Kinase- and phosphatase-anchoring proteins: harnessing the dynamic duo

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Protein–protein interactions contribute to the spatial and temporal resolution of many signal transduction cascades. Here, we discuss recent advances in our understanding of phosphatase-targeting subunits, kinase-anchoring proteins and the multiprotein signalling networks that they assemble.

Cellular equilibrium is maintained by the continual adjustment of catabolic, anabolic and signal transduction events. The ebb and flow of these cellular processes is often mediated through the phosphorylation and dephosphorylation of proteins. Two enzyme groups directly control protein phosphorylation: protein kinases, which catalyse the phosphotransfer reaction, and protein phosphatases, which catalyse the hydrolysis reaction. As there are more than 500 kinases and approximately 150 phosphatases encoded by the human genome, sophisticated regulatory mechanisms have evolved to control their activation state, substrate specificity and subcellular location. Signalling networks of kinases and phosphatases frequently orchestrate the synchronization of phosphorylation/dephosphorylation events. Core components of these networks are kinase-anchoring proteins and phosphatase-targeting subunits that co-ordinate the bound enzymes and orient them towards specific substrates[1,2]. Advances in proteomic and interaction screening strategies have revealed a high level of complexity in these molecular scaffolds[3,4]. This review will discuss the evolving field of anchoring proteins and targeting subunits, with particular emphasis on the biological role of signalling networks that include serine/threonine phosphatases and second-messenger-regulated protein kinases.

Protein-phosphatase-targeting subunits

The concept of phosphatase targeting was proposed as a mechanism to generate substrate specificity well before the notion of kinase anchoring became popular[5]. Since then, the targeting proteins that localize the catalytic subunits of most serine/threonine protein phosphatases have been identified. As recent reviews have comprehensively discussed targeting mechanisms for protein phosphatase 2A (PP2A) and novel protein phosphatases[6,7], we will primarily focus on compartmentalization of the type 1 phosphatase, PP1.

In the mid-1980's, the first PP1-targeting subunit, glycogen-particle-associated protein (GM), was identified[8]. Subsequent analyses have demonstrated that GM and a functionally related molecule called PTG (protein targeted to glycogen) co-ordinate signalling complexes that influence glycogen metabolism[9,10]. However, GM is the predominant glycogen-targeting subunit, as it is present at significantly higher concentrations in membranes and the glycogen particles of sarcovesicular structures[9]. Association of PP1 with the GM protein network results in up to an eightfold enhancement of the dephosphorylation of glycogen synthase, glycogen phosphorylase or glycogen phosphorylase kinase[9]. Modulation of targeted PP1 activity involves a Lys/Arg-Val/Ile-X-Phe motif (the RVXF motif, where X is any amino acid) in GM that inserts into a groove distal to the active site of the enzyme[11]. Peptides encompassing this region are sufficient to displace the PP1 catalytic subunit from GM and abolish the preferential dephosphorylation of glycogen-associated substrates[9]. This resulted in the proposal that GM not only targets PP1, but also allosterically regulates phosphatase activity. Similarly, the muscle-specific phosphatase holoenzyme (PP1-M) contains a targeting subunit, M110/MBS, which contains an RVXF motif[12]. This targeting subunit has two roles: it directs phosphatase activity towards a select group of muscle proteins, including myosin and possibly moesin, and it nucleates a signalling complex with the cGMP-dependent kinase (PKG) and the low-molecular-weight GTPase Rho[13–15] (Fig. 1a). Elevated concentrations of the second messenger cGMP activate anchored PKG which, in turn, phosphorylates M110/MBS to trigger events that result in relaxation of smooth muscle[15]. In addition, Rho-GTP activates a Rho-dependent protein kinase that phosphorylates M110/MBS to inhibit the tethered PP1 activity, which favours contraction of smooth muscle (Fig. 1a)[16]. Thus, the M110/MBS-targeting subunit co-ordinates the activities of several signalling enzymes that contribute to the regulation of smooth muscle signalling events.

