An emerging class of A-Kinase Anchoring Proteins are R2D2-like

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Abstract

A precise cellular response to both internal and external stimuli is crucial for the normal growth and development of all organisms. Almost all such responses are now known to be carried out by signal transduction pathways that involve signaling molecules such as receptors, second messengers, modifiers, effectors such as kinases, transcription factors, etc. Of critical importance are the modifiers such as scaffold proteins that impart spatial and temporal regulatory features to the response. A-Kinase Anchoring Proteins (AKAPs) are one such well-studied scaffold proteins that bind to cAMP-dependent protein kinase (PKA) and an array of signaling proteins. AKAPs are known to amplify, accelerate, localize and bring about specificity to the response. The AKAP-PKA complex is well studied and it has been found that regulatory subunits of PKA bind to the amphipathic helix of the AKAPs *via* their dimerization and docking (D/D) domains with what is commonly referred to as the RII-fold. This domain and fold is a characteristic feature of all the known regulatory subunits of PKA. However, recently, some molecules with an RII-fold have become known to bind to the amphipathic helices of AKAPs *via* their D/D-like domains; among others, these include several sperm proteins such as ropporin, AKAP-associated sperm protein (ASP), Sperm Protein-17 (SP-17) and fibrosheathin II (FSII) and others like DPY-30, RSP9, RSP11 and Myc-Binding Protein-1. The list of these types of proteins is growing and is referred to here as the atypical RII proteins (R2D2-like proteins).

Keywords: cyclic Adenosine Monophosphate (cAMP), RII, Protein Kinase A, Dimerization and docking domain

1. Introduction

A cell communicates with its surroundings using a battery of signaling molecules. The first such molecule to recognize signals (ligands) are the receptors, invariably followed by the release of second messengers such as cAMP, Ca^{2+}, cGMP, etc. Such a second messenger-signaling normally exerts its effects by activating its effector protein, which invariably is a protein kinase. Protein kinases use the γ-phosphoryl group of ATP to phosphorylate different target proteins thereby transducing the signal. The amino acid residues normally phosphorylated are serine, threonine or tyrosine, and in some cases it might be a histidine or a lysine. The number of different types of protein kinases, for example, in a typical eukaryotic genome like yeast is 130 (Zhu *et al*., 2000); while that in the human genome is 540 (Manning *et al*., 2002), and, this number varies from species to species. Considering the sheer numbers and the types of second messengers that activate these enzymes, nine subfamilies have now been categorized. The CGA family of protein kinases consists of the protein kinase C (PKC), cGMP-dependent protein kinase (PKG) and the cAMP-dependent Protein Kinase (PKA). Discovered in 1968 by Fisher and Krebs (Walsh *et al*., 1968), work on the PKA enzyme has come a long way. The residues commonly phosphorylated on the substrate protein by PKA are serine and threonine. The inactive PKA holoenzyme is a tetramer (R2C2 complex) composed of two regulatory (PKA-R2) and two catalytic subunits (PKA-C2). The bilobed structure of the catalytic subunit was first determined in 1991 (Knighton *et al*., 1991).

The second messenger, cAMP is involved in a variety of cellular functions. When its cellular level increases, four molecules bind to the two regulatory subunits subsequently causing the dissociation of the two free monomeric catalytic subunits from the holoenzyme; the latter is activated and available for phosphorylation of substrates that surround the site of kinase activation. It may be emphasized here that several cellular processes use the cAMP-mediated PKA activation and substrate phosphorylation events to mediate their responses. The moot question therefore is that of substrate specificity *vis-à-vis* the ligand that triggers the event. It is believed that PKA is anchored on to scaffold proteins that localize the phosphorylation events to cellular niches in the vicinity of specific substrates. The scaffold protein that tethers PKA is the A-Kinase Anchoring Protein (AKAP). This design helps PKA to co-localize with its specific target substrate and maintain a specific cellular niche. Evidence for such co-localizations and attachments to subcellular structures have been shown (Wong and Scott, 2004).

