringsmechanismes controleren verschillende corresponderende chemische netwerken in cellen en dus, uiteindelijk, ons lichaam. Een van deze is het zelf onderdrukkende fosforylatie-mechanisme dat we voorstelden in **hoofdstuk vier**. De binding van PKA op smAKAP is geblokkeerd doordat PKA het binding domein fosforyleert. Dit is het onmiddellijke effect van het mechanisme. Welke invloed heeft dit echter op het gehele chemische netwerk waarmee het in verbinding staat? Niet alleen smAKAP heeft een serine in het bindingdomein maar meerdere AKAPs hebben die ook. Dit suggereert dat het een wijd verspreid mechanisme is en niet alleen via PKA maar ook andere kinases. Het zou interessant zijn om andere AKAPs (zoals AKAP4 en AKAP11) te bekijken. Van deze is het namelijk al zeker dat de serine in het bindingdomein gefosforyleerd is. Dit is aangetoond door middel van massaspectrometrie. Hier waren we getuige van de fosforylatiemoment en hebben we de initiële functie ervan bestudeerd. Door verdere biofysische, biochemische en celbiologische experimenten (bij voorbeeld surface Plasmon resonance, phosphomimetics en mutagenesis) kunnen de exacte functie en dynamica van deze mechanismen tot in detail worden bekeken.

Niet elke AKAP is tot nu toe ontdekt. In dit proefschrift zijn twee nieuwe AKAPs ontdekt via chemical proteomics, bioinformatica en diverse andere technieken. De meeste van de recent ontdekte geïdentificeerde AKAPs zijn echter op andere wijzen gevonden, bijvoorbeeld door zogenoemde pull-downs. Een groot probleem met deze techniek is echter dat sommige AKAPs niet worden ontdekt. Dit komt bijvoorbeeld doordat ze te sterk binden aan onoplosbare membraanrijke regio's in de cel, vanwege geringe aanwezigheid zodat

het onmogelijk is om ze te detecteren of omdat hun interactie met PKA te snel verloren gaat. Een van de top tien hits die we vonden via THAHIT was het PKA binding motief van *vl*AKAP (**hoofdstuk vijf**). Toevallig hebben we *vl*AKAP via chemical proteomics ook in ratweefsel gevonden. Dit suggereert dat het zeer waarschijnlijk is dat andere top hits ook een AKAP zijn. Door middel van de voorgestelde methoden in **hoofdstuk vijf** moeten deze hits ook gescreend worden om te verifiëren of ze ook echt AKAPs zijn. Dit zou ook meer inzicht geven op het PKA-binding-motief en kan daardoor dus ook de methode voor het voorspellen van dit motief verbeteren.

De conclusie is dat we twee nieuwe AKAPs hebben ontdekt: smAKAP en *vl*AKAP. Niet alleen dat, maar we hebben ook een lijst van eiwitten die zeer veelbelovende PKA-RIα- en/of PKA-RIIα-bindende AKAPs zijn. Dit werd gedaan via de tool THAHIT die gebaseerd is op structurele data van vorige studies en de kristalstructuur van smAKAP gebonden aan PKA-RIα die wij hebben opgelost. Tenslotte hebben we een nieuw inhibitory mechanisme ontdekt dat voorkomt dat PKA zich bindt aan bepaalde AKAPs vanwege een fosforylatie in het PKA-binding-domein.

Chapter 7

Curriculum Vitae

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Acknowledgements

Curriculum Vitae

Pepijn Burgers was born on November 30th, 1986, in Amersfoort, the Netherlands. He obtained his high school diploma in 2003 from Fredericton High School in Fredericton, Canada. After this he attended the University of New Brunswick, Canada where he started off with a major in mathematics. However, after a year he switched to the combined program Biology/Chemistry. In 2007 Pepijn moved back to the Netherlands to broaden his scientific background by starting the master Drug Innovation at Utrecht University, the Netherlands. During his first master project at the department of Pharmaceutics, under supervision of Dr. Ed Moret, he studied how a nanovesicle consisting of peptides stayed intact. Applying molecular dynamics simulations to the nanovesicles and using self-written analysis tools coded in the Python programming language he found that water bridges between the peptides assisted in keeping the nanovesicle intact. In the final year of his master Pepijn attempted to find the structural interaction between the Protein Kinase A (PKA) catalytic subunit and an inhibitor using crystallography in the laboratory of Prof. Dr. Susan S. Taylor at the University of California San Diego, USA. Although learning how to culture cells, purify proteins using affinity assays and chromatography the crystals would not grow sufficiently. Pepijn was however very interested in PKA and its interactors. Therefore in 2009 he joined the Biomolecular Mass spectrometry and Proteomics group of Prof. Dr. Albert Heck in Utrecht (NL) under the supervision of Dr. Arjen Scholten to focus on the group of proteins named A-Kinase Anchoring Proteins (AKAPs) which anchor PKA. The research was mainly focussed on the discovery of novel AKAPs and further elucidating the structural interaction between AKAPs and the PKA regulatory subunits. A part of this work was performed in the laboratory of Susan Taylor in San Diego. The results of these projects are presented in this doctoral thesis.