The RVXF motif is now a recognized hallmark of most PP1-targeting subunits. A recent review has identified this motif in over 50 known and potential PP1-targeting subunits in a variety of organisms[17]. However, several targeting subunits, including PNUTS and AKAP220, interact with PP1 through sites outwith their RVXF-like motifs[18,19]. Interestingly, the competitive inhibitors of PP1, Inhibitor-1 and DARPP-32, are mechanistically related to the targeting subunits[20]. They contain a RVXF motif and a second structurally similar sequence that docks with the active site of the enzyme[20]. Hence, PP1 modulatory proteins contain a variety of

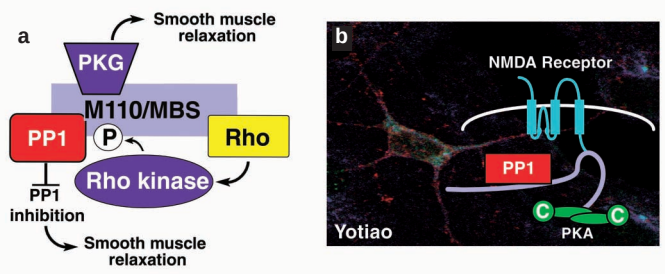

Figure 1 **Type-1 phosphatase-targeting subunits**. **a**, A schematic diagram of the PP1M signalling complex that is maintained by M110/MBS. Activation of a Rho-dependent kinase results in phosphorylation of M110/MBS, resulting in an inhibition of PP1 activity. Associated enzymes and signalling proteins are indicated. **b**, Yotiao directs a phosphatase/kinase signalling complex towards its substrate, the NMDA receptor. The foreground shows a schematic diagram of the Yotiao signalling complex. Associated enzymes and signalling proteins are indicated. The background shows the cellular distribution of Yotiao (green), PKA (blue) and PP1 (red) in cultured hippocampal neurons.

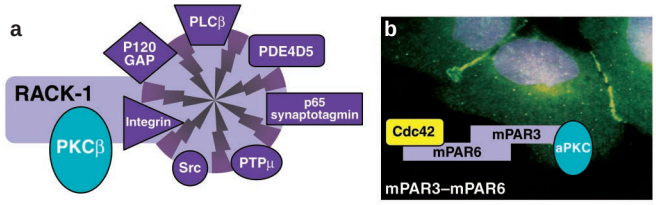

Figure 2 **PKC-interacting proteins. a**, A schematic diagram of RACK-1-binding partners. Associated enzymes and signalling proteins are indicated. **b**, The foreground shows a schematic diagram of the mPAR3–mPAR6 signalling complex, which co-ordinates the location of atypical PKCs and Rho family GTPases. Associated enzymes and signalling proteins are indicated. The background shows the cellular distribution of mPAR3 (green) in CHP-100 neuro-epithelial cells. Nuclei are shown in blue. This image was kindly provided by D. Linn and T. Pawson (Simon Lunenfeld Research Institute Toronto, Canada).

protein phosphatase interaction sites that regulate phosphatase activity and maintain the enzyme at defined subcellular locations.

Several PP1-targeting subunits also interact with protein kinases. The proximity of both enzymes provides a mechanism to precisely regulate the phosphorylation status and activity of substrate proteins[21]. Under basal conditions, these substrates are predominantly dephosphorylated because the targeted phosphatase is active. Therefore, pulses of substrate phosphorylation occur only when the activated kinase overwhelms the phosphatase activity. For example, yotiao, an *N*-methyl-D-aspartate (NMDA)-type glutamate-receptor-associated protein, binds PP1 and the protein kinase A (PKA) holoenzyme[22] (Fig. 1b). The tethered phosphatase is active, limiting channel activity, whereas activation of PKA overcomes the constitutive PP1 activity to enhance NMDA receptor currents[22]. Similarly, spinophilin/neurabin 2, another PP1-targeting subunit, may regulate striatal AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors in a similar manner[23,24]. This targeting subunit assembles a phosphatase/kinase signalling complex that includes PP1 and the p70 S6 kinase[25]. Although a direct association with AMPA receptors has yet to be demonstrated, *spinophilin* knockout mice fail to exhibit PP1-mediated down-regulation of channel activity. This suggests that spinophilin may anchor constitutively active PP1 to maintain the AMPA receptor in a dephosphorylated 'low activity' state. These examples demonstrate the utility of phosphatase-targeting subunits to assemble signalling complexes that work in a co-ordinated manner.

