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Protein Phosphatases: Regulating the Regulators

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Signalling through the modulation of protein phosphorylation is accepted as a dynamic process in all cells, yet protein dephosphorylation continues to receive relatively less attention. Recent reviews have served to correct this balance and, often through catchy titles (e.g. 'Protein phosphatases come of age' (1) or 'protein tyrosine phosphatases take off' (2) or 'Protein phosphatase 2A: a panoply of enzymes' (3)), have raised the awareness and profile of research focussed on the role and regulation of protein phosphatases in cellular signalling.

It is clear that phosphatases once considered the 'off switches' are now perceived more as the 'rheostats' of phosphorylation systems, providing fine or complete control necessary for the specificity of a particular signal. Consequently phosphatases are now recognised for their role in controlling a diverse array of cellular processes and disease. Nevertheless the fundamental question of how specificity is achieved from a limited number of broadly specific and widely distributed enzymes remains largely unanswered.

Protein phosphatase research has historically had 2 principal approaches; one that focussed on understanding the molecular regulation of the phosphatases directly and the other that 'stumbled' into the area from analysis of a favourite biological system. The latter approach has been, and continues to be, facilitated to a large extent by the use of cell permeable inhibitors, such as okadaic acid and calyculin A. Although these inhibitors are highly specific for serine/threonine protein phosphatases they have somewhat limited and variable capacity to distinguish between individual phosphatases *in vivo*.

Thus whilst their use can point to important functional roles it is necessary to determine exactly how and what phosphatases are being affected in the cell before the phosphatase involved can be determined. For example, concentrations of okadaic acid known to clearly inhibit both PP1 and PP2A in liver cells and adipocytes (4) fail to inhibit PP1 in mast cells (5) and fibroblasts (6), rendering okadaic acid a specific inhibitor of PP2A in these systems. Clearly, continued research focussed on determining principles of phosphatase regulation is necessary for improved understanding of their roles in specific systems or processes.

Specificity through association

In contrast to protein kinases which have diverged through numerous variations of the catalytic subunits, the serine/threonine protein phosphatases have a relatively small number of highly conserved catalytic subunits with broad and overlapping substrate specificity *in vitro*. It is now accepted that specificity of action is mainly derived from subcellular targeting of distinct pools of phosphatases through dynamic binding to relevant partners.

Whilst the catalytic subunits are also subject to post-translational modification (phosphorylation and methylation), the principal role of these modifications seems to be to influence the association with certain binding partners. A wide range of proteins also interact with protein kinases and, in many cases, both kinases and phosphatases are targeted by the same protein in a complex which often also contains a common substrate (7). These multi-enzyme signalling complexes are a rational mechanism for directing specific control of substrate phosphorylation but also add to the exquisite complexity that is inherent in cell signalling.

Whilst these principles hold true for most serine/threonine phosphatases, it is impossible to present details for all within the space of this brief review. Rather, by way of illustration, focus will be given to protein phosphatase 2A (PP2A), which together with PP1 has been estimated to account for >80% of all cellular protein dephosphorylation.

Protein phosphatase 2A – the promiscuous phosphatase

PP2A, a predominantly cytosolic enzyme, exists as a heterotrimer comprised of a conserved catalytic subunit (C), conserved scaffold subunit (A) and a variable B subunit (3). The B subunit, comprises 3 main families, each with multiple isoforms such that approximately 50 different trimeric configurations are theoretically possible.

The different B subunits confer modulation of catalytic activity and, at least potentially, contribute to the specificity of PP2A action. Despite the originally recognised cytosolic nature of the enzyme, some years ago we described a pool of trimeric PP2A in brain which was specifically associated with the cell membrane (8). Subsequently, nuclear (9) and cytoskeletal (10) pools of PP2A were also shown to exist.

More recently, we and others have shown that, at least in some systems, subcellular pools of PP2A are dynamically regulated in response to extracellular signals and the movement of PP2A between different subcellular loci is critical for a number of cell functions (see below). Whilst some of these individual pools comprised different B subunits, the same B subunits can be found in each subcellular location, suggesting that the B subunit alone is not responsible for directing the subcellular localisation of PP2A.

This has led to a search for additional binding partners and a bewildering array of proteins which bind PP2A is emerging. In most cases the identification of these binding proteins has been the key to identifying new functions for PP2A in regulating cell signalling.

PP2A binding partners

Proteins which bind PP2A have been discovered either as a result of a deliberate search for binding partners or through discovering PP2A as a binding partner for another protein of interest. Crystal structure analysis of the A subunit indicates that it is a hook-shaped protein binding both C and B subunits via a hydrophobic surface created by loops connecting 15 imperfect, 39 amino acid repeats (so called HEAT repeats).

