Regulation of the phosphatase PP2B by protein–protein interactions

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Protein dephosphorylation is important for regulating cellular signaling in a variety of contexts. Protein phosphatase-2B (PP2B), or calcineurin, is a widely expressed serine/threonine phosphatase that acts on a large cross section of potential protein substrates when activated by increased levels of intracellular calcium in concert with calmodulin. PxIxIT and LxVP targeting motifs are important for maintaining specificity in response to elevated calcium. In the present study, we describe the mechanism of PP2B activation, discuss its targeting by conserved binding motifs and review recent advances in the understanding of an A-kinase anchoring protein 79/PP2B/protein kinase A complex’s role in synaptic long-term depression. Finally, we discuss potential for targeting PP2B anchoring motifs for therapeutic benefit.

PP2B targeting by substrate interaction motifs and anchoring proteins

The present study focuses on how protein–protein interactions between protein phosphatase-2B (PP2B), also known as calcineurin, and substrates and anchoring proteins influence physiological processes controlled by this enzyme.

Reversible protein phosphorylation is perhaps the most well-studied post-translational modification [1]. It has been shown to be vital for almost all cellular signaling pathways, regulating signaling events in response to input from extracellular cues or from modulatory signaling cross-talk. Attachment of phosphate groups to protein substrates by kinases is reversed by the action of protein phosphatases, which are often specific for phosphotyrosine or phosphoserine/threonine residues.

PP2B is a ubiquitous serine (Ser)/threonine (Thr) phosphatase that is activated by elevated calcium levels and subsequent activation of calmodulin (CaM) [2]. The PP2B holoenzyme is a heterodimer that consists of a catalytic A subunit and a regulatory B subunit. When activated, this holoenzyme includes CaM in its calcium-bound state. Elevation of calcium and activation of CaM cause a structural rearrangement that allows CaM to bind to an α-helix in the A subunit of PP2B and remove an autoinhibitory helix from the active site of the phosphatase (Figure 1A) [3].

Because PP2B has the capacity to promiscuously dephosphorylate most solvent-exposed Ser/Thr residues, the controlled activation and inactivation by calcium transients is important for signaling specificity [4]. Moreover, substrate specificity is controlled by a variety of protein–protein interactions with anchoring proteins and allosteric recognition sequences on PP2B substrates [5–7]. In particular, the A-kinase anchoring protein 79 (AKAP79, AKAP150 in rodents; products of the AKAP5 gene), interacts with both PP2B and protein kinase A (PKA) [8,9]. This co-localization of a kinase and a phosphatase permits exquisite control of post-translationally mediated signaling events [10,11].

PP2B’s interactions with binding partners ensure that this broad spectrum phosphatase can have specific roles in diverse physiological contexts, such as neurotransmission, cardiac signaling, immune responses, and insulin signaling. We will describe the molecular basis for interactions between PP2B and anchoring proteins/substrates, and review how these interactions control cellular signaling events.
Anchoring and substrate interactions

The catalytic subunit of PP2B resembles the catalytic subunit of protein phosphatase-1 (PP1) [5,12]. However, while PP1 holoenzymes are formed via interactions with a large variety of regulatory and targeting subunits [13], PP2B holoenzymes can be formed with different combinations of three catalytic A-subunit isoforms (α, β, and γ) and two regulatory B-subunit isoforms (type 1 and type 2) [3,14,15]. These isoforms lack both the structural and functional diversity of targeted PP1 holoenzymes; therefore, the varied roles of PP2B in biological processes are often mediated by a similarly diverse range of interacting proteins.

The majority of these interactions are through two distinct surfaces on PP2B. The best-characterized surface is composed of a β-sheet on the catalytic subunit. This β-sheet interacts with a conserved motif known as the PxIxIT motif. The PxIxIT motif forms a short β-strand that interacts with β-strand 14 of the catalytic subunit and extends the β-sheet (Figure 1A) [16,17].

A more recently characterized PP2B-interacting motif is termed the LxVP motif. This motif binds near the interface of the catalytic and regulatory subunits of PP2B and forms important contacts with both subunits (Figure 1A) [6,22]. This interface is also spatially removed from the active site of the phosphatase, although it has been suggested that it serves to optimally place phosphosubstrates for efficient dephosphorylation [22]. This is supported by the observation that LxVP motifs only bind to activated PP2B (in the presence of calcium and CaM). In addition, the LxVP-binding site overlaps with the binding site for the immunosuppressant/immunophilin complexes cyclosporin/cyclophilin and FK506/FKBP12 [22]. A recent study suggests that the main mechanism of action for these immunosuppressants is to compete for binding with the LxVP motif present in NFAT. This conserved LxVP motif seems to have a looser consensus sequence than the PxIxIT, and the key requirements for binding have not yet been fully defined [5,22].