2. A-Kinase Anchoring Proteins

First discovered in 1991, AKAPs are proteins that seldom show primary sequence homology across species. With over 70 different AKAPs identified and isolated from several species, these diverse proteins are not direct mediators, but, are modifiers of signal propagation. Although diverse, there are two common denominators across all AKAPs, (i) all bind to the PKA, and (ii) this PKA can phosphorylate its dedicated substrate, also bound to the same AKAP. This gives rise to an interesting design that provides a spatial and temporal regulation of the signal. Both, the PKA and its substrate become co-localized and this proximity brings about a rapid amplification of the signal. Given this common role across all AKAPs, there are three common features (domains), (i) the Amphipathic helix which is a 14-18 amino acid stretch that binds to the dimerization and docking

domain present in all the regulatory subunits of PKA, (ii) unique targeting domain that allows positioning of the AKAP-PKA complex in close proximity to the substrate, and (iii) binding sites for other signaling proteins. In addition, several AKAPs are also known to anchor the entire complex to various organelles or locations within the cell (e.g., plasma membrane, mitochondria, etc.). The principle of this unique design is therefore to localize the signaling molecules, bring about specificity in the interaction of only those molecules that are tethered to the AKAP, accelerate the speed and magnify the response. Taken together, this creates a very efficient signaling hub within a certain niche of the cell. For example, an AKAP located on the Golgi apparatus and centrosome (AKAP350; Schmidt *et al*., 1999; Takahashi *et al*., 1999; Witczak *et al*., 1999; Shanks *et al*., 2002a; b; Carnegie et al., 2009), in the golgi apparatus (AKAP450, Takahashi *et al*., 2002), another in the muscle (muscle-selective AKAP) located near the nucleus of cardiomyocytes has been shown to bind to PKA and a phosphodiesterase, yet another employs an interesting strategy to switch PKA between the mitochondria and the endoplasmic reticulum (D-AKAP1; Ma and Taylor, 2008), ciliary AKAPs are present in the radial spokes and central pair of the '9+2' cilium as well as the flagella of sperms. For an exhaustive list and more information on AKAPs, extensive review material is available (Torres-Quesada *et al*., 2017; Calejo and Taskén K, 2015; Han *et al*., 2015).

3. Cyclic AMP-dependent Protein Kinase

3.1. Discovery

While working on the mechanism of hormone action in 1958, Earl Sutherland discovered cyclic AMP as a second messenger (Sutherland and Rall, 1958). A few years later, while working on glycogen metabolism, Fisher and Krebs isolated and thoroughly characterized the regulatory role of an enzyme that was dependent on cAMP for its protein kinase activity (Fisher and Krebs, 1955; Walsh *et al*., 1968). This discovery earned them the 1992 Nobel Prize in Physiology or Medicine. Now known popularly as cAMP-dependent protein kinase (PKA), subsequent work from laboratories the world over showed that it carries out phosphorylation of several cellular substrates in many organisms (Sim *et al*., 1999; Tasken *et al*., 2004; Carnegie *et al*., 2009). The genes for the enzyme are also expressed in a cell- and tissue-specific manner. This was the second protein kinase to be discovered and with its discovery, the rabbit and bovine enzyme complexes were the first to be thoroughly studied by several investigators. The holoenzyme of PKA was found to be a tetramer consisting of two catalytic (**C**) and two regulatory (**R**) subunits (Gill and Garren 1970; Tao *et al*., 1970; Brostrom *et al*., 1971). When bound to the **R** subunits, the **C** subunits are always inactive. It is only after the binding of one cAMP molecule per **R** subunit that releases the **C** subunits for activity. This aided in the ready availability of the subunits in their free and soluble forms and work on this enzyme moved at a faster pace. And, came the idea that the **R** subunits are the receptors of cAMP with high-affinity binding sites for the second messenger (Gill and Garren 1970; Tao *et al*., 1970; Brostrom *et al*., 1971). Taken together, two major regulatory mechanisms were then depicted and an entire paradigm shift was brought about in the understanding of cellular signaling, this being, phosphorylation by a protein kinase and activation by a second messenger. The concepts of protein oligomerization and allostery were evident in PKA signalling. Additionally, PKA was originally discovered as a kinase that phosphorylated glycogen phosphorylase kinase, an enzyme

involved in glycogen metabolism. It was named as phosphorylase kinase kinase and with this came about the concept of kinase cascade (Walsh *et al*., 1968).

