REVIEW ARTICLE

Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling

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Protein phosphatase 2A (PP2A) comprises a family of serine/threonine phosphatases, minimally containing a well conserved catalytic subunit, the activity of which is highly regulated. Regulation is accomplished mainly by members of a family of regulatory subunits, which determine the substrate specificity, (sub)cellular localization and catalytic activity of the PP2A holoenzymes. Moreover, the catalytic subunit is subject to two types of post-translational modification, phosphorylation and methylation, which are also thought to be important regulatory devices. The regulatory ability of PTPA (PTPase activator), originally identified as a protein stimulating the phosphotyrosine phosphatase activity of PP2A, will also be discussed, alongside the other regulatory inputs. The use of specific PP2A inhibitors and molecular genetics in yeast, *Drosophila* and mice has revealed roles for PP2A in cell cycle regulation, cell morphology and development. PP2A also plays a prominent role in the regulation of specific signal transduction cascades, as witnessed by its presence in a number of macromolecular signalling modules, where it is often found in association with other phosphatases and kinases. Additionally, PP2A interacts with a substantial number of other cellular and viral proteins, which are PP2A substrates, target PP2A to different subcellular compartments or affect enzyme activity. Finally, the de-regulation of PP2A in some specific pathologies will be touched upon.

Key words: cell cycle, dephosphorylation, methylation, PP2A, tumour suppressor.

INTRODUCTION

Reversible protein phosphorylation is an essential regulatory mechanism in many cellular processes. In general, cells use this post-translational modification to alter the properties (activity, localization, etc.) of key regulatory proteins involved in specific pathways. While in the past much attention has been paid to the regulation of protein kinases, it is now apparent that protein phosphatases (PPases) – like the kinases – are highly regulated enzymes that play an equally important role in the control of protein phosphorylation. This review focuses on the structure, regulation and biological role of protein phosphatase type 2A (PP2A), a major serine/threonine PPase in eukaryotic cells.

STRUCTURE OF PP2A

Several holoenzyme complexes of PP2A have been isolated from a variety of tissues and have been extensively characterized. The core enzyme is a dimer ($PP2A_D$), consisting of a 36 kDa catalytic subunit ($PP2A_C$) and a regulatory subunit of molecular mass 65 kDa, termed PR65 or the A subunit. A third regulatory B subunit can be associated with this core structure. At present, four different families of B subunits have been identified, termed the B, B′, B″ and B‴ families (Figure 1). The association of these third subunits with $PP2A_D$ is mutually exclusive [1].

Although the core dimer has been purified from many different tissues, its presence *in vivo* has long been the subject of debate: whereas some believed that it represented an artifact of enzyme purification, generated from the trimeric forms by dissociation or proteolysis of the B subunit [2,3], others argued that the dimer, with its specific properties, was already present early in the purification [4]. Later, by the use of monoclonal antibodies generated against specific holoenzyme complexes, $PP2A_D$ was shown to represent at least one-third of the total cellular PP2A [5].

The catalytic subunit

Molecular cloning revealed the existence of two mammalian $PP2A_C$ isoforms, α and β, which share 97% identity in their primary sequence [6–8]. Both isoforms are ubiquitously expressed, and very high levels are found in brain and heart.

Abbreviations used: AKAP, muscle A-kinase anchor protein; AP-1, activator protein-1; APC, adenomatous polyposis coli protein; CAK, Cdk-activating kinase; CAPP, ceramide-activated protein phosphatase; Cdk, cyclin-dependent kinase; CG-NAP, centrosome- and Golgi-localized protein kinase N-associated protein; CREB, cAMP regulatory element binding protein; Dsh, Dishevelled; 4E-BP1, eukaryotic initiation factor 4E binding protein 1; eRF, eukaryotic release factor; ERK, extracellular-signal-regulated kinase; GSK, glycogen synthase kinase; HEAT, huntingtin/elongation/A subunit/TOR; HSF, heat-shock transcription factor; I_1^{PP2A} and I_2^{PP2A}, inhibitors of PP2A; IκB, inhibitor of nuclear factor-κB; JAK2, Janus kinase 2; LCMT, leucine carboxyl methyltransferase; Lef, lymphoid enhancer binding factor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; MPF, M-phase-promoting factor; (m)TOR, (mammalian) target of rapamycin; NF-κB, nuclear factor-κB; OA, okadaic acid; PAK, p21-activated kinase; PHAP, putative histocompatibility leucocyte antigen class II-associated protein; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PKR, double-stranded RNA-dependent kinase; Plx1, polo-like kinase; PME-1, PP2A methylesterase 1; PP2A, protein phosphatase type 2A; $PP2A_C$, catalytic subunit of PP2A; $PP2A_D$, dimeric form of PP2A; PPase, protein phosphatase; PTPase, phosphotyrosine phosphatase; PTPA, PTPase activator; RTS1, rox three suppressor 1; SCR, sex combs reduced; SET, Suvar3-9, enhancer of zeste, trithorax; SG2NA, S/G_2 nuclear autoantigen; SIT4, suppressor of *His4* transcription 4; SV40, simian virus 40; Tap, two A phosphatase-associated protein; TCF, T-cell factor; TGF-β, transforming growth factor-β; TNFα, tumour necrosis factor α; TPD3, tRNA production defect; TRIP-1, TGF-β receptor II interacting protein 1; YY1, Yin Yang 1.

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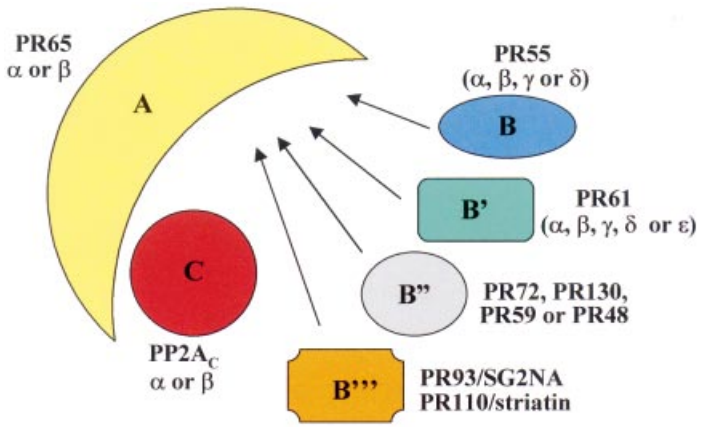

Figure 1 Structure of PP2A

C is the catalytic subunit, A is the second regulatory or structural subunit, and B/B′/B″/B‴ are the third variable subunits, which are structurally unrelated. In Mammalia, A and C are encoded by two genes (α and β); the B/PR55 subunits are encoded by four related genes (α, β, γ and δ); the B′/PR61 family are encoded by five related genes (α, β, γ, δ and ϵ), some of which give rise to alternatively spliced products; the B″ family probably contains three related genes, encoding PR48, PR59 and the splice variants PR72 and PR130; SG2NA and striatin comprise the B‴ subunit family.

However, PP2A$_{C\alpha}$ is about 10 times more abundant than PP2A$_{C\beta}$ [9]. These isoforms are encoded by different genes [8,10], localized to human chromosome 5q23–q31 for α and to 8p12–p11.2 for β [11]. Expression from the PP2A$_{C\alpha}$ gene promoter is 7–10-fold stronger than from the PP2A$_{C\beta}$ gene promoter, which may explain the difference in protein levels between the isoforms [10]. The molecular cloning of PP2A$_C$ from lower organisms, such as *Xenopus* [12,13], *Drosophila* [14], the plants *Brassica napus* [15] and *Arabidopsis thaliana* [16], and the yeasts *Schizosaccharomyces pombe* [17] and *Saccharomyces cerevisiae* [18], has revealed that the structure of PP2A$_C$ has remained remarkably constant throughout evolution, and may even be the most conserved of all known enzymes [19].

Attempts to overexpress functional PP2A$_C$ in mammalian cells with standard gene transfer techniques have long been unsuccessful [7], making the study of PP2A in mammalian cells rather frustrating. However, modifying the N-terminus by addition of a peptide sequence derived from the influenza haemagglutinin protein (HA-tag) appeared to be sufficient to stabilize the PP2A$_C$ translation product in a functional, active form [20]. Nevertheless, despite the availability of this expression vector, it was still not possible to stably overexpress PP2A. Apparently, the expression of PP2A$_C$ within cells is tightly controlled to ensure that relatively constant levels of PP2A are present. This autoregulatory control is exerted at the translational level and does not involve transcriptional mechanisms [21].

