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Review Article

Pseudoscaffolds and anchoring proteins: the difference is in the details

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Pseudokinases and pseudophosphatases possess the ability to bind substrates without catalyzing their modification, thereby providing a mechanism to recruit potential phosphotargets away from active enzymes. Since many of these pseudoenzymes possess other characteristics such as localization signals, separate catalytic sites, and protein–protein interaction domains, they have the capacity to influence signaling dynamics in local environments. In a similar manner, the targeting of signaling enzymes to subcellular locations by A-kinase-anchoring proteins (AKAPs) allows for precise and local control of second messenger signaling events. Here, we will discuss how pseudoenzymes form 'pseudoscaffolds' and compare and contrast this compartment-specific regulatory role with the signal organization properties of AKAPs. The mitochondria will be the focus of this review, as they are dynamic organelles that influence a broad range of cellular processes such as metabolism, ATP synthesis, and apoptosis.

Introduction: protein kinase A/cyclic AMP, pseudoenzymes, and mitochondria

Pseudoenzymes as 'pseudoscaffolds'

An emerging aspect of cell signaling is the role of pseudoenzymes as active participants in signal transduction cascades. These interesting signaling elements control the ebb and flow of metabolic information without fulfilling a catalytic function. Pseudokinases and pseudophosphatases, in particular, contain a substrate-binding domain similar enough to the active site of their relative enzymes to bind substrates, but do not typically possess detectable levels of catalytic activity [1–5]. Previous work has challenged this latter assumption by identifying catalytic activity in some pseudoenzymes, although with much lower activity than their canonical enzyme counterparts [6–9]. Another emergent role for pseudoenzymes is to act as an inhibitory anchor by recruiting substrate proteins into a pseudoenzyme scaffold, or 'pseudoscaffold.' This mechanism can serve to reduce the availability of free substrate protein in the vicinity of active enzymes (Figure 1A). Additionally, pseudoenzymes often possess functional domains such as localization motifs, other functional catalytic sites, and protein interaction domains (Figure 1A). Utilization of these additional features expands the repertoire of pseudokinases and pseudophosphatases as context-specific modulators of local protein phosphorylation events. Interestingly, these features of pseudoenzymes bear some resemblance to another family of non-catalytic signal organizing proteins, the A-kinase-anchoring proteins (AKAPs; Figure 1B).

cAMP signaling and AKAPs

The role of signaling enzyme anchoring has been most thoroughly investigated as a means to restrict and focus the action of second messenger-regulated kinases and phosphatases [10,11]. Historically, this field was born out of an interest in how the ubiquitous second messenger cyclic AMP (cAMP) can be used to process chemical signals in parallel such that different clusters of cAMP effector enzymes can be simultaneously activated at discrete locations in the cell.

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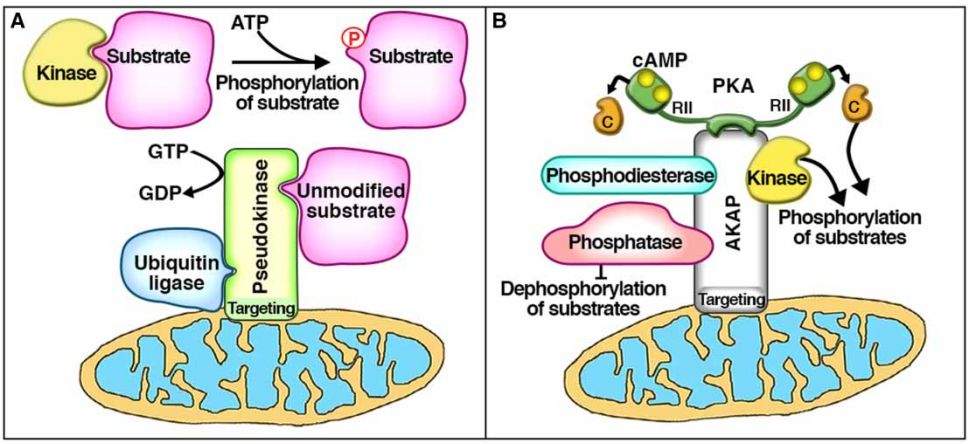

Figure 1. Comparison of pseudoenzyme scaffolds and AKAPs.

