More on target with protein phosphorylation: conferring specificity by location

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One important regulatory mechanism in the control of phosphorylation events is the subcellular location of phosphatases or kinases. Several serine/threonine phosphatases and kinases have now been shown to be associated with targeting subunits; these contribute to the organization and specificity of signal transduction pathways by favoring the accessibility of their enzymes to certain substrates.

IN THE WORDS of one colleague, 'compartmentalization is the last refuge of a scoundrel'. This typifies the prevailing view of many classical biochemists that the cell is just a bag of enzymes that float around in a cytoplasmic soup searching for their substrates. However, more sophisticated models are required to account for the specific phosphorylation events that occur following activation of second messenger signaling pathways. In particular, a recent idea has been that protein phosphatases and kinases are compartmentalized to restrict subcellular location and enhance specificity. More recently, Hubbard and Cohen integrated their work and that of others into a 'targeting hypothesis' in their review 'On target with a mechanism for reversible phosphorylation'[1]. This simple, but deceptively complicated concept proposes that phosphorylation events are controlled not only by the balance of kinase and phosphatase activity, but also by where these enzymes are placed in the cell. Subcellular targeting enhances the selectivity of multifunctional serine/threonine phosphatases and kinases by favoring their accessibility to certain substrate proteins.

Compartmentalization is achieved through a 'targeting moiety', which is defined as that part of a phosphatase or kinase that directs the catalytic subunit to a certain subcellular environment[1]. The nature of this targeting moiety can be quite varied. In some cases, separate targeting subunits direct the location of the catalytic subunit (Fig. 1a), in others, the targeting function is achieved through a specialized domain fused to the catalytic core of the enzyme (Fig. 1b). In either case, the targeting moiety restricts the location of a phosphatase or kinase through association with a 'targeting locus'[1]. As the name suggests, targeting loci are generally structural proteins that are components of membranes, the cytoskeleton or cellular organelles. The scope of this review is to compare and contrast the properties of targeting proteins that localize broad-specificity protein phosphatases, protein kinase C (PKC) and the cAMP-dependent protein kinase (PKA), as well as to describe a new class of multivalent adapter proteins that coordinate the location of more than one kinase or phosphatase.

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Phosphatase targeting

Serine/threonine protein phosphatases control a variety of physiological events such as cell proliferation and the cell cycle. However, regulation of these complex cellular processes proceeds through only a limited number of phosphatases. Three prominent types of phosphatase catalytic (C) subunit have been identified, termed PP-1, PP-2A and PP-2B, which are classified according to their substrate preferences, mechanisms of activation and sensitivity to inhibitor proteins or naturally occurring toxins (for review, see Ref. 2).

PP-1. How can any degree of specificity be achieved in the regulation of many phosphorylation events by relatively few phosphatases? The idea of targeting subunits originated with studies on glycogen metabolism in skeletal muscle, where it was recognized that a substantial proportion of PP-1 is associated with glycogen particles[2]. It is now clear that each phosphatase type is regulated by targeting subunits (Fig. 2a). The PP-1 targeting subunits serve two functions: (1) to localize the C subunit; and (2) to adapt the phosphatase activity to favor the dephosphorylation of certain substrates. The glycogen-associated enzyme PP-1G is a heterodimer composed of a 37 kDa C subunit and a targeting subunit (G), sometimes called RG (Ref. 3). The muscle form, GM, is a 124 kDa protein, whereas the liver form, GL, is a distinct, but related protein of 33 kDa (Refs 4, 5). In yeast, the G subunit is an 85 kDa protein, GAC-1, which coordinates glycogen levels[6]. The mammalian GM subunits direct PP-1 to either glycogen particles or to the membranes of the sarcoplasmic reticulum, while GL directs PP-1 only to glycogen[7]. The glycogen-binding and PP-1-binding sites are in the amino-terminal regions of GM, whereas the membrane-binding domain includes a stretch of hydrophobic residues at the carboxyl terminus[4]. Additional mapping studies have identified peptides from the G subunit that bind PP1-C (P. Cohen, pers. commun.).