The regulatory A subunit (PR65)

The A subunit is a structural subunit that is tightly associated with PP2A$_C$, forming a scaffold to which the appropriate B subunit can bind. Different B subunits interact via the same or overlapping sites within the A subunit of the core dimer, which explains why binding of the B subunits is mutually exclusive [22,23].

As is the case for the catalytic subunit, in Mammalia two distinct PR65 isoforms are present, α and β, which share 86% sequence identity; both are ubiquitously expressed [24]. In general, PR65β appears to be much less abundant than PR65α, except in *Xenopus* oocytes [25]. In the latter, the PR65β mRNA is highly expressed in the ovary during oogenesis, meiotic maturation and fertilization, up to stage 35 of embryogenesis. From that stage on, the β/α ratio starts to decrease gradually [26]. Interestingly, PR65β has been identified as a putative human tumour suppressor [27,28]. Somatic alterations in the gene encoding PR65β were discovered in 15% of primary lung and colon tumour-derived cell lines. These alterations, including entire gene deletions, internal and C-terminal protein deletions, and missense and frame shift mutations, are likely to disrupt the structure of the core PP2A heterodimer. More recently, in the gene encoding the PR65α isoform, mutations were detected in human melanomas and breast and lung carcinomas, albeit with lower frequency when compared with the PR65β studies [29]. Aberrant levels of A/PR65 may severely compromise the functional activity of PP2A to regulate the cell cycle (see below), a notion supported by the finding that rat fibroblasts overexpressing PR65α, become multinucleated [30].

The structure of PR65 is unusual, since it is entirely composed of 15 tandem repeats of a 39-amino-acid sequence, termed a HEAT (huntingtin/elongation/A subunit/TOR, where TOR is target of rapamycin) motif. Tandem repeats of HEAT motifs are found in a variety of proteins (reviewed in [31]), including the huntingtin protein, an elongation factor required for protein synthesis and the TOR kinase. The crystal structure of PR65α [32] revealed that the fundamental architecture of each repeat is virtually the same, being composed of two superimposed α-helices. The particular stacking of these repeats within the PR65 molecule gives rise to a stable protein with an overall asymmetrical and elongated architecture, reminiscent of a hook (C-shape). The evolutionarily conserved PR65 residues and the exposed hydrophobic surfaces are localized to the intra-repeat turns, connecting the two helices of each HEAT motif, and are likely to constitute the sites of interaction with the catalytic subunit and the B subunits. As such, the structural data seem to confirm the biochemical data obtained from reconstitution experiments with wild-type and mutated PP2A subunits [22,23,33]. Similarly, the Lys$^{416} \rightarrow$ Glu substitution, known to reduce the affinity between PR65 and PP2A$_C$ by 100-fold [34], could be rationalized by the structural data [32].

The regulatory B subunits

Two striking features of the B subunits are their diversity, stemming from the existence of entire subunit families, and the total lack of sequence similarity between these gene families, even though they recognize similar segments of the A subunit.

B or PR55 family

In Mammalia, the 55 kDa subunit is encoded by four genes (*PR55*α, *PR55*β, *PR55*γ and *PR55*δ), which are expressed in a tissue-specific manner [35–38]: PR55α and PR55δ have a widespread tissue distribution, whereas PR55β and PR55γ are highly enriched in brain. Analysis of the spatial and temporal expression patterns of PR55α, PR55β and PR55γ in the brain revealed that levels of PR55α are high in striatum, those of PR55γ are high in hindbrain, and those of PR55β are low in cerebellum [39]. PR55α and PR55β also have distinct localization patterns within neurons: PR55α is distributed primarily in the cell body and nucleus of Purkinje cells, whereas PR55β is excluded from the nucleus and extends into dendrites. PR55α and PR55β are mainly cytosolic, and PR55γ is enriched in the cytoskeletal fraction. In contrast with PR55α, PR55β and PR55γ are developmentally

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regulated, with PR55β levels decreasing and PR55γ levels increasing sharply after birth [39]. As the A and C subunits are present uniformly, these data indicate that the B subunits confer subcellular localization, developmental regulation and cell specificity to the PP2A holoenzyme in brain. Recently, in subjects with spinocerebellar ataxia (a neurodegenerative disorder), an expanded CAG repeat has been identified immediately upstream of the *PR55*β gene (extending into the 5′ untranslated region) that might affect PR55β expression and might be implicated in the aetiology of the disease [40].

A structural feature of the PR55 subunits is the presence of five degenerate WD-40 repeats [41]. WD-40 repeats are minimally conserved sequences of approx. 40 amino acids that typically end in tryptophan–aspartate (WD) and are thought to mediate protein–protein interactions (reviewed in [41]). In this respect, PR55α and PR55β have been shown to interact with the cytoplasmic domain of kinase-active type I transforming growth factor-β (TGF-β) receptors and to be a direct target for their kinase activity [42]. Previously it had been demonstrated that TGF-β receptor II interacting protein-1 (TRIP-1), a protein largely composed of WD-40 repeats, can associate with the closely related type II TGF-β receptors [43], thereby suggesting that binding of both PR55 and TRIP-1 to the TGF-β receptors could be mediated by their WD-40 repeats.

B′ or PR61 family

The B′ family contains at least five distinct gene products, denoted α, β, γ, δ and ϵ [44–50], which have been localized to human chromosomes 1q41, 11q12, 3p21, 6p21.1 and 7p11.2–p12 respectively [51]. The human B′β gene encodes two isoforms, β1 and β2. At least three different splice variants exist of the human B′γ form (γ1, γ2 and γ3; in addition, γ4 and γ5 may be predicted from rabbit cDNA sequences [45]). All B′ family members contain a highly conserved central region (80% identical), while both the C- and N-termini are significantly more divergent. This suggests that the conserved region is required for interaction with the A and possibly the C subunit, whereas the ends may perform different functions, such as regulation of substrate specificity and subcellular targeting. Specifically, PR61α, PR61β and PR61ϵ localize to the cytoplasm, whereas PR61γ1, PR61γ2 and PR61γ3 are concentrated in the nucleus, and PR61δ is found in both the nucleus and the cytoplasm. Moreover, all isoforms except one (PR61γ1) are phosphoproteins [48]. PR61δ, for instance, which was purified initially from human erythrocytes [52], can be phosphorylated *in vitro* by protein kinase A (PKA) [52,53] (see below).

Northern analyses indicate tissue-specific expression of the isoforms. PR61α and PR61γ1–γ3 are widely expressed, and are extremely abundant in heart and skeletal muscle [44,46]. PR61β and PR61δ are expressed predominantly in brain [44,45]. Interestingly, upon retinoic acid-induced differentiation of neuroblastoma cells, the expression of the brain-specific PR61β and PR61δ forms increases, whereas the isoforms that are less abundant in brain show a slight or no increase in expression [48]. This suggests that the expression of PR61β and PR61δ may be developmentally regulated in the brain.

B″ or PR72 family

A trimeric PP2A holoenzyme containing the PR72 subunit was purified from rabbit skeletal muscle and its third subunit cloned from a human heart muscle library [54]. A related clone, PR130, was isolated from a human brain library and contains a different N-terminus. It was suggested that PR72 and PR130 might arise from the same gene by alternative splicing. PR72 is expressed exclusively in heart and skeletal muscle, whereas PR130 was detected in almost all tissues analysed, but with highest levels in heart and muscle [54].

Recently, two new members of the B″ family were identified by a yeast two-hybrid screen approach. PR59 shares 56% identity and 65% similarity with PR72, and was identified as an interaction partner of the retinoblastoma-related p107 protein [55]. Its expression pattern differs completely from that of PR72, being detected in testis, kidney, liver, brain, heart and lung, but not in skeletal muscle. Interestingly, overexpression of PR59 results in inhibition of cell cycle progression and accumulation of cells in G_1. This phenomenon is probably related to an increase in the amount of hypophosphorylated – and thus active – p107 that was observed when PR59 and p107 were co-expressed.