(**A**) Pseudoenzymes can act as an inhibitory anchor by binding and recruiting substrate protein away from active kinases and into a pseudoenzyme scaffold or 'pseudoscaffold,' thereby preventing substrate phosphorylation. This pseudoscaffold can possess additional features or functionalities such as (1) organelle-targeting motifs (i.e. mitochondrial, such as ADCK3), (2) separate catalytic sites (i.e. GTPase, such as MTMR5/13), or (3) other protein–protein interaction domains (i.e. heterodimerization, such as HER3; or other regulatory enzymes and their substrates, such as Trib2). Similarly, (**B**) AKAPs are a family of non-catalytic scaffolding proteins that, by definition, anchor the kinase PKA. In addition to PKA anchoring, AKAPs have also been shown to interact with a variety of other signaling molecules such as PDEs, phosphatases, and even other kinases. Many AKAPs also contain organelle-targeting motifs (i.e. mitochondrial, such as D-AKAP1). Through these additional features, both of these non-catalytic protein scaffolds can exhibit exquisite control of subcellular microdomains.

The second messenger cAMP is a versatile chemical signal [12] that is manufactured in response to ligand occupancy of G-protein-coupled receptors and the concomitant activation of adenylyl cyclases (ACs) [13]. The cellular accumulation of cAMP is strictly regulated by the superfamily of proteins known as phosphodiesterases (PDEs) [14]. The effects of cAMP in the cytosol have been exhaustively documented. However, distinct compartments or 'pools' of cAMP have also been identified at various subcellular locations, including, but not limited to, both at and within the mitochondria [15–17]. The presence of these distinct cAMP pools is indicative of tightly regulated cAMP signaling both at the mitochondrial outer membrane and within this vital organelle. A large portion of activities carried out by cAMP signaling is done so by the activation of the serine kinase protein kinase A (PKA). Four molecules of cAMP bind the regulatory subunits of the PKA holoenzyme causing the catalytic subunits to dissociate and become active, allowing the phosphorylation of PKA substrates [4,12,18–21]. In addition to PKA activation, cAMP can also activate Epacs (exchange proteins directly activated by cAMP) [22] and CNG (cyclic nucleotide-gated) channels [23].

Compartmentalization of PKA activity occurs through interactions with the large family of proteins termed AKAPs [4]. This diverse group of proteins anchors PKA via a canonical amphipathic helix and can recruit PKA to specific subcellular locations [24–26]. Interestingly, most AKAPs also create a scaffold by anchoring other important signaling molecules such as PDEs, phosphatases, and other kinases to create unique signaling microdomains that respond to cAMP and a variety of other chemical signals (Figure 1B). The clustering of enzymes with potentiating (i.e. substrates) and opposing (i.e. phosphatases) effects provides an efficient mechanism to confer bi-directional control of cell signaling at very precise locations [4,27]. Accordingly, AKAP signaling complexes and 'pseudoscaffolds' are both examples of macromolecular complexes that use protein–protein interactions to exert exquisite control over enzyme activity [28]. This shared mechanism affords the ability to deliver and transfer chemical information at discrete subcellular microdomains to generate local cellular responses.

Mitochondria and their disorders

Although AKAP-mediated signaling occurs at a variety of cellular organelles, the present study will focus on the role and regulation of enzymatic events, with an emphasis on cAMP-mediated phosphorylation occurring

at the mitochondria. Mitochondria are often referred to as 'the powerhouse of the cell' because of their critical role in oxidative phosphorylation (OXPHOS) and cellular ATP production. Disorders of mitochondrial physiology, genetics, and proteins have been attributed to an array of diseases including cancer, Parkinson's disease, Alzheimer's disease, lung disease, and cardiomyopathy [29–33]. In addition to OXPHOS, an increasing number of reports supports the idea that the mitochondria are important signaling hubs for a variety of cellular events [34,35]. The regulation of many post-translational modifications, such as phosphorylation and ubiquitination, often occurs at the mitochondria and can have a significant impact on not only mitochondrial function, but also overall cellular health. Although both PKA and Epacs have been identified at the mitochondria, only PKA activation has been associated with changes in mitochondrial function and dynamics [36].