This arrangement leaves an external face capable of interacting with other proteins. The B subunit also provides poten-

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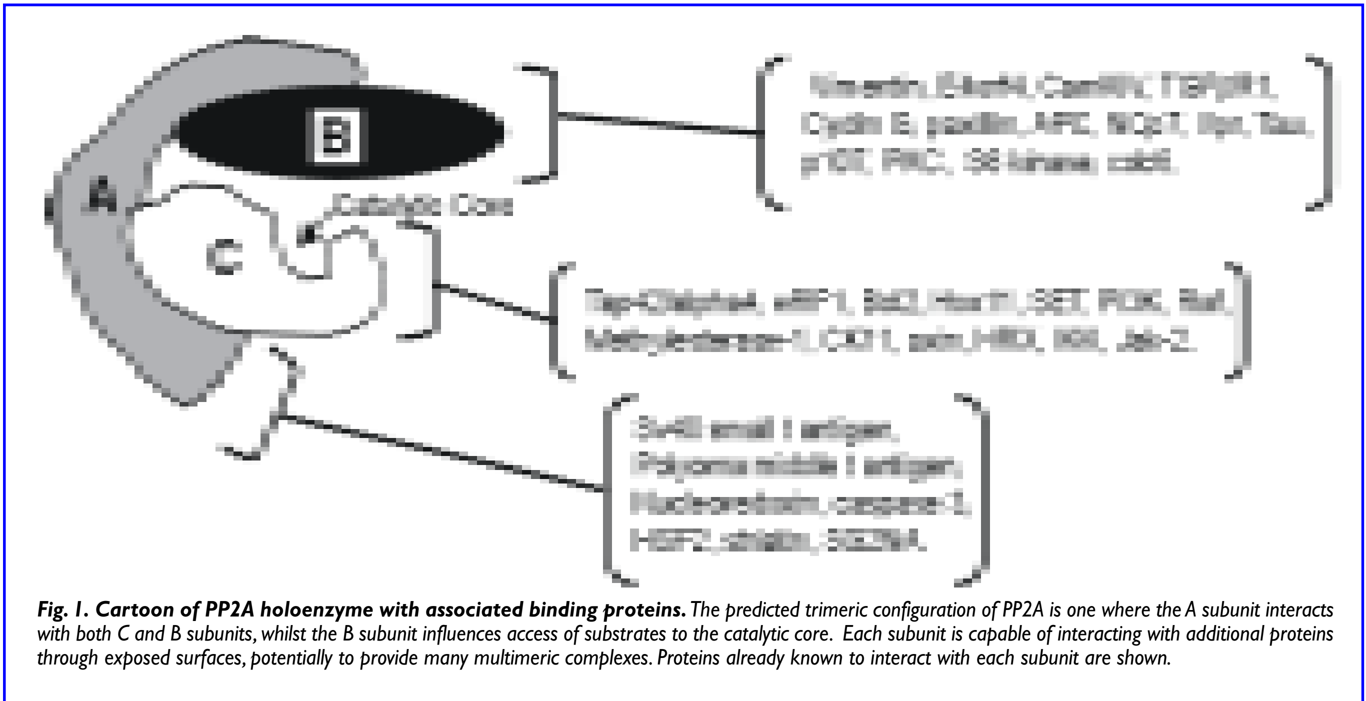

Fig. 1. Cartoon of PP2A holoenzyme with associated binding proteins. *The predicted trimeric configuration of PP2A is one where the A subunit interacts with both C and B subunits, whilst the B subunit influences access of substrates to the catalytic core. Each subunit is capable of interacting with additional proteins through exposed surfaces, potentially to provide many multimeric complexes. Proteins already known to interact with each subunit are shown.*

tial binding sites for interacting proteins. One family of B subunit contains 5 WD repeats (conserved sequences of around 40 amino acids which typically end in trytophan-aspartate and are thought to mediate protein-protein interactions) whilst the other B subunits (B' and B") do not (11). It is likely that the WD repeats are responsible for interacting with additional proteins (rather than the A or C subunits).

This information has led to a cartoon (**Fig. 1**) of the trimeric complex illustrating the capacity of each subunit to confer differential access of various substrates to the catalytic core of the C subunit. Furthermore, the capacity can be conceptualised for many additional proteins to interact with the trimeric complex, through binding not only the A subunit but also the C and B subunits.

In some cases the binding partner replaces the native B subunit thereby modifying substrate specificity or enzyme location. For example, the small t antigen of SV40 tumour virus inhibits PP2A by displacing the B subunit (12). Interestingly, another B subunit (B') can displace SV40 small t antigen and reverse its cellular effects. These findings are important in linking PP2A to DNA replication and cell transformation. They also suggest that dynamic exchange of B subunits and binding proteins can have dramatic effects on cell function.

Moreover, the capacity to manipulate native trimeric configuration through expression of SV40 small t antigen, or B subunit constructs (sense and antisense) to displace or down regulate endogenous B subunits, provides a useful tool to investigate PP2A function.

The trimeric holoenzyme is capable of binding at least 3 other proteins such that multimeric PP2A complexes can be formed. Perhaps the most extreme form of PP2A complex formation is seen in the wnt signalling pathway where low levels of β-catenin are maintained by phosphorylation-dependent degradation by the ubiquitin proteasome pathway. The adenomatous polyposis coli (APC) protein binds the B' subunit (13) whilst axin has been shown to interact with the C subunit of PP2A (14). B'-subunit binding to APC results in reduced β-catenin and this is believed to be due to inhibition of PP2A and elevation of phosphorylation levels. The kinase responsible, GSK3β, also binds axin, is a substrate for PP2A and has been found in the same complex as PP2A, axin and β-catenin. This implies the formation of a hexameric complex where the A subunit of PP2A represents the cornerstone, and the B' subunit modulates the activity of the C subunit towards the other 3 complex members.