PR48 shares 68% homology with PR59, and was identified as an interaction partner of Cdc6, a protein required for the initiation of DNA replication [56]. PR48 localizes to the nucleus, and Cdc6 seems to be a selective substrate for the PR48-containing PP2A trimer. As with PR59, overexpression of PR48 causes a G_1 arrest. It is believed that PP2A keeps Cdc6 in the dephosphorylated form, a prerequisite for binding to origins of DNA replication.

B‴ or PR93/PR110 family

Based on a conserved epitope, shared with the B′ subunits, striatin (PR110) and S/G_2 nuclear autoantigen (SG2NA; PR93) were identified as new members of a potential B‴ subunit family [57]. Like the B/PR55 family, striatin and SG2NA contain WD-40 repeats and interact with $PP2A_D$. Both proteins also bind to calmodulin in a calcium-dependent manner. Striatin is localized to the post-synaptic densities of neuronal dendrites, whereas SG2NA is nuclear. Striatin–$PP2A_D$ and SG2NA–$PP2A_D$ complexes contain several additional unidentified proteins, suggesting that striatin and SG2NA may function as scaffolding proteins involved in Ca^{2+}-dependent signalling [57].

REGULATION OF PP2A

Holoenzyme composition

Although there are only two C subunit isoforms of PP2A, the number of potential combinatorial associations of the different A and B/B′/B″/B‴ regulatory subunits is very large. Given the occurrence of two A, two C, four B, at least eight B′, four B″ and two B‴ isoforms, a total of about 75 different dimeric and trimeric PP2A holoenzymes can be generated. This specific holoenzyme composition provides many possibilities for regulation. First, due to their specific cellular and subcellular localizations, the third subunits can target the PPase to different tissues and cellular compartments. Secondly, the presence of different regulatory subunits has been shown to determine the substrate specificity of PP2A holoenzymes *in vitro* [58–64], and probably also *in vivo*. For instance, the presence of PR55 is a prerequisite for PP2A to efficiently dephosphorylate the intermediate filament protein vimentin *in vitro* and *in situ* [65]. In addition, PR55-containing trimers can efficiently dephosphorylate substrates phosphorylated by $p34^{cdc2}$ kinase [62,64]. Thirdly, the presence or absence of additional subunits can modulate the response to agents that modify PP2A activity *in vitro* (such as protamine and heparin) [34,66], and affects the catalytic activity of PP2A towards the same substrate. For instance, the C subunit as such is more active towards phosphoprotein substrates than is the core enzyme ($PP2A_D$) *in vitro* [34], whereas the reverse is true for activity towards phosphopeptides [60]. On the other hand, in the presence of polycations, A/PR65 stimulates $PP2A_C$ activity

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towards phosphoproteins [34], whereas polycations are without effect on $PP2A_D$ activity towards phosphopeptides [60]. Similarly, the B subunits also modulate the catalytic activity of $PP2A_D$, suppressing activity towards some substrates, but greatly enhancing the dephosphorylation of others [52,58,59,61,62,67,68].

Post-translational modifications

Phosphorylation

In vitro, the catalytic subunit of PP2A can be phosphorylated by the tyrosine kinases $pp60^{v\text{-}src}$, $pp56^{lck}$, and the epidermal growth factor and insulin receptors [69]. The phosphorylation occurs on Tyr^{307}, which is located in the conserved C-terminal part of $PP2A_C$, and results in inactivation of the enzyme. Tyrosine phosphorylation of $PP2A_C$ is enhanced in the presence of the phosphatase inhibitor okadaic acid (OA), suggesting that, under normal conditions, PP2A can rapidly re-activate itself in an autodephosphorylation reaction. This observation has the important implication that PP2A can also act as a phosphotyrosine phosphatase (PTPase) (see below). *In vivo*, tyrosine phosphorylation of $PP2A_C$ was detected in a small PP2A fraction recovered from activated human T cells, and in fibroblasts overexpressing $pp60^{v\text{-}src}$ [70]. Moreover, growth stimulation of cells in response to epidermal growth factor or serum [71], in response to interleukin-1 or tumour necrosis factor α (TNFα) [72], or in response to insulin [73–75] also promoted a transient tyrosine phosphorylation and inactivation of PP2A. Thus the increased phosphorylation of PP2A in intact cells by growth factors and cell transformation implies *in vivo* regulation of PP2A. Moreover, the concomitant transient inactivation of PP2A could be an accelerating factor during the transmission of signals through kinase cascades. This attractive model places PP2A in a pivotal position to modulate signal transduction cascades.

In addition to phosphorylation on Tyr^{307}, $PP2A_C$ can also be phosphorylated *in vitro* on threonine(s) by an autophosphorylation-activated protein kinase [76]. The precise location of the Thr phosphorylation site(s) remains to be determined. Interestingly, this phosphorylation leads to the inactivation of both the phosphoserine/threonine [76] and the phosphotyrosine PPase activities of PP2A [77]. In this case also, PP2A can reactivate itself by autodephosphorylation. The physiological implications of the inactivation of PP2A by threonine phosphorylation remain elusive.

Not only is $PP2A_C$ subject to phosphorylation, but also the regulatory B subunits – notably those of the B′ family – can be phosphorylated. Phosphorylation of PR61δ might regulate PP2A activity *in vivo*, since phosphorylation of PR61δ by PKA *in vitro* changes the substrate specificity of the trimeric enzyme, without dissociating the B′ subunit from PP2A [53]. More recently, the double-stranded-RNA-dependent protein kinase (PKR) was shown to interact with and phosphorylate PR61α, both *in vitro* and *in vivo* [78]. Phosphorylation of PR61α by PKR increased the activity of the AB′αC trimer towards protein kinase C (PKC)-phosphorylated myelin basic protein and PKR-phosphorylated eukaryotic translation initiation factor 2α *in vitro*. Indirect evidence was obtained to confirm this PKR-mediated alteration of PP2A activity *in vivo* [78].

Methylation

All $PP2A_C$ sequences identified to date have a $T^{304}PDYFL^{309}$ motif at their C-terminus. This motif not only contains Tyr^{307}, but is also the recognition site for carboxymethylation by a specific carboxyl methyltransferase [79–81]. Methylation occurs on the carboxy group of the C-terminal residue Leu^{309} and is reversible *in vivo*, due to the presence of a specific methylesterase [82,83]. OA inhibits methylation of PP2A [84,85], probably by binding to the C-terminus and thereby preventing access of the transferase to its target site. In *Xenopus* oocyte extracts, cAMP moderately stimulates PP2A methylation, whereas Ca^{2+} and calmodulin have no effect [85]. In contrast with these observations, it was reported that, in rat pancreatic cells, Ca^{2+} stimulated methylation and cAMP had no influence [86]. Interestingly, methylation of $PP2A_C$ varies during the cell cycle. In general, $PP2A_C$ is methylated throughout the cycle, but temporary decreases in methylation are observed at the G_0/G_1 boundary in the cytoplasm, and at the G_1/S boundary in the nucleus [87]. The mechanism and the physiological consequences of this oscillating methylation are unknown.

The human methyltransferase and methylesterase have recently been cloned. LCMT-I (leucine carboxyl methyltransferase-I) was purified from porcine brain, and the human homologue was cloned based on tryptic peptides from the porcine form [88]. Database screening revealed the existence of a putative homologue with a long C-terminal extension (LCMT-II) containing five Kelch-like repeats, but it remains to be determined whether this protein actually possesses methyltransferase activity towards PP2A [88]. PME-1 (PP2A methylesterase-1) was identified as a protein that associated specifically with two catalytically inactive mutants of PP2A [89]. Like $PP2A_C$, PME-1 is highly expressed in brain and testis.