Part I: importance of cAMP signaling at the mitochondria

Outer mitochondrial membrane

The outer mitochondrial membrane (OMM) is known to be a hub for cAMP signaling, with multiple AKAPs responsible for anchoring PKA to the membrane, including D-AKAP1/S-AKAP84, Rab32, and WAVE-1 [37–40]. Since the OMM serves as a barrier between the cytosol and the inner workings of the mitochondria, anchored cAMP signaling molecules at this location serve to control mitochondrial function and dynamics, as well as cellular health signaling.

It has been observed that some soluble ACs (sACs) translocate to the mitochondria under ischemic conditions in cardiomyocytes [41,42]. This allows localized production of cAMP at the mitochondria, leading to the PKA-dependent phosphorylation, activation, and translocation of pro-apoptotic Bax to result in cell death [41,42]. Additionally, cAMP signaling at the OMM is responsible for the regulation of mitochondrial fission and mitochondrial membrane potential ($\Delta\Psi$m; discussed further in OMM-anchored signaling) [43,44].

Intermembrane space, inner mitochondrial membrane, and matrix

The OMM is considered to be permeable to small molecules such as cAMP, allowing cytosolic cAMP to diffuse into the intermembrane space (IMS) and activate local PKA signaling. A few AKAPs have been identified in the IMS sphingosine kinase-interacting protein (SKIP) and optic atrophy 1 (OPA1), which confirm localized PKA signaling [45–47].

Conversely, the inner mitochondrial membrane (IMM) has been shown to be largely impermeable to external cAMP [16]. Therefore, any presence of cAMP within the mitochondrial matrix can probably be attributed to its production by resident sACs [48]. Interestingly, although there is some support for cAMP/PKA signaling cascade occurring in the matrix [48–50], no mechanism to import PKA has been identified to date. In fact, Lefkimmiatis et al. [16] were unable to detect the presence of endogenous mitochondrial matrix PKA activity using matrix-targeted FRET reporters. Therefore, the existence of PKA-dependent cAMP signaling within the mitochondrial matrix remains disputed.

Part II: AKAP anchored cAMP/PKA signaling at mitochondria

OMM-anchored signaling

The localization of kinases and other signaling molecules at the mitochondria has been determined to play a crucial role in regulating mitochondrial physiology, health, and dynamics. One such instance is the activation of PKA by the second messenger cAMP at the mitochondria. Activation of PKA localized to the OMM by D-AKAP1 (and its isoforms AKAP149, AKAP121, and S-AKAP84) [37,38] has been attributed to an inhibitory phosphorylation of the mitochondrial fission enzyme, dynamin-like protein 1 (Drp1) [51]. This phosphorylation of Drp1 inhibits mitochondrial fission, allowing for unopposed mitochondrial fusion. Additionally, the overexpression of D-AKAP1 leads to hyperelongated mitochondria and has been attributed to protection from cell death by promoting the PKA-dependent phosphorylation and inhibition of the pro-apoptotic Bad protein [51–53]. Interestingly, the depletion or displacement of D-AKAP1 from the mitochondria has also been associated with a decrease in $\Delta\Psi$m in cardiomyocytes and HEK293 cells [43,44]; however, any role of cAMP/PKA signaling remains unclear.

The mitochondrially targeted Rab32, a member of the Ras superfamily of small G-proteins, was identified as a dual-function protein that acts as both a GTPase and an AKAP [39]. Its function as a GTPase has been attributed to play a role in mitochondria–microtubule organization and the synchronization of mitochondrial

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fission events [39]. Interestingly, D-AKAP1 and Rab32 are localized to the OMM by different mechanisms. D-AKAP1 contains an N-terminal mitochondrial targeting motif, whereas Rab32 contains a pair of C-terminal cysteine residues that are required for mitochondrial targeting [38,54–56].