3.2. Typical R subunits

The typical **R** subunits of PKA are multifunctional proteins and participate in myriads of cellular processes. Till date, the **R** subunit that essentially controls the **C** subunit of PKA is known to exist in two forms (or types), RI and RII (Hofmann *et al*., 1975; Erlichman *et al*., 1974; Beebe and Corbin, 1986). Both these differ in their elution profiles on ion exchange chromatography; and, each have two subunits alpha and beta. Therefore, there exist four PKA isozymes, PKA-RIα (Lee *et al*., 1983), PKA-RIIα (Clegg *et al*., 1988), PKA-RIβ (Scott *et al*., 1987), and PKA-RIIβ (Jahnsen *et al*., 1986) and all these show different biochemical properties and subcellular localizations. For example, when compared with PKA-RII, the PKA-RI dissociates readily upon cAMP binding. All known R subunits have well-defined domains, the dimerization and docking domain (D/D) at the N-terminus is used to bind to the amphipathic helix of AKAPs, followed by an autoinhibitory site, the two-tandem highly conserved cAMP-binding domains and the C-terminus high affinity binding of the C subunit (Weber *et al*., 1987). While their C-termini are well conserved in RI and RII, the N-terminus differ significantly and makes them unique. AKAPs that bind to both RI and RII (Huang *et al*., 1997a; Huang *et al*., 1997b) and those that only bind to RI have been reported (Angelo and Rubin, 1998). Accumulating evidence also indicates that RI and RII subunits are not only multifunctional but are also very distinct in their functions as well (Cummings *et al*., 1996). With accumulating mutational and structural studies of these subunits, it is evident what certain key residues contribute towards the working of these isoforms. In particular, the determinants on RIIα that are crucial in AKAP binding have been well established. Nevertheless, how individual PKA isoforms organizes itself to deliver a specific response remains unknown.

3.3. Atypical R subunits (R2D2-like proteins)

It is noteworthy that AKAPs bind not only to PKA and their substrates, but also to other signaling molecules such as protein phosphatases, protein kinase C, calmodulin and calcineurin (Coghlan *et al*., 1995; Klauck *et al*., 1996; Nauert *et al*., 1997; Sarkar *et al*., 1984; Schillace and Scott 1999). The AKAP-PKA interaction is well studied biochemically leading to the design and synthesis of peptide inhibitors. Such inhibitors have been used to determine the physiological consequences of AKAP-PKA interaction in several somatic cells whose cellular events are controlled by cAMP. Referred commonly as anchoring inhibitor peptides (AIPs), these inhibitors were designed to encompass the amphipathic helix of the AKAPs (Carr *et al*., 1992). Combined with PKA **C** subunit inhibitors (H-89 or S-PKI), their usefulness in physiological experiments to successfully depict AKAP interaction with **R** subunit or target the **C** subunit to relevant substrates is well-established. Over the past two decades, several sperm AKAPs have been identified and characterized from bovine, monkey, rat, mouse and humans (Carrera *et al*., 1994; Erlichman *et al*., 1999; Horowitz *et al*., 1988; Lin *et al*., 1995; Mei *et al*., 1997; Miki and Eddy, 1999; Pariset and Weinman, 1994; Reinton *et al*., 2000; Vijayaraghavan *et al*., 1997; 1999). Exhaustive experiments conducted using a combination of APIs and H-89 or S-PKI show that neither the typical **R** nor the **C** subunits are required for motility in