Conflicting data exist with regard to the effect of $PP2A_C$ methylation on its catalytic activity, with one group observing a moderate increase in phosphatase activity [90], another seeing no direct effect on phosphatase activity [88], and a third observing a decrease in activity [91]. Therefore it is believed that methylation of $PP2A_C$ may affect other characteristics of PP2A. In this respect it was demonstrated that, in order for the B/PR55α subunit to bind, not only do the seven C-terminal residues of $PP2A_C$ have to be intact [92], but also the C-terminal Leu^{309} residue has to be methylated [93]. Moreover, $PP2A_D$, as isolated from tissues, was found to be fully demethylated [88,93], whereas trimers containing B‴/striatin/SG2NA [57] or B″/PR72 [88] were fully methylated, and trimers containing B/PR55 were sometimes methylated and sometimes not [88]. Taken together, the reversible carboxymethylation of $PP2A_C$ may affect the holoenzyme composition of PP2A. *In vivo* evidence for this hypothesis is provided by a transient and reversible interconversion of holoenzyme forms (from a B′-containing trimer to a B-containing trimer) during the initial stage of retinoic acid-induced granulocytic differentiation, which coincides with increased methylation of $PP2A_C$ [91]. However, whether the increased methylation of $PP2A_C$ is the consequence rather than the cause of this interconversion remains to be determined. Moreover, disruption of the *Saccharomyces cerevisiae* homologue of LCMT-I (but not LCMT-II) results in decreased methylation of PPH21 (one of the budding-yeast homologues of $PP2A_C$) and in decreased binding of TPD3 (tRNA production defect 3; a PR65 homologue), Cdc55 (a PR55 homologue) and, to a lesser extent, RTS1 (rox three suppressor 1; a PR61 homologue) [94]. This suggests that, in yeast, methylation of PPH21 is important for the formation of trimeric and dimeric PP2A complexes *in vivo*.

Second messengers: activation of PP2A by ceramide

In many signalling pathways, one of the early intracellular biochemical responses to extracellular stimulation is the generation of lipid-like second messengers, such as ceramide. Depending on the cell type, ceramide can induce differentiation, cell

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proliferation, growth arrest, inflammation or apoptosis (reviewed in [95,96]). A number of direct cellular targets for ceramide have been identified, including a ceramide-activated protein kinase, a ceramide-activated protein phosphatase (CAPP) and PKCζ [95,96].

CAPP was initially identified as a cytosolic PPase that was cation-independent and inhibited by OA [97]. Later studies identified the enzyme as a type 2A PPase [98]. Ceramide activation of PP2A was initially reported to require the presence of the B subunit [98], but a more recent study has indicated that ceramide is also able to activate $PP2A_D$ and the catalytic subunit alone [99]. Purification and characterization of CAPP from rat brain revealed that it is composed predominantly of ABC and AB′C, as well as AC complexes [100]. The role of PP2A as a ceramide effector is conserved in yeast [101]. In mammalian cells, some downstream targets of CAPP include c-*myc*, Bcl2 and c-Jun. In HL-60 cells, activation of the TNFα receptor results in the generation of ceramide, down-regulation of c-*myc* and eventually apoptosis. Since OA inhibits these effects, it was suggested that CAPP activity is important for the ceramide-induced down-regulation of c-*myc* in these leukaemia cell lines [102]. Moreover, in these cells, ceramide was found to specifically activate a mitochondrial PP2A, which rapidly and completely induced the dephosphorylation and inactivation of Bcl2, a potent anti-apoptotic protein [103]. Further, in TNFα-treated A431 cells, rapid hydrolysis of sphingomyelin was accompanied by c-Jun dephosphorylation; OA inhibited this effect [104]. Moreover, a partially purified CAPP preparation could dephosphorylate c-Jun *in vitro*, suggesting that it may be a direct substrate of CAPP *in vivo*.

Inhibitory proteins

Two specific, non-competitive and heat-stable inhibitors of PP2A were purified from bovine kidney and termed, by analogy with the PP-1-specific inhibitors, I_1^{PP2A} and I_2^{PP2A} [105]. Both proteins inhibit all holoenzyme forms of PP2A, probably by binding directly to the catalytic subunit. In intact cells, overexpression of I_2^{PP2A} results in increased expression, DNA-binding and Ser^{63} phosphorylation of c-Jun, and in higher transcriptional activity of activator protein-1 (AP-1) [106]. These effects are reversed by overexpression of haemagglutinin-tagged $PP2A_C$, consistent with I_2^{PP2A} acting as a PP2A inhibitor *in vivo*. Interestingly, in the presence of near-physiological concentrations of Mn^{2+}, I_1^{PP2A} and I_2^{PP2A} also associate with and markedly stimulate the activity of PP-1 towards some substrates, whereas Mn^{2+} does not affect the inhibition of PP2A by I_1^{PP2A} and I_2^{PP2A} [107]. This might suggest a novel role for I_1^{PP2A} and I_2^{PP2A} in the co-ordination of PP-1 and PP2A activities within cells.

Based on some amino acid sequences, I_1^{PP2A} was identified as PHAP-I (putative histocompatibility leucocyte antigen class II-associated protein-I) [108] and I_2^{PP2A} as a truncated cytoplasmic form of PHAP-II, also termed SET (Suvar3-9, enhancer of zeste, trithorax) or TAF-Iβ (template-activating factor-Iβ) [109]. SET, in its complete form, is a nuclear protein and contains an additional highly acidic C-terminal region that is involved in chromatin remodelling. SET itself is also a potent and specific inhibitor of PP2A [109], and exerts its inhibitory activity via its N-terminal part [110]. SET is phosphorylated *in vivo* on two serine residues, probably by PKC [111]. Interestingly, in acute non-lymphocytic myeloid leukaemia, SET is found to be fused to CAN (nucleoporin Nup214), apparently as a result of a chromosomal translocation [112]. As such, the formation of this SET–CAN fusion protein may impair the normal regulation of PP2A and contribute to leukaemogenesis. In this respect, it was also shown that HRX leukaemic fusion proteins (resulting from another common genetic alteration in human acute leukaemia) associate with SET and co-immunoprecipitate PP2A [113], suggesting that HRX fusion proteins may function in conjunction with SET and PP2A to de-regulate cell growth.

Modulation of the levels of expression of PP2A and its subunits

Although expression of $PP2A_C$ is tightly controlled by an autoregulatory translational mechanism [21], there are some reports describing changes in $PP2A_C$ levels, for instance during all-*trans*-retinoic acid-induced differentiation of HL-60 cells [114,115], during adipocyte differentiation induced by peroxisome proliferator-activated receptor-γ [116], and in macrophages stimulated by colony-stimulating factor 1 [117]. Also, the expression of some of the PP2A subunits is developmentally regulated [26,39,48]. The underlying mechanisms for these differences in regulation of expression remain unknown.

PP2A as a PTPase

It has long been known that, apart from their apparent phosphoserine/threonine PPase activity, PP2A enzymes also exhibit low but detectable PTPase activity *in vitro* [118,119]. This PTPase activity can be regulated independently from the Ser/Thr PPase activity. The characterization *in vitro* of the 'dual specificity' of PP2A indicates that the phosphoserine and phosphotyrosine PPase activities exhibit distinct catalytic properties and thermostability [118,119]. They are either conversely affected by free ATP or pyrophosphate [120,121], or stimulated concurrently by tubulin [122]. A third regulatory mechanism for the PTPase activity of PP2A involves a specific protein factor, the PTPase activator (PTPA).

A few years ago the PTPA protein was isolated from rabbit skeletal muscle, *Xenopus laevis* oocytes, dog liver, pig brain and budding yeast [4,123–126]. PTPA specifically stimulates the PTPase activity of the dimeric form of PP2A, and to a lesser extent that of the free catalytic subunit, without affecting the serine/threonine PPase activity. The PTPase activity of the trimeric AB″C and ABC enzymes is not, or is much less, affected by PTPA. The exact activation mechanism is currently unclear, but requires the presence of physiological concentrations of ATP/Mg^{2+}. The latter might suggest that PTPA is a kinase, but this possibility could be excluded, due to (1) the lack of phosphate incorporation into either $PP2A_D$ or PTPA itself, (2) the lack of kinase activity ascribable to PTPA using exogenous substrates, and (3) the lack of a canonical kinase motif in the primary PTPA sequence. The low ATPase activity detected appeared to be a consequence rather than a direct cause of the activation [123,127]. In contrast with the basal, ATP-stimulated and tubulin-stimulated PTPase activity, which remains stable over a long period of time, the PTPA-induced PTPase activity of $PP2A_D$ is transient, and decreases rapidly during phosphotyrosine hydrolysis. It seems that PTPA induces a reversible conformational change in PP2A, so that the same catalytic site becomes accessible by the larger tyrosine phosphate [4]. In this respect, a weak interaction between PP2A and PTPA has been observed [123].