Protein-kinase-C-interacting proteins

Signalling pathways that emanate from G-protein-coupled receptors, growth factor receptors and tyrosine kinases often converge on the activation of protein kinase C (PKC)[26]. This family of calcium/phospholipid-dependent isozymes is divided into the classical, novel and atypical subtypes on the basis of sequence homology and sensitivity to activators[26]. Although most cell types express a selection of PKC subtypes, discrete activation signals can promote the translocation of individual isoforms to distinct subcellular locations[27]. Compartmentalization of PKC isoforms often involves an association with a variety of interacting proteins, including RACKs (receptors for activated C kinase), PICKs (proteins that interact with C kinase) or STICKs (substrates that interact with C kinase). Although each class of interacting protein binds the enzyme through a slightly different mechanism, common functions include influencing the substrate specificity of PKC and recruiting the enzyme into signalling networks.

RACKs are not substrates for the enzyme, but bind activated PKC isozymes in a selective and saturable manner[28,29]. Although at least ten PKC isozymes are known, only two RACKs have been identified: RACK1, which binds to PKC-β, and RACK-2/β′-COP, which interacts with PKC-ε. Several unrelated reports have implicated RACK-1 as a promiscuous scaffolding protein that could possibly bring PKC-β together with a variety of signalling molecules, including PLC-γ1, the integrin β-subunit, p120 GAP, the cAMP-specific phosphodiesterase PDE4D5, p65 synaptotagmin and the tyrosine phosphatase PTP-μ (Fig. 2a). A β-propeller-like structure, formed by seven WD-repeat sequences, seems to be required for the interaction of RACK1 with many of its binding partners. For example, the src-homology 2 (SH2) domain of the Src tyrosine kinase recognizes a phosphotyrosine motif in the sixth WD repeat[30]. Activation of PKC increases the amount of Src associated with RACK-1 and provides an example of crosstalk between tyrosine kinase and serine/threonine kinase signalling cascades[31]. However, the physiological relevance of the RACK-1 interaction with other putative binding partners has yet to be determined.

Unlike RACKs, STICKs associate with a variety of PKC isoforms[31]. Most STICKs, such as β-adducin, MARCKs and gravin/sseCKs/clone72, are cytoskeletal substrates for PKC that target the enzyme to the cortical cytoskeleton[32,33]. Another protein that could be placed in this class of interacting proteins is PAR3, a scaffolding protein that interacts with atypical PKCs. *Caenorhabditis elegans* PAR3 and its *Drosophila melanogaster* homologue, Bazooka, are important in asymmetric division and the establishment of epithelial cell polarity[34,35]. The mammalian form, mPAR3, also called ASIP, contains three PDZ domains and a region that binds to the catalytic domain of atypical PKCs[36]. Each PDZ domain interacts with a variety of binding partners. These include the junctional adhesion protein (JAM), which recruits the complex to tight junctions in epithelial cells and mPAR6, a protein that couples to the low-molecular-weight GTPases Cdc42 or Rac[37–40]. Thus, the mPAR3–mPAR6 interaction generates a cellular locus for the efficient transmission of signals through Rho family GTPases and atypical PKCs (Fig. 2b). Currently, it is unclear whether these distinct signalling pathways behave synergistically or function independently in response to developmental cues.

A-kinase-anchoring proteins

Most effects of the second messenger cAMP are mediated through activation of PKA. The PKA holoenzyme is a tetramer, consisting of a regulatory (R) subunit dimer and two catalytic (C) subunits. The spatial organization of this enzyme is achieved through the interaction of the R subunits with AKAPs (A-kinase-anchoring proteins)[41–43]. High-affinity interaction with the type 1 or type II PKA isozymes is mediated by an amphipathic helix on the AKAP, which inserts into a hydrophobic pocket formed by the R subunit dimer[44,45]. Targeting determinants within the anchoring protein confers the targeting of PKA–AKAP complexes to specific organelles[42].