Mammalian GM function is hormonally regulated. Insulin-stimulated phosphorylation of GM favors glycogen synthesis by enhancing PP-1 activity towards glycogen synthase and phosphorylase kinase. By contrast, adrenaline stimulates glycogenolysis through

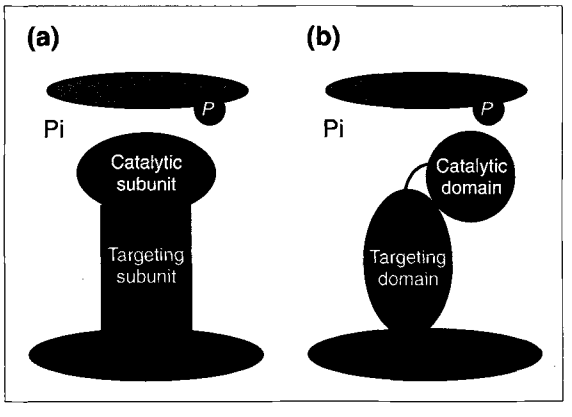

Figure 1
Schematic diagram depicting the subcellular targeting of enzymes through targeting moieties. **(a)** Localization of the catalytic subunit to a targeting locus through association with a targeting subunit. **(b)** Localization of enzymes through a distinct targeting domain.

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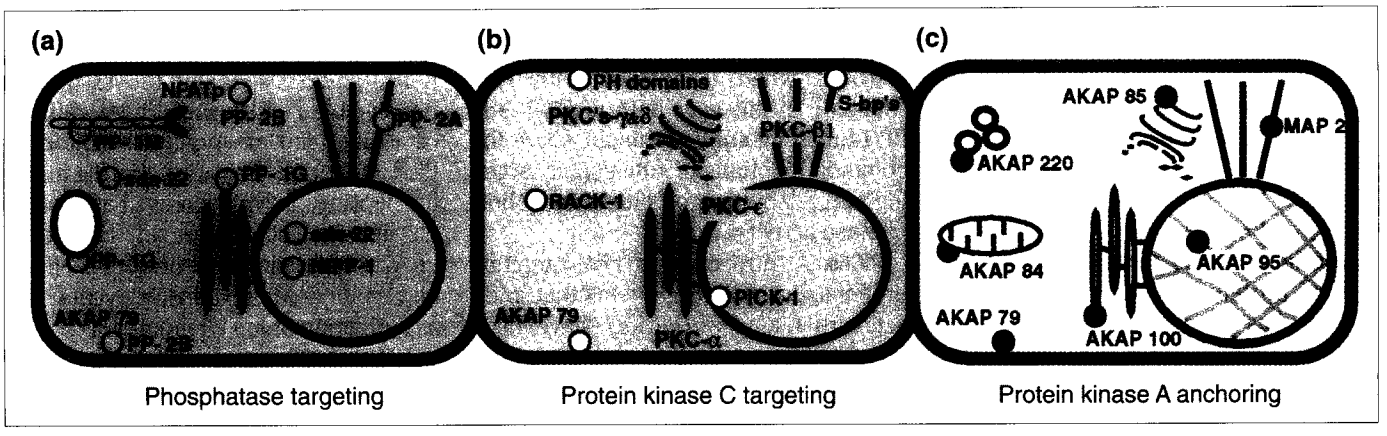

Figure 2

The cellular location of targeting subunits. The subcellular location of known targeting subunits that attach their respective enzymes to specific organelles are indicated. **(a)** Targeting of protein phosphatases; PP-1G associates with glycogen particles and/or sarcoplasmic reticulum, PP-1M with myosin, PP-1 with sds-22 and NIPP-1 in the nucleus, PP-2a associates with microtubules, PP-2B with AKAP79 at the membrane. **(b)** Targeting of protein kinase C (PKC); PKC associates with RACK-1 in the cytoplasm, with PICK-1 at the nuclear membrane, with substrate-binding proteins associated at the cyoskeleton and membrane, and with AKAP79 at the membrane. **(c)** cAMP-dependent protein kinase (PKA); PKA is targeted to the endoplasmic reticulum through association with AKAP100, to the membrane through AKAP79, to mitochondria through AKAP84, to peroxisomes through AKAP220, to the golgi through AKAP85 and to microtubules through MAP 2.

PKA phosphorylation of GM, which permits release of PP-1 (Ref. 1). PP-1 is not restricted to association with glycogen particles and sarcoplasmic reticulum membranes, but is found in other cellular organelles, which raises the question of how PP-1 is targeted to many different locations.