The possible relevant physiological role played by PTPA is suggested by: (1) its cellular concentration, which is sufficiently high (micromolar) to play an important role in the regulation of PP2A, (2) the specific substrate specificity of PTPA-stimulated PP2A, which differs from that of the authentic PTPases *in vitro* [128], and (3) its ubiquity and abundance in differentiated and proliferating tissues, in organisms ranging from yeast to humans [123]. The molecular cloning of PTPA from rabbit and

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human has revealed 96.6 % identity within their primary structures [126]. PTPA homologues have been found in *Xenopus*, *Drosophila*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [124]. From an alignment of these various PTPA cDNAs, some highly conserved 'boxes' of amino acids can be determined, which appear to be essential for PTPA activity. Interestingly, one of these boxes contains the consensus motif for a type B ATP-binding site, which could explain the ATP-dependence of PTPA activity [124].

Human PTPA is encoded by a single gene that has been mapped to chromosome 9q34 [129] and gives rise to seven alternatively spliced transcripts (PTPAα–PTPAη), four of which encode functional proteins [130]. A classical promoter analysis revealed that basal expression of the gene requires the activity of the ubiquitous transcription factor Yin Yang 1 (YY1), which positively regulates basal promoter activity through binding to two functional *cis* elements in the minimal promoter [131]. Interestingly, the tumour suppressor protein p53 can significantly down-regulate PTPA expression, both in normal conditions and in conditions where p53 is activated by UVB irradiation. This p53-mediated suppression occurs through an as yet unknown mechanism involving the negative control of YY1 [132].

So far, information on the role of PTPA *in vivo* comes solely from studies applying molecular genetics in yeast. In *Saccharomyces cerevisiae*, PTPA is encoded by two genes, *YPA*1 and *YPA*2. Deletion of both genes is lethal [133,134], but this lethality can be rescued by overexpression of PPH22, one of the yeast homologues of $PP2A_C$ [134]. In general, single disruption of *YPA*1 results in a more severe phenotype than single disruption of *YPA*2 [133,134,134a], suggesting that the two proteins are not completely redundant. The phenotype of single *YPA*1 mutants is pleiotropic and resembles the phenotype of PP2A-deficient strains in specific aspects, such as aberrant bud morphology, abnormal actin distribution and similar growth defects in various growth conditions [134a]. *YPA*1 mutants are also defective in at least two mitogen-activated protein kinase (MAPK) pathways (the osmosensing or HOG1 pathway, and the PKC/MPK1 pathway) [134] and progress more rapidly to S-phase after G_1-arrest [133]. Additional data on the role of YPA1 *in vivo* came from a report by Ramotar et al. [135], who showed that *YPA*1 mutants display hypersensitivity to oxidative DNA damage. This implies a function for PTPA in the pathway(s) signalling the repair or in the repair itself of this type of DNA damage. The authors suggest that these effects occur independently of PP2A, but the data provided to sustain this claim are debatable. Finally, *YPA*1 mutants are rapamycin-resistant [133,134], suggesting that PTPA may be implicated in the TOR pathway (see below).

Although a genetic interaction is observed between *YPA* and *PPH*, and although yeast PTPA stimulates the PTPase activity of rabbit $PP2A_D$ *in vitro*, the effect of yeast PTPA on yeast PP2A is less clear. Moreover, tyrosine phosphorylation is a rare event in yeast. This could indicate that PTPA might affect other characteristics of PP2A. In this respect, it has been shown that a *YPA*1 deletion strain contains much more trimeric ABC PP2A than a wild-type strain, which contains much more of the dimeric AC form [134a]. Thus it is possible that PTPA may affect the subunit composition of PP2A, not favouring association of the dimer with a third subunit. Another interesting observation came from the discovery of an inactive PP2A fraction that can be isolated during the purification of PP2A holoenzymes from rabbit skeletal muscle or pig brain (J. Goris, unpublished work). For unknown reasons, this inactive form is always found in association with PME-1, but, more importantly, its serine/threonine PPase activity can be completely restored by the addition of PTPA. Since it is obvious that inactivation and re-activation of PP2A could be an important event *in vivo* (occurring for instance during the cell cycle; see below), PTPA may be an important player in this equilibrium.

BIOLOGICAL ROLE OF PP2A

Use of specific, cell-permeable phosphatase inhibitors

The discovery of many naturally occurring phosphatase inhibitors, which are able to penetrate living cells, has been a breakthrough in the study of the functions of PPases *in vivo*. The best studied, and most widely used, of these is OA, a polyether fatty acid produced by marine dinoflagellates and the causative agent of diarrhoetic shellfish poisoning [136]. OA inhibits the serine/threonine PPases to differing extents: PP2A is inhibited most strongly (K_i 0.2 nM), followed by PP-1 (K_i 2 nM); PP2B is even less sensitive (K_i 10 μM) and PP2C is not inhibited at all [137]. In addition to these four major PPases, PP4/PPX [138,139] and PP5 [140] were shown to be almost as sensitive to OA as PP2A, whereas PP7 is completely insensitive [141]. Interestingly, the PTPase activity of PP2A is also potently inhibited by OA, with the K_i values lying in the same nanomolar range [142,143]. Since this activity is relatively insensitive to vanadate, a potent inhibitor of the classical PTPases, this criterion can help to distinguish PP2A PTPase activity from other cellular PTPases.

In spite of these different sensitivities, care has to be taken when using OA to discriminate between the activities attributable to the actions of various PPases *in vivo*. Indeed, OA does not penetrate cell membranes rapidly, but accumulates slowly, making it difficult to control the actual concentration of the compound *in vivo*. Also, the efflux of OA can vary considerably between different cell lines [144]. Moreover, the amount of OA needed to inhibit specific PPases depends on the concentration of the PPases within the cell. Since, for instance, the cellular concentration of PP2A is estimated to be in the micromolar range [145], complete inhibition may only be achieved with relatively high concentrations of OA, and these concentrations may also affect the activities of PPases, which are less sensitive to OA, but which are present at lower cellular concentrations. Nevertheless, conditions for the selective inhibition of PP2A in intact cells, which take into account the penetration kinetics, have been established [146]. Moreover, in cell-free extracts, the former limitations do not exist, and OA is a valuable tool for distinguishing between different PPases acting upon a given substrate.

With regard to its mechanism of action, OA has been shown to bind directly to the catalytic subunits of PP2A and PP-1, albeit with different affinities [147]. Resistance to OA in Chinese hamster ovary cells has been associated with a more rapid efflux of OA, and with a $Cys^{269} \rightarrow Gly$ mutation in the C-terminal region of $PP2A_{C\alpha}$ [144]. This cysteine residue is conserved in OA-sensitive PPases (PP2A and PP4), but not in PP-1 and PP2B. Moreover, substitution of residues 274–277 (Gly-Glu-Phe-Asp) of $PP\text{-}1_C$ with the corresponding $PP2A_{C\alpha}$ residues 267–270 (Tyr-Arg-Cys-Gly) results in a chimaeric mutant that shows a 10-fold increase in OA-sensitivity, indicating that this region may determine the specificity of the PPase–OA interaction [147].

OA induces various biological effects *in vivo*, including promotion of tumour growth in mouse skin [148], stomach and liver [149], prolonged smooth muscle contraction [150] and promotion of genomic instability [151–153]. Intriguingly, in some systems OA promotes malignant transformation [154], whereas in others it inhibits transformation and induction of cell growth [155–157], and promotes differentiation [158]. Despite the diversity of these effects, all are likely to result from the de-regulation of OA-sensitive PPases.