Notably, each of these mitochondrial PKA-anchoring proteins also binds a variety of other signaling molecules. For example, D-AKAP1 has been found to interact with PDE4A, PP1 (protein phosphatase 1), Drp1, and calcineurin [57–60]. The recruitment of such molecules to the OMM allows for precise control of the cAMP signaling microenvironment by localizing not only kinases, but also cAMP-degrading enzymes (PDEs), phosphatases, and important substrates to one discrete location.

IMM, IMS-anchored signaling

While the role of anchored cAMP/PKA signaling at the OMM has been broadly studied over the past few decades, signaling within the mitochondria has proved to be more difficult. PKA signaling was first proposed to occur inside the mitochondria with the observation of a PKA-dependent phosphorylation of ChChd3 (coiled-coil–helix–coiled-coil–helix domain-containing 3) [61]. This protein is a ChCh family member protein that is an important regulator in cristae maintenance and is found in the IMM, facing the IMS [61,62]. Interestingly, ChChd3 was also found to be a binding partner of the IMM protein OPA1, which was later identified as an AKAP [45,59]. PKA signaling inside the mitochondria was further confirmed by the identification of the type-I PKA-specific AKAP, SKIP, in the IMS [45,46]. Although the PKA-dependent phosphorylation of ChChd3 was critical in confirming the presence of a functional pool of PKA in the mitochondria, it remains unknown if this phosphorylation affects cristae maintenance or architecture.

Part III: pseudokinases and pseudophosphatases as localized ‘pseudoscaffolds’

General description

As mentioned earlier, pseudoenzymes can serve as an ‘inhibitory anchor’ by binding substrates, but not catalyzing their modification. This interaction can serve two purposes: (1) to reduce free substrate, preventing the substrate from being modified by active enzymes or (2) to localize substrate to particular subcellular locations. Since pseudoenzymes have the ability to carry out these actions simultaneously, they can be considered an inhibitory anchor.

In a paradigm-changing study published in 2008, Mukherjee et al. [7] found that the pseudokinase CASK (Ca^{2+}/calmodulin-activated Ser-Thr kinase) was not catalytically inactive, as its classification as a pseudokinase suggested. Rather it possessed low but significant levels of kinase activity [7]. Since then, more proteins originally classified as pseudoenzymes have also been shown to possess low levels of enzymatic activity [6]. However, since their classification as pseudoenzymes often stems from their lack of important amino acid residues involved in catalysis, their enzymatic activity is often lower or distinct from their canonical enzyme counterparts [6–9]. Regardless, the implications of low-activity pseudoenzymes as inhibitory anchors represent a key cellular function that may be implicated in certain human diseases of defective cell signaling.

Examples of pseudoenzyme scaffolding

Interestingly, pseudoenzymes often affect more than just the localization of their trapped substrates. They have also been shown to (1) act as a scaffold for other active molecules and enzymes and (2) facilitate other protein–protein interactions, and some pseudoenzymes even (3) possess additional domains that *are* catalytically active. An example of this third classification of multifunctionality can be seen in the myotubularin-related (MTMR) pseudophosphatases MTMR5 and MTMR13 [3]. In addition to containing a catalytically inactive phosphatase domain, these pseudophosphatases have been found to contain DENN (‘Differentially Expressed in Neoplastic vs. Normal cells’) domains, which act as an exchange factor to activate Rab GTPases [3,63]. Both of these MTMR proteins display subcellular localization to different areas of the cell, MTMR5 to the nucleus [63] and MTMR13 to endosomes [64]. Knocking out either of these genes in mice leads to visible pathology: MTMR5 caused impaired spermatogenesis and infertility in male mice [65] and MTMR13 knockout can be used as a mouse model of Charcot–Marie–Tooth disease [66].