In muscle cells, PP-1 is associated with myofibrils through regulatory M subunit(s). This not only determines the subcellular location, but also modifies the substrate specificity of the enzyme[1]. The exquisite specificity achieved with analogous PP-1-targeting subunits is highlighted when the skeletal and smooth muscle enzymes are compared. While the smooth muscle PP-1M enhances the dephosphorylation of smooth muscle myosin, but not skeletal muscle myosin; the skeletal muscle PP-1M enhances the dephosphorylation of skeletal muscle myosin to a much greater extent than smooth muscle myosin. PP-1M in smooth muscle is a heterotrimeric complex adapted for the dephosphorylation of myosin consisting of the 37 kDa C subunit and regulatory subunits of 130 kDa and 20 kDa, called M-130 and M-20 (Refs 8–10). The regulatory targeting subunits specifically direct the enzyme to dephosphorylate myosin. Myosin and PP-1 bind to the amino-terminal third of M-130 within a region containing eight ankyrin repeats[9]. The M-20 subunit contains a leucine zipper motif in the carboxyl terminus, which might be involved in protein–protein interactions[8–10]. Interestingly, leucine-rich repeats can form the PP-1-binding site on another targeting subunit, sds-22, which facilitates the metaphase–anaphase transition during mitosis in yeast. The human sds-22 homolog retains the leucine-repeat sequences and is equally distributed in the cytoplasm and nucleus of liver cells[11]. The best characterized PP-1-binding protein in the nucleus is NIPP-1, a 38.5 kDa protein, which is a potent and specific inhibitor of PP-1 (Ref. 12). Targeting of PP-1 also proceeds through association with the tumor suppressor accessory protein, p53-binding protein or p53bp2 (Ref. 13). This protein modifies the specificity of PP-1 and, like M-130, it contains ankyrin-repeat sequences, which have been implicated in PP-1 binding. While p53 is known to be regulated by phosphorylation, it is not yet clear if PP-1 modulates p53 tumor suppressor activity.

PP-2A and PP-2B. Recent studies suggest protein phosphatase 2A (PP-2A) might be targeted in an analogous way to PP-1. PP-2A is a heterotrimer consisting of a 36 kDa catalytic (C) subunit, a 65 kDa structural subunit (A) and a third protein, termed B or phosphatase regulatory (PR) subunit. The A and C subunits form a core enzyme, whereas there are several families of B subunit, which increases the complexity of PP-2A tissue-specific production and subcellular targeting[14–16]. For example, the Bα subunit is localized to the centrosome and microtubule network in CV-1 cells, the ABαC holoenzyme form of PP-2A interacts with microtubules and the dimeric AC form of PP-2A, termed PP-$2A_2$ binds to the SV40 small tumor antigen[17]. In addition, immunofluoresence studies suggest that other pools of PP-2A are localized in the endoplasmic reticulum (ER), golgi and nucleus[18]. No doubt future studies will establish whether the subcellular location of PP-2A is directed solely through the B subunit or whether additional contacts are mediated through the core enzyme.

A proportion of protein phosphatase 2B (PP-2B) or calcineurin is targeted to the membrane. In bovine brain, the particulate form of PP-2B is inactive[19]. This could be explained by evidence that the phosphatase is inhibited and targeted to sub-membrane sites through association with AKAP75, an anchoring protein that also binds PKA (Ref. 20). Other studies suggest that cytosolic PP-2B associates with one of its physiological substrates, the transcription Factor NFATp (Ref. 21). However, PP-2B binding does not depend upon the phosphorylation state of NFATp, thus suggesting a role in targeting.

PKC-targeting proteins

Protein kinase C (PKC) is a family of at least 11 enzymes activated by Ca^{2+} and/or diacylglycerol (for review, see Ref. 22). The role of individual PKCs in cell function is not clear, as most cells synthesize multiple isoforms with nearly identical ligand-binding and substrate specificities. Recently, compartmentalization of individual PKC isoforms has been suggested as a means to attain some measure of selectivity towards their physiological substrates (Fig. 2b). Accordingly, studies with isoform-specific antisera demonstrate that PKC subtypes do have distinct subcellular locations in

specified cell types. For example, phorbol ester treatment causes a rapid redistribution of several PKC isoforms overproduced in 3T3 fibroblasts. PKC-α accumulates in the ER and cell margins, PKC-βII associates with the actin cytoskeleton, PKC-γ is concentrated in the golgi and PKC-ε is found at the nuclear membranes[23]. PKC targeting clearly involves protein–lipid interactions: the kinase has two membrane-targeting domains, C1 and C2, which contain diacylglycerol-/phorbol ester- and acidic lipid- and/or Ca^{2+}-binding sites, respectively[22]. However, it is becoming increasingly apparent that protein–protein interactions also contribute to compartmentalization[22]. Wolfe and Sahyoun[24] were the first to demonstrate that PKC bound to a variety of cytoskeletal and postsynaptic density (PSD) proteins in a phosphatidylserine (PS)-dependent manner. Since then, the laboratories of Jaken and Mochly-Rosen and Olson have significantly advanced our understanding of PKC-targeting proteins (for review, see Ref. 25). Using a variety of techniques at least three classes of PKC-binding proteins have been identified (Fig. 2b).