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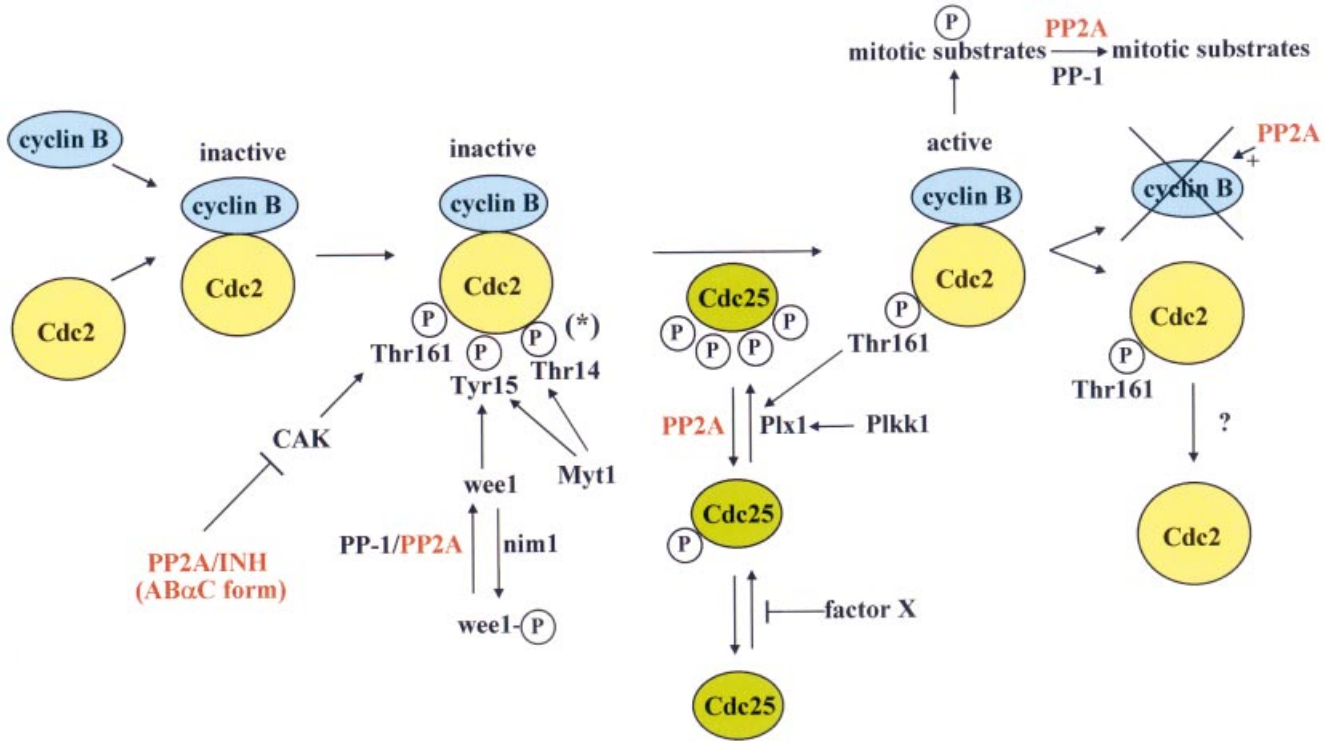

Figure 2 PP2A and regulation of the G_2/M transition

The most important kinases and phosphatases implicated in the activation of MPF (Cdc2/cyclin B), governing the G_2/M transition of the cell cycle, are depicted. In early G_2, PP2A (INH) is required to keep MPF in its inactive precursor form by inhibiting the activities of both CAK and Wee1. PP2A also inhibits complete Cdc25 phosphorylation (and activation) by counteracting the Plx1 kinase. Finally, PP2A is also positively implicated in the exit from mitosis, through its role in cyclin B destruction and by dephosphorylating specific mitotic substrates of activated MPF. * For reasons of clarity the intermediate Thr^{14}/Tyr^{15}-*P*/Thr^{161}-*P* phospho form has been omitted (see the text).

Following these studies on OA, several other PPase-inhibiting compounds have been identified, including calyculin A [159], microcystin-LR [160], tautomycin [161], nodularin [162], cantharidin [163], and their respective derivatives. The K_i values of these inhibitors are similar for PP-1 and PP2A, with the exceptions of cantharidin, which, like OA, can be used to distinguish between PP2A (IC_{50} 0.16 μM) and PP-1 (IC_{50} 1.7 μM), and of tautomycin, which is more specific for PP-1 [149].

PP2A and the cell cycle: an OA-sensitive phosphatase negatively regulates entry into mitosis

At present, one of the best-documented cyclin-dependent kinase (Cdk)–cyclin complexes is MPF (M-phase-promoting factor), which consists of the $p34^{cdc2}$ kinase (Cdc2 or Cdk1) and cyclin B, and is involved in the G_2/M transition (see [164] for a review). In G_2, Cdc2 associates with freshly synthesized cyclin B and is phosphorylated on three sites (Figure 2): Thr^{161} is phosphorylated by CAK (Cdk-activating kinase), which itself consists of Cdk7 and cyclin H; Thr^{14} is phosphorylated by the dual-specificity kinase Myt1; and Tyr^{15} becomes phosphorylated by the Wee1 kinase and/or by Myt1. This triple-phosphorylated complex is inactive and is called pre-MPF. Final activation of pre-MPF occurs when the inhibitory Thr^{14} and Tyr^{15} are dephosphorylated by the dual-specificity PPase Cdc25, the activity of which, in its turn, is regulated positively by phosphorylation (Figure 2). MPF phosphorylates specific substrates, such as histone H1, lamins, vimentin, cyclins and microtubule-associated proteins, explaining the initiation of mitotic processes such as nuclear breakdown, chromosome condensation and spindle formation. At the end of mitosis, MPF is inactivated by cyclin B destruction and by dephosphorylation of Thr^{161}. The exit from mitosis is also promoted by dephosphorylation of MPF substrates [164] (Figure 2).

The implication of PP2A in the regulation of the G_2/M transition was initially suggested through experiments using OA. Injection of OA into *Xenopus* [142,165] or starfish [166] oocytes induces the formation of active MPF, resulting in meiotic maturation. In starfish oocytes, activation of MPF requires a nuclear component that inhibits PP2A, and which can be bypassed by addition of OA [167]. OA, but not the PP-1 inhibitors I-1 and I-2, induces Cdc2 kinase activation in interphase extracts [168]. Similarly, OA treatment of BHK21 cells synchronized early in S-phase results in the induction of mitosis-specific events and in a significant rise in Cdc2 kinase activity towards histone H1 [169]. Together, these data suggest that PP2A is required to maintain MPF in its inactive precursor form. INH, originally identified as an activity that could inhibit activation of pre-MPF, was indeed shown to be a form of PP2A [170], more particularly a trimer containing the Bα/PR55 subunit [171]. *In vitro*, PP2A can dephosphorylate a specific site on Cdc2 [170], later identified as Thr^{161} [172], resulting in Cdc2 inactivation. Subsequently it was found that PP2A inhibits the pathway leading to phosphorylation of Thr^{161}, rather than being involved in the direct dephosphorylation of Thr^{161} [171]. PP2A may therefore play a role in the control of CAK activity, since CAK is the kinase responsible for Thr^{161} phosphorylation. Furthermore, genetic

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evidence indicates that PP2A may also positively regulate the activity of the Wee1 kinase, responsible for the inhibitory Tyr^{15} phosphorylation, possibly by direct dephosphorylation of Wee1 [173]. In contrast, PP2A can dephosphorylate Thr^{14} *in vitro* without dephosphorylating Tyr^{15}, even in the presence of PTPA, leading to a partial activation of Cdc2 [174]. This observation could be part of an explanation for a sequential dephosphorylation of Thr^{14} and Tyr^{15} by PP2A and Cdc25 respectively. However, dephosphorylation of Cdc2 by Cdc25 *in vitro* also occurs successively (first Thr^{14}, then Tyr^{15}), as observed *in vivo* during starfish oocyte maturation [174]. Moreover, in the presence of vitamin K3, an inhibitor of Cdc25, the two inhibitory sites on Cdc2 remain phosphorylated and activation of Cdc2 is prevented [174]. Taken together, the observations with OA [165–169] and vitamin K3 [174] make a positive role for PP2A in the activation of Cdc2 very unlikely.