Another example of multifunctional pseudoenzymes is the tyrosine kinase epidermal growth factor receptor family member HER3 (human epidermal growth factor receptor 3, also known as ERBB3) [3,4,67,68]. This pseudokinase has the ability to bind ligand, but does not homodimerize, rendering it inactive. However, HER3

can heterodimerize with HER2, which itself has no known ligands, and together the heterodimer is able to function as a signaling entity [69]. This HER2/3 heterodimer signaling is said to function as an oncogenic unit [69]. Notably, HER3 also displays very low levels of catalytic activity *in vitro*, nearing 1000-fold less activity than HER1 [6]. However, further analyses failed to identify any significant cellular effect of this low catalytic activity [70]. HER3 displays altered expression in breast cancer and other cancers [71–74], and knockdown of HER3 in breast cancer cells decreases proliferation, migration, and invasive potential [75]. In this example, HER3 can act as a pseudokinase inhibitory anchor by binding ligand but not promoting signaling, thus reducing free ligand to also reduce the activation of other ligand-binding enzymes, such as the enzymatically active HER1. Additionally, HER3 also modulates HER-family signaling by interacting with HER2 to generate a functional heterodimer from two individually nonfunctional units.

In addition to their roles in protein phosphorylation pathways, 'pseudoscaffolds' can also participate in other regulatory processes. For instance, the Tribbles pseudokinase, Trib2, contains a highly unusual catalytic loop that not only abolishes kinase activity, but also plays a critical role in recruiting the COP1 ubiquitin ligase substrate C/EBP for ubiquitination [76,77]. Interestingly, C/EBP interacts with the modified amino terminal catalytic loop of Trib2, whereas COP1 interacts with a carboxy-terminal domain, thus anchoring both ligase and substrate to the same location via this Trib2 scaffold [76–78]. The disruption of this recruitment by Trib2 has been attributed to the development of acute myelogenous leukemia in mice [77]. Trib2 is a perfect example of how protein–protein interactions of a 'pseudoscaffold' can anchor and regulate additional cellular events separate from kinase activity.

Mitochondrial pseudoenzymes and potential significance

Since healthy mitochondrial function is crucial to cell survival, the anchoring of signaling microdomains often plays a major role in regulating their action [29–33]. Recent work has begun to highlight a potential role for pseudoenzymes at the mitochondria; however, much remains to be investigated. Protein tyrosine phosphatase non-receptor type 21 (PTPN21 or PTPD1) contains a Cys-to-Ser mutation in its catalytic motif [79], and therefore sometimes is classified as a pseudophosphatase [3], although it does not display altered levels of phosphatase activity [79]. PTPN21 was also shown to be a binding partner of the mitochondrial D-AKAP1 [44,79,80]. Interestingly, PTPN21 was found to interact with the non-receptor tyrosine kinase Src, and, through this interaction, recruit Src to the mitochondria [44]. This D-AKAP1–PTPN21–Src complex increases Src-dependent phosphorylation of mitochondrial substrates and enhances cytochrome *c* oxidase activity [44]. Overexpressing a D-AKAP1 mutant that cannot bind the PTPN21/Src complex led to a decrease in $\Delta\Psi m$ and concurrent decrease in ATP production that was comparable with a D-AKAP1ΔPKA mutant [44]. However, the mechanism of how either PKA or the PTPN21/Src complex drives $\Delta\Psi m$ decrease has not yet been fully elucidated.

The depletion of another pseudophosphatase, MK-STYX, in the human cervical cancer cell line HeLa was found to protect cells from initiating apoptosis with treatment by various chemotherapeutics [81]. This group identified MK-STYX as a catalytically inactive phosphatase with significant homology to the mitogen-activated protein kinase (MAPK) phosphatases [81]. However, MK-STYX-depleted HeLa cells were unable to initiate cytochrome *c* release by the pro-apoptotic signaling by BCL-2 family proteins (Bax, Bid, and Bim) at the mitochondria, suggesting that the activity of this pseudophosphatase is occurring at the mitochondrial outer membrane [81]. The perturbation of MK-STYX expression with RNAi did not reveal any significant effect on MAPK signaling in the present study. Therefore, it is likely that this particular pseudophosphatase is not exerting its effects as an inhibitory anchor for MAPK phosphatase substrates, but is acting through a different, unknown mechanism. Since evasion of apoptosis is a significant concern in many cancer types, the exact role of this pseudophosphatase may be interesting to follow up for the design of future cancer therapeutics.