Figure 1. (A) Schematic of PP2B (gray) activation by CaM (green) and calcium. Structural rearrangement of the autoinhibitory helix (red) allows catalytic activity. PxIxIT (blue) and LxVP (orange) binding surfaces are indicated, as well as the approximate location of the active site (red circle). (B) AKAP79/SAP97-mediated complex of GluA1-containing AMPA receptors, PP2B and PKA. Upon elevation of calcium, PKA and PP2B work in a distinct manner to promote long-term depression through regulation of AMPA receptors.
The LxVP motif was originally identified in a region of the RII\(\alpha\) regulatory subunit of PKA that was used as a substrate peptide for the phosphatase [23]. However, the LxVP sequence was not recognized as a consensus motif at that time. Subsequent analyses revealed that NFAT contains an LxVP motif, as do other PP2B substrates. In addition, a peptide derived from a viral protein that contains both an LxVP motif and a PxIxIT motif was co-crystallized with PP2B, and the resulting structure has shed light on the binding site of the LxVP motif (Table 1) [22].

Proteins that contain LxVP motifs have been shown to use them for various purposes. NFAT family members use the LxVP motif to stimulate dephosphorylation of phosphosites located between the LxVP motif and the PxIxIT motif [5]. The scaffold protein KSR2 appears to only have an LxVP motif and lacks a PxIxIT, and this promotes dephosphorylation of upstream distal Ser and Thr residues [24]. Because of the flexibility inherent in the LxVP motif, it is likely that most proteins contain regions of disorder surrounding the LxVP motif that act analogously to the disorder in RII, allowing a range of nearby phosphosites to occupy a constrained yet flexible conformational space so that multiple sites can be dephosphorylated by a single-anchored PP2B molecule [22].

As more LxVP motifs are identified in other proteins, undoubtedly the function and consensus of this sequence will be more well understood. The combination of multiple binding motifs for a single phosphatase allows fine-tuning of binding affinities under different enzymatic states, while also possibly influencing substrate selectivity. The potential for the LxVP motif to fine-tune the activity and specificity of PP2B may be important for understanding how anchored PP2B acts in regulating synaptic transmission.

### Postsynaptic signaling

Synaptic plasticity — the ability of synapses to undergo changes in the strength of their response to neurotransmitter release — is believed to underlie aspects of contextual learning and memory [25,26]. Many of these changes are mediated by N-methyl-d-aspartate (NMDA) and \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors on the postsynaptic neuron, which are modulated by phosphorylation events. These covalent modifications promote changes in the number of receptors at the synapse, as well as changes to receptor ion permeability [27]. AMPA receptors are tetrameric channels that are composed of some combination of four different subunit isoforms — GluA1—4. Homomeric GluA1/1 receptors are calcium permeable, while heteromeric receptors that contain GluA2 are considered to be calcium impermeable [28].

PP2B has been suggested to be particularly important for modulating the phosphorylation state of Ser845 of the GluA1 subunit of the AMPA receptor in the hippocampal region of the brain [29–31]. Phosphorylation of Ser845 increases ion permeability and also results in increased trafficking to the postsynaptic density [29]. PP2B has long been implicated in dephosphorylation of this site in response to high-frequency stimulation and is targeted to AMPA receptors by a protein complex that involves AKAP79 and, the membrane-associated

### Table 1 PP2B-binding motifs in anchoring proteins and substrates

<table>
<thead>
<tr>
<th>PP2B-binding partner</th>
<th>Demonstrated phosphosubstrate</th>
<th>PxIxIT motif identified</th>
<th>LxVP motif identified</th>
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guanylate kinase protein, synapse-associated protein 97 (SAP97) [29,32]. SAP97 is a scaffolding protein that interacts with the GluA1 subunit of the AMPA receptor and with AKAP79 to form a macromolecular complex that serves as an important signaling node in the postsynaptic density [33,34].