Another factor contributing to the negative effect of PP2A on the G_2/M transition is situated at the level of Cdc25, the activity of which is required for dephosphorylation of the inhibitory Thr^{14} and Tyr^{15} sites on Cdc2 in the Cdc2–cyclin B complex (Figure 2). At a certain threshold of cyclin B, Cdc25 is activated through phosphorylation. This is followed by a positive-feedback loop between Cdc2 and Cdc25: Cdc25 activates Cdc2, and Cdc2 in turn contributes to the further phosphorylation and activation of Cdc25. This mechanism explains the rapid activation of MPF, preceding M-phase (reviewed in [164]). A major question is how this positive-feedback loop is initially triggered. Karaïskou et al. [175,176] provide evidence that MPF auto-amplification depends upon a two-step mechanism. In a first step, Cdc25 activates Cdc2 with linear kinetics, and no auto-amplification takes place. In G_2, an unknown inhibitory factor is present (PP2A could be excluded) that prevents the phosphorylation and activation of Cdc25. Therefore the precise trigger of this first activation step remains elusive. In a second step, a polo-like kinase (Plx1) catalyses the hyperphosphorylation of Cdc25. Interestingly, this step occurs *in vitro* only when PP2A is inhibited by OA. This shows that PP2A antagonizes the action of Plx1, and strongly suggests that PP2A is the physiological PPase that catalyses the dephosphorylation of the Plx1-phosphorylated residues of Cdc25. This is in line with the previous observation that, *in vitro*, PP2A can dephosphorylate the hyperphosphorylated form of Cdc25, keeping it in a low-activity state [177].

Finally, PP2A, together with PP-1, may also be implicated in the exit from mitosis, since cyclin degradation and the subsequent inactivation of MPF at the metaphase/anaphase transition are affected by an OA-sensitive PPase [168,169]. In support of this, Vandré and Wills [178] showed that low OA concentrations result in a metaphase-like mitotic block of a pig kidney cell line, suggesting the involvement of PP2A in the transition from metaphase to anaphase. Moreover, maintenance of cyclin B destruction during G_1 requires the activity of a PP2A-like PPase, since OA, but not I-2, blocked destruction of cyclin B in G_1 extracts [179]. In addition, PP2A (more specifically, the trimeric ABC complex) seems to be the major enzyme that dephosphorylates several physiological substrates of $p34^{cdc2}$ [62,64], such as histone H1 [62,64,180], the high-mobility group protein I(Y) [180], caldesmon [64,180], vimentin [65] and the *cis*-Golgi matrix protein GM130 [181].

The regulatory functions of PP2A at the G_2/M transition are summarized in Figure 2. However, it is clear that, in order to fulfil these functions, the activity of PP2A has to be tightly regulated as well. In particular, for progression into mitosis PP2A has to be inactivated, and at the exit from mitosis it has to be re-activated. Intriguingly, both the expression and the activity of PP2A were found to be constant throughout the cell cycle when using phosphorylated myosin light chains as a substrate [182]. In contrast, the microtubule-associated PP2A pool is regulated during the cell cycle [183], as is the methylation state of PP2A [87]. However, in both cases the underlying mechanisms that affect the activity of PP2A remain to be elucidated.

Use of molecular genetics

Yeast

The biological role of PP2A in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* has been investigated extensively by genetic analyses of deletion mutants of the different PP2A subunits, with the exception of the B″ subunits (of which no yeast homologues seem to exist) (reviewed in [184]). Recently it was demonstrated that the 'yeast system' can also be very useful for the identification of catalytically impaired and dominant-negative mutants of human [185–187] and *Arabidopsis* [186] $PP2A_C$. The direct use of these mutants in human or *Arabidopsis* cells is likely to be a useful tool with which to study the biological role of PP2A in the future.

In *Schiz. pombe*, $PP2A_C$ is encoded by two genes, *ppa*1 and *ppa*2, and their double disruption is lethal [17]. In contrast with the single *ppa*1 disruption, the single *ppa*2 disruption results in a particular phenotype: $ppa1^+$ $ppa2^-$ cells exhibit retarded growth and decreased cell size, indicating premature entry into mitosis. This observation suggests that ppa1 represents a minor fraction of the PP2A activity in the cell and only partially substitutes for ppa2 [17]. The effects of *ppa*2 disruption on cell size and growth can be mimicked by OA [173]. Apparently, *ppa*2 interacts genetically with the cell cycle regulators *cdc*25 and *wee*1: *ppa*2Δ can suppress the *cdc*25–22 mutation, but is lethal in combination with the *wee*1–50 mutation, indicating that, in fission yeast, PP2A is a negative regulator of the G_2/M transition, interacting with the machinery involved in Cdc2 activation [173]. Thus these data confirm results obtained in higher eukaryotes.

In *Sacch. cerevisiae* the situation is slightly different. $PP2A_C$ is encoded by two genes, *PPH*21 and *PPH*22, which are linked genetically and encode polypeptides that share 74% amino acid sequence identity with mammalian PP2A [18]. Disruption of either *PPH* gene alone is without any major effect, but the double disruption causes a very severe growth defect and is lethal in the absence of *PPH*3, a related PPase gene [18,188]. Thus, in this case, both gene products perform essential cellular functions that are largely or even completely overlapping. *pph*21/*pph*22 mutants have a highly abnormal morphology, with cells and buds being shrunken and pear-shaped, possibly resulting from de-regulation of the cytoskeleton [188]. The generation of yeast strains expressing temperature-sensitive forms of PPH21 [189] or PPH22 [190], in a genetic background where the other *PPH* gene is deleted, has yielded further evidence for roles of PP2A in morphogenesis and mitosis. In either case, bud morphology was perturbed at the restrictive temperature, resulting from a disturbed organization of the actin cytoskeleton. Another defect in these strains is blockage of the cells in G_2, due to: (1) the fact that the mitotic spindles are not able to form or not able to extend, and (2) decreased activity of Cdc2/cyclin B, necessary for the advancement from G_2 into mitosis [189,190]. Therefore these data suggest a positive role for PP2A in the G_2/M transition, and as such oppose the data obtained in *Schiz. pombe* and in higher eukaryotes. The reason for this is still unclear.

In *Sacch. cerevisiae*, the A/PR65 subunit is encoded by *TPD*3, a gene identified as being required for production of tRNA [191]. The tRNA production defect in *tpd*3 mutant strains could be overcome by the addition of TFIIIB (but not TFIIIC), despite normal levels of TFIIIB and RNA polymerase III in the mutant

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extracts [192]. The data suggest that dephosphorylation of some element of the transcriptional machinery by PP2A is important for regulating RNA polymerase III transcription. Another phenotype of these *tpd*3 mutant strains results from defects in cytokinesis, since most cells become multi-budded and multi-nucleated [192]. Similarly, disruption of the *paa*1 gene (encoding the *Schiz. pombe* homologue of PR65) causes anomalies in microtubule and actin distribution [193].

Mutations in *CDC55*, encoding the *Sacch. cerevisiae* B/PR55 homologue, result in highly elongated, multiply budded cells, indicative of delayed cytokinesis [36]. *CDC55* has multiple roles in mitosis. *cdc55* mutants are hypersensitive to nocodazole and thus lack a functional kinetochore/spindle assembly checkpoint, whereas their cell cycle progression in response to DNA damage or an inhibitor of DNA synthesis is not affected [194,195]. The spindle assembly checkpoint contributes to the accuracy of mitosis by delaying the onset of anaphase until the spindle has been fully assembled and each pair of sister chromatids is attached to it. This defective spindle assembly checkpoint in *cdc*55 mutants allows inactivation of Cdc2–cyclin B by tyrosine phosphorylation (instead of cyclin B destruction) and sister chromatid separation in cells that lack spindles [194]. Similarly, in *Schiz. pombe* disruption of *pab*1 (encoding the PR55 homologue) causes reduced growth, morphological abnormalities (such as defects in cell wall synthesis, sporulation and cytoskeletal distribution) and delayed cytokinesis [193].