Over the past decade, numerous AKAPs have been identified, and represent a functionally related group of proteins that bind to PKA. This resulted in the hypothesis that AKAPs direct PKA to certain substrates and position the kinase, where it can efficiently respond to elevations in cAMP[46]. Two recent studies using fluorescent imaging techniques lend support to this hypothesis. The first study involves the use of a cAMP sensor protein. A chimeric protein has been constructed that contains a consensus PKA substrate site and a phosphopeptide-binding module fused to the yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) moieties. PKA-mediated phosphorylation of the sensor generates intramolecular changes that increase the rate of fluorescence resonance energy transfer (FRET). Introduction of an AKAP sequence enhances the rate of cAMP-induced FRET in HeLa cells, because PKA is recruited to the sensor protein[47]. In a second study, intermolecular FRET was used to demonstrate that micro-gradients of cAMP emanate from sites of synthesis at the plasma

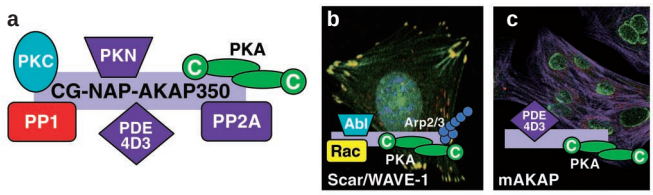

Figure 3 **A-kinase-anchoring proteins. a**, A schematic diagram of the CG-NAP/AKAP350 signalling complex. Associated enzymes and signalling proteins are indicated. **b**, Scar/WAVE-1 maintains an actin-associated signalling complex. The foreground shows a schematic diagram of the Scar/WAVE-1 signalling complex. Associated enzymes and signalling proteins are indicated. The background shows the cellular distribution of WAVE-1 (green) and actin (red) in Swiss 3T3 fibroblasts. Nuclei are shown in blue. **c**, mAKAP co-ordinates a cAMP signalling module. The foreground shows a schematic diagram of the mAKAP signalling complex, showing associated PKA and PDE4D3. The background shows the cellular distribution of mAKAP (green), PDE4 (red) and actin (blue) in rat neonatal cardiomyocytes.

membrane. Hormonal stimulation of cardiomyocytes induces changes in the rate and magnitude of local cAMP gradients, with concomitant effects on the activation of anchored PKA pools[48]. Both studies implicate AKAPs as the physiological mediators of PKA localization.

In 1995, it was shown that AKAPs simultaneously associate with multiple signalling enzymes[49]. Since then, numerous AKAP signalling complexes have been identified[50–52]. For example, the centrosomal-anchoring protein CG-NAP/AKAP350 interacts with PKA, PKC-ε, the Rho-activated kinase PKN and the protein phosphatases PP1 and PP2A[53–55]. This multi-enzyme scaffold integrates a variety of second messenger signals to alter the phosphorylation status of proteins that synchronize cell division (Fig. 3a). Other AKAP signalling complexes mediate a variety of cytoskeletal events[56]. Scar/WAVE-1 is a member of the Wiskott-Aldrich-syndrome family of scaffolding proteins that binds to PKA, Abl tyrosine kinase and the Arp2/3 complex, a group of seven proteins that control actin remodelling[56–58]. The dynamic assembly of this complex at sites of lamellapodial extension occurs in response to growth factor signals that activate Rac[57]. Therefore, Scar/WAVE probably directs PKA and Abl towards cytoskeletal substrates, and probably controls cell movement and shape by ensuring efficient transmission of Rac-mediated signals to the actin remodelling machinery (Fig. 3b). However, Abl also induces formation of F-actin through other mechanisms[59]. Analogous signalling networks participate in the formation of actin stress fibres. AKAP-lbc, a splice variant of the Lbc oncogene, encodes a chimeric molecule that anchors PKA and functions as a Rho-selective guanine nucleotide exchange factor[60]. Application of lysophosphatidic acid or selective expression of Gα12 enhances the cellular activation of AKAP-lbc and results in the formation of actin stress fibres in fibroblasts[60]. Each of the examples cited above suggest that AKAP provides a molecular platform to permit the convergence or segregation of signalling pathways. In the case of CG-NAP/AKAP350, there is a convergence of second messenger signalling events, as PKA and PKC often phosphorylate the same substrate (Fig. 3a). By contrast, Scar/WAVE and AKAP-lbc assemble separate signalling pathways at the same cellular locus. The segregation of these enzyme activities permits the parallel processing of information.