PKC substrate/binding proteins bind PKC in the presence of PS and have been detected by a gel-overlay procedure. A distinguishing feature is that phosphorylation regulates their association with PKC (Ref. 26). It has been proposed that PS supports a ternary complex of PKC and the substrate/binding protein. Phosphorylation simultaneously abolishes PKC-substrate/binding-protein interaction and PS-binding[26]. So far, cDNAs for 11 substrate/binding proteins have been isolated that encode proteins involved in membrane–skeleton linker functions, such as vinculin and talin, or structural components that link to the actin cytoskeleton such as MARCKS-related protein, adducins, annexins and desmoyoki[27].

Receptors for activated C-kinase (RACKs) also bind PKC, but are not substrates for the kinase[28]. Unlike the substrate-binding proteins, RACKs bind at a site that is distinct from the substrate-binding pocket of the kinase and in a PS-independent manner[25]. Peptides to a prospective PKC-binding site on annexin I inhibit PKC–RACK interaction and block insulin-induced translocation of PKC in *Xenopus* oocytes[29]. One RACK, called RACK-1, has been cloned and shows some similarity to the β subunits of G proteins, as it contains seven WD40 motifs.

A second proposed function of RACK-1 is to potentiate the phosphorylation of histones when complexed with PKC (Ref. 30). In this way, both RACK-1- and PP-1-targeting subunits might share the common functions of subcellular targeting and adaptation of kinase and phosphatase activity.

PICKs. A third class of PKC targeting proteins, designated 'proteins that interact with C-kinase' (PICKs), have been identified by two-hybrid screening. One of these proteins, PICK-1, is a perinuclear PKC substrate that exclusively binds the catalytic core of PKC-α (Ref. 31). The failure of PICK-1 to bind native PKC or the regulatory domain has led to the suggestion that this targeting protein recognizes determinants that are masked in the dormant holoenzyme[31]. Unlike the substrate/binding proteins both phosphorylated and dephosphorylated forms of PICK-1 bind equally well to the kinase. Furthermore, PICK-1 does not contain any regions with homology to the RACKs.

A fourth class? Potentially, a fourth class of targeting protein might exist, as recent evidence suggests that pleckstrin-homology (PH) domains on the Burton tyrosine kinase and RAC kinase bind PKC. PH domains might modulate the substrate specificity of PKC because binding to the 14-3-3-like protein, stratifin, enhances the phosphorylation of histones[32].

PKA anchoring

Although cAMP is the principle determinant for PKA activation it has become evident that additional regulatory mechanisms are in place to restrict the subcellular location of active enzyme. Gradients of cAMP are formed through the opposing actions of adenylate cyclase and phosphodiesterases[33,34], which might activate pools of anchored PKA. In order to facilitate this process, up to 75% of type II PKA is targeted through association of the regulatory subunit (RII) with A-kinase anchoring proteins, called AKAPs (for review, see Ref. 35). Additionally, there is now some indication that the type I PKA is also compartmentalized. RII dimerization is a prerequisite for anchoring and the AKAP-binding surface includes two isoleucine sidechains on each RII promoter[36]. By contrast, the corresponding binding site on the AKAP is a sequence of approximately 20 residues and is likely to form an amphipathic helix[37]. As a result, the topology of the RII-/AKAP-binding site includes protein–protein contacts between three polypeptide chains. Despite the complexity of these interactions, peptides spanning the AKAP helix region bind RII with high affinity (K_d = 4 nM) and are potent inhibitors of PKA anchoring. These 'anchoring inhibitor peptides' have been used as tools to disrupt the localization of the type II PKA *in vivo* and have profound effects on certain cAMP-responsive events such as activation of ion channels[38,39].

AKAPs are targeting loci. According to the targeting hypothesis, AKAPs should really be considered as targeting loci because they bind and attach a targeting subunit, RII, to subcellular structures. In order to fulfill this function each AKAP contains a unique targeting domain. In essence, the targeting domain dictates the specificity of the PKA by placing the anchored holoenzyme complex close to a particular subset of substrate proteins (Fig. 2c). Data from immunochemical and subcellular fractionation experiments have identified AKAPs at the centrosomes, ER, golgi, mictrotubules, mitochondria, membranes, nuclear matrix and secretory granules (for review, see Ref. 40). More biochemical and molecular approaches have been used to map a few AKAP-targeting sequences. A carboxy-terminal octadecapeptide-repeat sequence serves as an intracellular targeting domain for the microtubule associated protein MAP 2 (Ref. 41), two non-contiguous amino-terminal basic regions, called T1 and T2, facilitate sub-membrane attachment of AKAP 75 (Ref. 42) and a carboxy-terminal peroxisomal-targeting signal 1 (PTS-1) can target AKAP 220 to the peroxisomes (Ref. 43). Current work is under way to identify additional AKAP-targeting domains.