Despite the existence of these, often elaborate, complexes, their discovery and characterisation represents only the first step in understanding the molecular interactions underlying the cellular processes with which they are associated. Further progress has largely been hampered by the fact that until recently no means of regulating PP2A in response to extracellular signals have yet been defined.

Signalling to PP2A

Recent work from our laboratory, and subsequently others, has indicated that the subcellular location and activity of PP2A can indeed be dynamically altered by extra-cellular signals. In collaboration with Dr Russell Ludowyke we have found that, in response to secretagogue stimulation of mast cells, a pool of PP2A is transiently translocated from the cytosol to the membrane (5). Moreover this translocation matches the peak rate of secretion of inflammatory mediators and okadaic acid blocks secretion.

Okadaic acid reduces exocytosis from a number of secretory cells, including neurons, where it has been shown to disrupt synaptic vesicle clustering (15). From these and other data, a picture is emerging of a role for PP2A in exocytotic vesicle targeting to release sites. Whilst neurotrans-

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mitter release is a highly specialised process, studies from yeast and other cell types suggest that the molecular mechanisms of intracellular vesicle trafficking are highly conserved. Analysis of signal-mediated PP2A translocation in model systems such as mast cells, where the process occurs over minutes, therefore holds great promise for understanding the role of PP2A in the rapid exocytosis that occurs in neurons.

Curiously, translocated PP2A is inactive and requires a signal generated by the influx of external calcium (5). In isolated synaptosomes, high levels of inactive membrane-bound PP2A also exist and the activity can be altered by increasing the extracellular calcium concentration (16). In addition, PP2A has been shown to form complexes with CaM-PKIV, further linking it with Ca^{2+} signalling (17). Thus far, no mechanism for calcium activation of PP2A has been found, but one recent, perhaps serendipitous, finding adds substance to the proposal.

Western blot analysis using a B'-specific monoclonal antibody revealed an additional protein which also appeared in PP2A immunoprecipitates. Peptide sequencing revealed that the protein was striatin, a known protein which also binds calmodulin in a calcium-dependent manner (18). Striatin shares a conserved epitope with B', contains several WD40 repeats and has been proposed to represent a new family of calmodulin-binding PP2A B subunit. Striatin-bound PP2A has lower activity than the native holoenzyme but the effect of Ca^{2+}-dependent calmodulin binding remains to be determined.

Whilst subcellular targeting of the AC dimer or ACB trimer appears to be the principal mechanism of regulating PP2A, the carboxyl terminus of the catalytic subunit seems to be a focal point for regulation. PP2A is subject to tyrosine phosphorylation (Y-307) and carboxymethylation (L-309) and this region also contains residues important for stable interaction with the regulatory subunits. Despite the close proximity of these residues they confer quite different properties on the enzyme and may indeed be mutually exclusive.

Tyrosine phosphorylation potently inhibits enzyme activity whilst carboxymethylation modestly increases activity. Tyrosine phosphorylation abolishes B subunit binding whilst carboxymethylation is essential for B subunit binding. Moreover, tyrosine phosphorylation blocks carboxymethyl-ation. Although reports of *in vivo* PP2A tyrosine phosphorylation are sparse, mutation studies clearly indicate a role for this modification in determining the ability of the AC core to bind other proteins (19).

Carboxymethylation is reversible and PP2A-specific methyltransferase and methylesterase enzymes have been purified and cloned (20). PP2A has been clearly shown to undergo cell cycle-dependent changes in methylation but the mechanism remains unknown. Whilst earlier reports suggested that PP2A carboxymethylation could be stimulated by cAMP, more recent reports suggest that the methyltransferase is constitutively active. Control may therefore reside with the methylesterases.

Interestingly, although PP2A is highly methylated in most cells, the PP2A recovered from mast cell membranes after stimulation is in the demethylated form. Since the demethylase is found in both cytosol and membrane, it remains to be seen if demethylation is required for translocation or occurs subsequent to it.

Summary and perspectives

This snapshot article illustrates the complexity that is PP2A. PP1 and other serine/threonine phosphatases are similarly regulated with an equally diverse array of interacting proteins. Although protein phosphatases are clearly implicated in the regulation of cellular signalling events, much remains to be determined in defining exactly how these complex enzymes are regulated in response to long and short term signals.

The mechanisms underlying the dynamic exchange of pools of PP2A between subcellular compartments are likely to be unique to each cell system, such that the binding proteins in each cell system will need to be identified and characterised. Nevertheless, broad principles of regulation underlying functions common to many cell types are likely to be gained in the near future utilising model systems, such as mast cells, which will allow focussed analysis of specific, perhaps more complex systems, such as neurons.

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