While the dephosphorylation of Ser845 has been known to be important for a form of synaptic plasticity called NMDA receptor-dependent long-term depression (LTD), the exact molecular mechanisms have not been clear [35,36]. Early work implicated a PP2B/PP1 cascade in regulating this type of LTD; however, previous studies suggest that PP2B directly dephosphorylates Ser845 [37–40]. Recent work highlights the importance of the AKAP79/150 complex containing both PKA and PP2B in inducing LTD in the CA1 region of the hippocampus [41–43]. It is also important to note that a role of AKAP79/150 interactions with GABA receptors at inhibitory synapses has also been proposed. Therefore, AKAP79/150 may be a more general synaptic anchoring protein that co-ordinates the differential localization of PP2B and a variety of other kinases to various synaptic sites [44].

After initial studies with AKAP150 knockout mice [36], several knock-in mouse models were generated which express forms of the anchoring protein that lack binding sites for PKA (ΔPKA) [35], protein kinase C (ΔPKC) [45], or PP2B (ΔPIX) [30]. These mouse models have been used to study a variety of physiological arenas, such as vascular smooth muscle [45,46], cardiac function [47,48], and glucose homeostasis [49]. Recently, the ΔPIX and ΔPKA mouse lines have been used to show that in the postsynaptic density, AKAP79/150-anchored PKA is necessary to phosphorylate homomeric GluA1 AMPA receptors at Ser845 and recruit them in rapid response to calcium influx through NMDA receptors. These homomeric calcium permeable GluA1 receptors are recruited to the synapse and augment the later calcium response to glutamate in order to efficiently activate AKAP79/150-anchored PP2B [42]. This PP2B activation promotes GluA1 dephosphorylation to remove both the homomeric GluA1 AMPA receptors and the heteromeric ionotropic glutamate receptors 1/2, resulting in an overall decrease in synaptic strength [42]. This activated PP2B has also been shown to be important in spine shrinkage and trafficking of AKAP79/150 and MAGUK protein complexes away from the postsynaptic density [19,33,50,51].

Another long-standing puzzle in neuronal signaling has been the relative activity of the Ca^{2+}/CaM-dependent protein kinase II (CaMKII) and PP2B in response to postsynaptic influxes of calcium. Both enzymes are activated by calcium/CaM and have many overlapping substrates, such as the GluA1 subunit of the AMPA receptor, the NMDA receptor, and other postsynaptic targets [50,52]. How these enzymes are differentially regulated may be explained not only by their differential sensitivity to calcium, but also by their localization within the postsynaptic density by anchoring and substrate interactions. In addition, previous studies have implicated neuronal pools of AKAP79-anchored PP2B in dephosphorylation of NFAT in response to calcium entry via the L-type calcium channel [16,19].

As more is learned about the functional effects of PP2B anchoring, light will be shed on these questions as well as on the role of PP2B in the other diverse physiological events it helps control.

**Conclusion: targeting PP2B for therapeutics**

PP2B is an important therapeutic target for cyclosporin and FK506, immunosuppressants used clinically for organ transplant patients as well as to treat psoriasis and rheumatoid arthritis [53–55]. However, its role in synaptic plasticity, as well as in other processes such as insulin signaling [49], and cardiac function [56–58] make PP2B an attractive potential target for other conditions. In order to effectively target specific pathways, PP2B’s interactions with anchoring proteins, such as AKAP79 or specific phosphosubstrates, need to be disrupted. This requires a detailed understanding of what the structural basis for these interactions is, as well as the relative functions of the PxIxIT and LxVP-binding motifs.

Several small molecules that disrupt PP2B interactions have already been developed, including the INCA compounds [59] and the VIVIT peptide [17,60], both of which disrupt interactions with the PxIxIT motif. The fact that binding sites for the LxVP motif and the immunophilin complexes overlap suggests that the targeted disruption of PP2B’s interaction with the LxVP motif is a promising avenue for specific therapeutic treatment [22]. Because PP2B has a wide range of potential targets, targeting the catalytic site of PP2B is likely to have a plethora of undesirable effects, whereas targeting interaction motifs will provide the best specificity. Only a more thorough understanding of the function and molecular details of these interaction motifs will allow such an approach to be successful.
Abbreviations
AKAP, A-kinase anchoring protein; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CaM, calmodulin; CaMKII, Ca2+/CaM-dependent protein kinase II; LTD, long-term depression; NFAT, nuclear factor of activated T-cells; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PP1, protein phosphatase-1; PP2B, protein phosphatase-2B; RCAN1, regulator of calcineurin 1; SAP97, synapse-associated protein 97; Ser, serine; Thr, threonine; TRESK, TWIK-related spinal cord potassium channel.

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

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