Multi-enzyme signaling complexes

In the past year, a new class of multivalent adapter proteins that coordinate the location of multienzyme signaling complexes has been identified. For example, the pheromone mating response in yeast is initiated through a G-protein-linked receptor that activates the STE 20 kinase. This leads to the stimulation of STE 11 (a MEKK homolog), which phosphorylates and activates STE 7 (a MEK homolog), which in turn phosphorylates and activates the mitogen-activated protein (MAP) kinase homologs (FUS 3 or KSS 1). This process proceeds efficiently because each enzyme associates with a scaffold protein called STE 5 (Ref. 44; see Fig. 3a). Clustering of successive members in

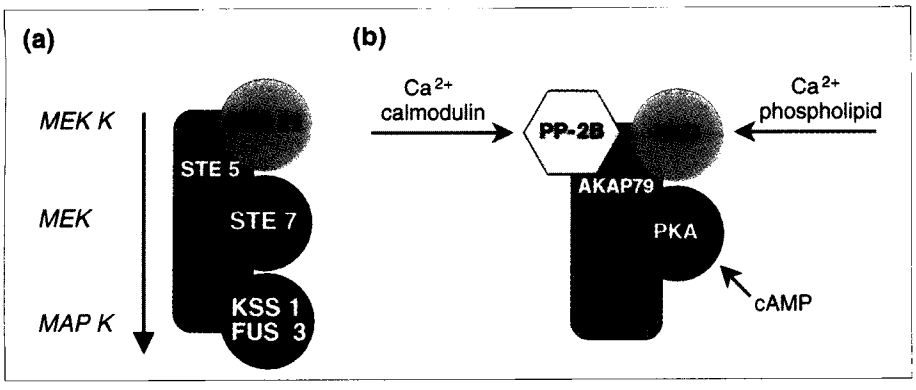

Figure 3

Multi-enzyme signaling complexes. **(a)** Coordination of yeast mitogen-activated protein (MAP) kinase cascade through STE 5. **(b)** The AKAP79 signaling complex targets PP-2B, protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) to sub-synaptic sites in neurons.

the MAP kinase cascade is optimal for the tight regulation of the pathway and prevents crosstalk between the six functionally distinct MAP-kinase modules in yeast. A considerable research effort is currently focused on determining if signaling scaffolds coordinate the mammalian MAP kinase cascade.

Another example of a multivalent adapter protein is the anchoring protein AKAP79, which seems to target PKA, PKC and PP-2B at the PSD of mammalian synapses[45]. Potentially this provides a mechanism to modulate the phosphorylation state of a single substrate in response to distinct second messenger signals (Fig. 3b). Possible substrates of the AKAP79-associated enzymes include the synaptic glutamate receptor/channels and Ca^{2+} channels, which have recently been shown to be modulated by AKAP-targeted PKA (Refs 38, 39) and NMDA receptors, which are activated by PKC (Ref. 46) and attenuated by PP-2B (Ref. 47).

Concluding remarks

Targeting subunits have emerged as a new class of regulatory molecule that contribute to the organization and specificity of signal transduction pathways. The broad-specificity phosphatases appear to use targeting subunits not only to direct the phosphatase C subunit to particular parts of the cell, but also to enhance dephosphorylation towards substrates. This might represent a mechanism to prevent indiscriminate dephosphorylation of phosphoproteins by these broad-specificity enzymes. The diversity of PP-1 and PP-2A targeting subunits might reflect the diversity of cellular locations where these enzymes act. Another feature for certain phosphatases is that phosphatase-targeting subunits are components of the holoenzyme. However, less is known about kinase targeting and it might be simply that we do not yet know how many kinases are targeted. For example, myosin light chain kinase is attached to myosin, Raf activation occurs at the membranes and many kinases containing PH domains are targeted to membranes. Undoubtedly additional kinase-targeting proteins will be identified.

Interestingly, those kinases that are localized through targeting subunits, such as PKC and PKA, have broad and overlapping substrate specificities. Now there is evidence that another multifunctional kinase, the Ca^{2+}–calmodulin kinase II, is targeted through association with anchoring proteins[48]. A variation on this theme seems to be the packaging of enzymes into signaling complexes. This might prove to be an efficient means for controlling the phosphorylation state of a given protein in response to multiple intracellular signals. No doubt the future will show that even more is on target with phosphorylation.

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