mammalian spermatozoa. And, yet other proteins that interact with sperm AKAPs regulate motility without any help from the **C** subunit of PKA (Vijayaraghavan *et al*., 1997). The search for these proteins which behaved like RIIs led to the discovery of two unique sperm proteins, ropporin and AKAP-associated sperm protein (ASP) that bound to a sperm AKAP110 in a manner that was similar to AKAP binding to RII (Carr *et al*., 2001), there being no involvement of the **C** subunit of PKA. Ropporin, a molecule that was earlier found to be a rhophilin-binding protein (Fujita *et al*., 2000) is 39% identical to ASP; both have strong sequence similarity with the conserved AKAP-binding D/D domain situated at the N-terminus. Mutations in these conserved residues prevent ropporin from binding to its AKAP, viz. AKAP110. This study also resulted in two more sperm proteins, SP-17 and fibrosheathin II (FSII) with properties similar to ropporin and ASP (Carr *et al*., 2001). It also led to the identification of one other hypothetical protein (F39H12.3) identifiable by sequence homology from *C. elegans*. It showed features of AKAP-binding domain but not a cyclic-nucleotide binding domain. The location of this protein in *C. elegans* remains elusive. These data strongly point out towards the presence of AKAP-binding proteins in sperm in a manner similar to RII and imply that AKAPs may have additional and perhaps unique functions in spermatozoa (Carr *et al*., 2001). Now commonly referred to as the R2D2-like proteins, these sperm orthologs use their D/D domain that they share with RII to bind to the amphipathic helix of AKAPs (Fiedler *et al*., 2008; Newell *et al*., 2008). Unlike RII, these sperm proteins do not bind cAMP and therefore might not invoke PKA signaling pathway. Another R2D2-like calcium binding tyrosine phosphorylation regulated (CABYR) protein was found to be present in the fibrous sheath of spermatozoa (Sen *et al*., 2003). CABYR gets phosphorylated when sperm capacitation occurs (Naaby-Hansen *et al*., 2002). It is through its D/D domain that CABYR binds to AKAP3 and AKAP4 present in the fibrous sheath. A recent study has shown that a CRISPR/Cas9 *Cabyr* knockout male mouse resulted in severe subfertility (Young *et al*., 2016). The total and progressive motility of the sperms were lowered significantly and there is impairment in the ability of the sperms to penetrate the egg. The fibrous sheaths of these mice were severely damaged and the axoneme in the principal portion of the flagellum compromised. Taken together, a *Cabyr* knockout indicates a structural role of this protein in the fibrous sheath of spermatozoa (Young *et al*., 2016). Additionally, knockout of ROPN1 and ROP1L, both R2D2-like proteins reveal defects in fibrous sheath integrity (Fiedler *et al*., 2013). The fourth R2D2-like protein, SPA17 has not yet been knocked out. These data indicate a very significant role for R2D2-like proteins in the fibrous sheath formation of spermatozoa.

Additionally, R2D2-like proteins were also found in the *Chlamydomonas* flagella. The radial spoke proteins, viz. RSP7 and RSP11 were found to harbour the D/D domains; both also bind to RSP3, an AKAP, also found in the radial spoke (Yang *et al*., 2006). As proposed by Yang and colleagues, a complex of RSP3, RSP7, RSP11 and RSP8 are formed in the radial spoke, of which RSP7 and 11 are radial spoke stalk proteins. While no human orthologue was found for RSP7, RSP11 was found to be weakly similar to the ASP sperm protein from humans and is now known to be the RPON1L orthologue. The rsp11 mutant (*pf25*) was found to exhibit very unusual motility phenotype, suggesting an important role for this R2D2-like protein (Huang *et al*., 1981). In yet another study of