Another key mitochondrial protein is the atypical pseudokinase ADCK3, also known as COQ8A [82,83]. ADCK3 is a member of the widespread but little understood UbiB protein kinase-like (PKL) family [82,83], and is localized to the matrix face of the IMM, the site of CoQ synthesis [84]. The crystal structure of ADCK3 has illuminated how the kinase activity of UbiB PKL proteins is physically self-inhibited [83], rendering ADCK3 (and therefore other related UbiB PKL proteins) an atypical pseudokinase. This structural analysis uncovered an unexpected selectivity for ADP, thus limiting the ATP binding of ADCK3 [83]. In keeping with this notion, ADCK3 knockout mice develop a slow-progressing cerebellar ataxia that closely models Purkinje cell dysfunction caused by hereditary CoQ deficiency in humans [85]. These mice also display abnormal mitochondrial morphology in skeletal muscle, although no gross changes in mitochondrial function were observed [85]. These studies went on to elucidate the mechanism of CoQ deficiency in this model, attributing

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it to the destabilization of the CoQ biosynthetic complex 'complex Q', which is normally stabilized in the presence of ADCK3 via its proposed ATPase activity [85]. This atypical pseudokinase is a great example of the importance of pseudoenzyme complexes and how their unique features can influence biological pathways.

Part IV: conclusions

It is increasingly obvious that anchoring proteins are crucial in fine-tuning localized signaling events to control a myriad of cellular functions. We have discussed the importance of anchored PKA in the mitochondria by AKAPs and attributed the precise control of cellular maintenance functions such as mitochondrial dynamics, apoptosis, ATP production, and even the concentration of small molecules to AKAP anchoring.

Pseudoenzymes, on the other hand, possess the intrinsic ability to act as an inhibitory anchor by recruiting substrates and preventing their modification by other enzymes, while tethering them to discrete locations into a unique 'pseudoscaffold'. Furthermore, this protein scaffold can itself possess certain abilities such as (1) organelle-targeting motifs (i.e. mitochondrial, such as ADCK3), (2) catalytic activity separate from its pseudoenzyme-binding pocket (i.e. DENN domains of MTMR5/13), or (3) the ability to interact with or activate other proteins to influence signaling events (i.e. HER3 heterodimerization; Trib2-anchoring COP1 and its substrate C/EBP).

Thus, pseudoenzymes are not simply evolutionary 'leftovers' of functional enzymes, but a unique and emergent class of proteins united in their ability to anchor, but not modify, substrates of their enzymatic counterparts.

Abbreviations

ACs, adenylyl cyclases; AKAPs, A-kinase-anchoring proteins; Bcl-2, B-cell lymphoma 2; cAMP, cyclic AMP; C/EBP, CCAAT-enhancer-binding proteins; ChChd3, coiled-coil–helix–coiled-coil–helixdomain-containing 3; DENN, differentially expressed in neoplastic vs. normal cells; Epacs, exchange proteins directly activated by cAMP; FRET, fluorescence resonance energy transfer; HER3, human epidermal growth factor receptor 3; IMM, inner mitochondrial membrane; IMS, intermembrane space; MAPK, mitogen-activated protein kinase; MTMR, myotubularin-related; OXPHOS, oxidative phosphorylation; PDEs, phosphodiesterases; PKA, protein kinase A; PKL, protein kinase-like; PTPN21, protein tyrosine phosphatase non-receptor type 21; sACs, soluble ACs; SKIP, sphingosine kinase-interacting protein; $\Delta\Psi m$, mitochondrial membrane potential.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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