A novel role for AKAPs is to anchor signal transduction enzymes with signal termination enzymes. A prototypic example is the muscle-specific anchoring protein mAKAP, which synchronizes the activities of cAMP-dependent and cAMP-metabolizing enzymes to maintain a hormonally regulated negative feedback loop[61]. mAKAP associates with PKA and an active cAMP-specific phosphodiesterase, PDE4D3, and targets them to the perinuclear regions of cardiomyocytes (Fig. 3c). This configuration helps to limit activation of PKA under basal conditions by hydrolysing cAMP within the immediate vicinity of the kinase[61]. After hormonal stimulation, the cAMP levels rise and overcome the local PDE activity, allowing for activation of the kinase. The relationship between both anchored enzymes involves several layers of regulation, as PKA-mediated phosphorylation of Ser 54 on PDE4D3 increases the rate of cAMP metabolism[62]. Recently, PDE4D3 has also been reported to associate with CG-NAP/AKAP350, suggesting that cAMP-responsive events may be regulated in a similar manner at other sites in the cell[63].

Conclusions

As we learn more about the proteins that target PP1, PKC or PKA, some common themes begin to emerge. Clearly, the principal function of these proteins is to influence the location and substrate specificity of the broad-spectrum enzymes that they anchor. This can be achieved in different ways. Phosphatase-targeting subunits, such as Gm or M110, not only target PP1, but also function as allosteric modulators of phosphatase activity. This implies that although a localized PP1 holoenzyme may have access to a range of substrates, its specificity is constrained by an interaction with the targeting subunit. By contrast, true anchoring proteins, such as CG-NAP/AKAP350 and mAKAP, do not alter enzyme activity, but rely solely on protein proximity to direct their bound enzymes towards individual substrates. This latter model suggests that the intracellular terrain may be composed of discrete pools of anchored kinases and phosphatases that are surrounded by select substrates. If this is the case, each constellation of enzymes must be positioned to efficiently receive activation signals from other parts of the cell.

Another common property of anchoring proteins is to position signalling enzymes in microenvironments, where they can easily access effector proteins. For example, AKAP-lbc, WAVE-1 and mPAR3 anchor kinases and recruit Rho family GTPases to initiate distinct actin remodelling events mediated by Rho, Rac and Cdc42, respectively[56]. Furthermore, unconventional modes of signalling to PKA may also be governed by AKAPs, as the testis-specific anchoring protein AKAP110 has been reported to interact with the heterotrimeric G protein subunit Gα13, which activates AKAP110-associated PKA through a cAMP-independent mechanism[64]. However, the majority of AKAPs co-ordinate signals from G-protein-coupled receptors through cAMP-dependent mechanisms. Gravin and AKAP79/150 maintain kinase complexes that respond to signals emanating from plasma membrane β2-adrenergic receptors through direct protein–protein interactions with these receptors[65,66]. A later report suggests that another AKAP, MAP2c, participates in a larger signalling complex that includes the β2AR, adenylyl cyclase, PKA and a substrate for the kinase, the class C L-type calcium channel[67]. This finding not only underscores the view that receptors, effector proteins, kinases and their substrates are spatially co-ordinated, but also emphasizes that cAMP may not have to diffuse very far from its site of synthesis to activate the PKA holoenzyme. However, the question of how far the active C subunit diffuses from it site of activation remains unanswered. Two factors are important in this equation, the cellular excess of R subunit that drives holoenzyme reformation and the influence of localized phosphodiesterase activities that metabolize cAMP. Certainly, the mAKAP and AKAP350 signalling complexes may factor into this equation by clustering PKA, PDE and phosphatase activities to ensure transient activation of PKA and the bidirectional control of protein phosphorylation events. All of the above examples highlight the sophisticated degree of spatial organization achieved by these anchoring proteins and emphasize a wider role for 'signal organizing molecules' as focal points for the reception, propagation and integration of distinct cellular signals. □

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