the proteomic characterization of the sperm radial spokes from the protozoan *Ciona intestinalis*, NDK/DPY26 with a Dpy30 motif was found to be the RSP7 orthologue (Satouh and Inaba, 2009). The Dpy30 domain resembles in structure to R2D2-like proteins and also harbors the D/D domains. A 100 amino acid protein, DPY30 is found in several eukaryotes and is a part of complexes involved in several cellular processes (Wang *et al*, 2009). It was first found in *Caenorhabditis elegans*, when the defective protein resulted in dumpy-shaped body of XO male worms (Hsu *et al*., 1995). DPY30 was later found to be very useful in the cell fate specification of mammalian embryonic stem cells (Jiang *et al*., 2011). DPY30 also plays a role in the assembly of *Chlamydomonas* cilia (Stolc *et al*., 2005). It is now quite evident that DPY30 is present in both unicellular and multicellular organisms; but, the domain is present in a handful of important molecules, most of which have not been vigorously studied and hence their roles in cellular processes remain largely elusive. The Radial Spoke Protein 2 (RSP2) also contains the Dpy30 domain and its mutant results in paralyzed flagella with several subunits missing in the radial spoke heads (Gopal *et al*., 2012). In fact, the C-terminal of RSP2 harbours calmodulin-binding domain and strains lacking this domain from RSP2 were no severely affected in motility. Those that lacked the Dpy30 domain resulted in paralyzed flagella, indicating that this R2D2-like domain was indeed very crucial for motility (Gopal *et al*., 2012). Another protein containing the Dpy30 domain is the Sdc1 protein from the Set1 molecular complex of *Saccharomyces cerevisiae*; a complex that is involved in the methylation of the lysine 4 on histone 3 (H3K4). Sdc1 interacts with Bre2 in the manner their human orthologs ASH2L (Absent, small or homeotic discs-like 2) and DPY30 (South *et al*., 2010). It is now evident that the DPY30 and ASH2L proteins are very important in the H3K4 methylation activity (Dou *et al*., 2006; Patel *et al*., 2011). Further evidence for the functionally divergent nature of DPY30 in interacting with several proteins such as BIG2, ASH2L, Sdc2 and HDAC1 implies its significance in several cellular processes (Tremblay *et al*., 2014). For example, the WRAD complex containing WD repeat containing protein 5 (WDR5), Retinoblastoma Binding Protein 5 (RbBP5), Absent-Small-Homeotic-2- Like protein (ASH2L) and Dumpy-30 protein (DPY30) exhibit conventional and non-conventional functions. When present as WRAD along with SET1, it behaves as a methytransferase to methylate H3 for the activation of the transcription of genes (Dou *et al*., 2006; Steward *et al*., 2006; Patel *et al*., 2011; Ernst and Vakoc 2012; Dharmarajan *et al*., 2012; Shinsky and Cosgrove 2015; Zhang *et al*., 2015). When present as WRAD with MLL, it regulates cell cycle (Ali *et al*., 2014). When present as RAD, the DPY30 subunit interacts with BIG1 to modulate endosomal transport (Xu et al., 2009; Ali and Tyagi, 2017). Taken together, these data indicate that R2D2-like proteins are not restricted to spermatozoa, and are now found widely in several tissues containing motile as well as primary cilia.

Another R2D2-like protein is the MYC-Binding Protein-1 (MYCBP-1). The transcription factor, c-MYC and MYCBP-1 form a complex that enhances the transcription of genes controlled by the E-Box element, leading to erythrocyte differentiation (Taira *et al*., 1998; Furusawa *et al*., 2000). Initial studies have shown that MYCBP-1 traffics between the nucleus and cytoplasm. It also forms a ternary complex with AKAPs and MYCBPAP in the nucleus. MYCBP-1 seems to utilize an R2D2-like domain to associate with AKAPs and its coiled-coil region to bind c-MYC and MYCBPAP (Yukitake *et al*., 2002a).

Additionally, it was proposed that MYCBP-1, PKA and AKAP95 form a ternary complex in the nucleus negatively regulating the PKA activity (Furusawa *et al*., 2002). When outside the nucleus, MYCBP-1 has been shown to interact AKAP149 in sperm mitochondria and its splice variant S-AKAP84 (Furusawa *et al*., 2001; Yukitake *et al*., 2002b), and BIG2 (an AKAP in the trans-Golgi network) (Ishizaki *et al*., 2006). We recently identified a MYCBP-1 orthologue from the *Chlamydomonas* flagella [annotated as Flagellar Associated Protein 174 (FAP174)]. FAP174 was 43-87% identical or similar in the N-terminal region when compared with MYCBP-1 from other organisms. This region also spanned the helix-turn-helix fold typical to all R2D2 proteins. We also found that the C-terminus is a helix with a strong propensity to form a coiled-coil, known for protein-protein interaction. Hence, we predict two molecular modules for FAP174, one for binding an AH and one for interacting with other proteins (Rao *et al*., 2016). We strongly believe that one of these modules is used to interaction with AKAPs (viz. AKAP240). We did use overlay assays and pull-down experiments to show a strong interaction between FAP174 and AKAP240 (Rao *et al*., 2016). The presence of FAP174 in the basal body or TZ could be an indication of the protein trafficking between the various locations. In these locations, it might be binding to additional AKAPs and the same has not yet been determined. In this regard, FAP174 is rather versatile and might be involved in the assembly of several molecular complexes in distinct cellular compartments just like the way D/D domain-containing proteins function to localize molecular modules for both the assembly as well as modulation of macromolecular complexes.

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