*RTS*1, which encodes the *Sacch. cerevisiae* homologue of the B′/PR61 subunit family, was isolated independently by two laboratories, using different screening approaches. The first group isolated *RTS*1 as a multi-copy suppressor of a *ROX*3 mutation [196]. The *ROX*3 gene encodes an essential nuclear protein that functions in the global stress-response pathway. Deletion of *RTS*1 caused temperature and osmotic sensitivity [196]. The second group isolated *RTS*1/*SCS*1 as a high-copy suppressor of *hsp*60^{ts} mutant alleles. Disruption of *RTS*1 causes a reduction in the mRNA levels of the Hsp60 chaperonin and thus reduces the normal heat-shock response [197]. Therefore *RTS*1 plays a prominent role in the global stress response. Interestingly, a more recent paper showed that *RTS*1 is also required for the correct regulation of the cell cycle. N-terminally truncated forms of Rts1p lose this cell cycle regulatory capacity, while maintaining stress-related functions [198]. Moreover, it was shown that, with regard to this cell cycle-regulating ability, Rts1p and Cdc55p are functionally not interchangeable [198,199]. In *Schiz. pombe*, two B′/PR61 genes have been identified, *par*1 and *par*2 [200]. Neither gene is essential, but double-deletion mutants show abnormal septum positioning, anomalous cytokinesis and defects in growth under stressful conditions, very reminiscent of the Δ*rts*1 phenotypes in *Sacch. cerevisiae*. Moreover, both genes can functionally complement *RTS*1 deletion in *Sacch. cerevisiae*.

Drosophila

The molecular cloning of PP2A subunits from *Drosophila melanogaster* has provided additional insights into the role of PP2A in developmental processes, cell cycle regulation and intracellular signalling. To date, only PP2A$_C$ [14], the A/PR65 [201], the B/PR55 [202] and the B′/PR61 [203] subunits have been cloned; in contrast with the situation in yeast and Mammalia, each of them is encoded by a single gene. Moreover, a homologue of the B″ subunits is present in the Fly Base, but it has not yet been functionally characterized.

PP2A$_C$ is expressed throughout *Drosophila* development, but is notably much more abundant in early embryos [14]. PP2A$_C$ mutants die in embryogenesis around the time of cellularization, exhibiting overcondensed chromatin and a block in mitosis between prophase and the initiation of anaphase. The most striking feature of these PP2A$_C$-negative embryos is that they possess multiple centrosomes with disorganized, elongated arrays of microtubules radiating from them in all directions, like a star [hence their name: *microtubule star* (*mts*) embryos] [204]. The data suggest that PP2A is required for the attachment of microtubules to chromosomal DNA at the kinetochore and the proper initiation of anaphase. Moreover, mutation of PP2A$_C$ affects the Ras1 signalling pathway controlling cell fate determination in the eye [205]. Ras1 and the downstream cytoplasmic kinases Raf, MEK (MAPK/ERK kinase, where ERK is extracellular-signal-regulated kinase) and MAPK comprise an evolutionarily conserved cascade that mediates the transmission of signals from receptors at the membrane to specific factors in the nucleus. A decrease in the dose of the gene encoding PP2A$_C$ stimulates signalling from Ras1, but impairs signalling from Raf. This suggests that PP2A regulates the Ras1 cascade both negatively and positively, by dephosphorylating factors that function at different steps in the cascade [205].

Like PP2A$_C$, A/PR65 is most abundant during early embryogenesis, and is expressed to a much lower extent in larvae and adult flies. Moreover, A/PR65 and PP2A$_C$ transcripts always colocalize. A/PR65 expression is high in oocytes, consistent with a high, equally distributed expression in early embryos. In later embryonic stages, expression remains high in the nervous system and the gonads, but the overall expression levels decrease. In third-instar larvae, high expression levels could be observed in brain, the imaginal discs and the salivary glands [201]. These results indicate that PP2A levels change during development in a tissue- and time-specific manner.

A cell cycle function of PP2A is again strongly suggested by the mitotic defects exhibited by two *Drosophila* mutants, termed *aar*1 (*abnormal anaphase resolution*) and *twinsP*, both of which are defective in the gene encoding B/PR55 (α and β). *aar1* mutants typically display: (1) intact lagging chromatids that have undergone separation from their sisters, but which remain at the position formerly occupied by the metaphase plate; and (2) anaphase figures that show bridging chromatin having two centromeric regions [206]. Apparently, these defects can be completely rescued by the re-introduction of intact B/PR55, but not of a truncated form lacking the C-terminal half of the B/PR55 coding region [202]. *twinsP* mutants typically contain morphologically abnormal imaginal discs: part of the wing imaginal disc is duplicated in a mirror-image fashion [207]. Since the differentiation fate of the cells comprising the disc is already so diversified as to produce an organ with a specific pattern, these data suggest that PP2A is crucial for the specification of tissue patterns. In both mutants, the reduced levels of B/PR55 correlate with reduced phosphatase activity towards p34^{cdc2}-phosphorylated substrates [64]. So far, these results, together with the previously described yeast data, suggest that PP2A not only is implicated in the negative regulation of mitosis, but also is involved in the control of structural events associated with mitosis. Also, it was shown in the *Xenopus* egg that PP2A is required to maintain the short-steady length of microtubules during mitosis, in part by regulating Op18/stathmin, a molecule involved in the control of microtubule dynamics [208].

The *Drosophila* homologue of B′/PR61 was picked up as a specific interaction partner of one of the Hox proteins, 'sex combs reduced' (SCR) [203]. Interaction occurs with the N-terminal arm of the DNA-binding homeodomain that was shown to be a target of phosphorylation/dephosphorylation by PKA and PP2A. Dephosphorylation of this arm is required for SCR DNA binding *in vitro* and for SCR activity *in vivo*. Ablation of

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B′/PR61 gene activity resulted in embryos without salivary glands, an SCR-null phenotype [203]. In another study, mammalian $PP2A_{C\alpha,\beta}$ as well as PP-1 have been shown to interact with Hox11, another homeobox transcription factor controlling genesis of the spleen and possessing oncogenic properties [209]. In this case, interaction occurs via the N-terminus of Hox11 and does not involve the homeodomain. Moreover, Hox11 suppresses PP2A/PP-1 activity *in vitro* and disrupts a G_2/M cell cycle checkpoint *in vivo*, since microinjection of Hox11 into *Xenopus* oocytes induces premature G_2-to-M progression, and expression of Hox11 in Jurkat T cells abrogates γ-irradiation-induced G_2 arrest [209].

Knock-out mice

To date, knock-out mice have only been established for the $PP2A_{C\alpha}$ gene [210]. Mice lacking $PP2A_{C\alpha}$ die around embryonic day 6.5, despite the fact that total levels of $PP2A_C$ are comparable with those in wild-type embryos. This indicates that $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ serve only partially redundant functions, since $PP2A_{C\beta}$ cannot completely compensate for the absence of $PP2A_{C\alpha}$. Degenerated embryos can be recovered even at embryonic day 13.5, indicating that, although embryonic tissue is still capable of proliferating, normal differentiation is significantly impaired. While the primary germ layers (ectoderm and endoderm) are present, mesoderm is not formed in degenerating embryos. The functional difference between $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ may be explained by their distinct subcellular localizations in the early embryo: while Cα was found predominantly in the plasma membrane, Cβ was localized mainly within the cytoplasm and the nucleus [211]. Moreover, at the plasma membrane, Cα forms a complex with E-cadherin and β-catenin, two components of the Wnt signalling cascade, which controls the epithelial–mesenchymal transition during vertebrate development. In $C\alpha^{-/-}$ embryos, both E-cadherin and β-catenin are redistributed to the cytoplasm, resulting in degradation of β-catenin in both the presence and the absence of a Wnt signal [210,211].

Additional evidence implicating PP2A in Wnt signalling came from the observed association of $PP2A_C$ with axin, a binding protein for β-catenin and glycogen synthase kinase-3β (GSK-3β) [212]. Axin promotes the phosphorylation of β-catenin by GSK-3β, resulting in β-catenin degradation and inhibition of the Wnt pathway. In the presence of a Wnt signal, however, Dishevelled (Dsh) inhibits GSK-3β activation, and thus permits the accumulation of unphosphorylated β-catenin, which can translocate to the nucleus to transactivate the Wnt target genes in cooperation with Lef (lymphoid enhancer binding protein)/TCF (T-cell factor) transcription factors (reviewed in [213]) (Figure 3). Moreover, all B′/PR61 isoforms interact with the adenomatous polyposis coli (APC) protein, which acts as a scaffolding protein for the assembly of β-catenin, axin and GSK-3β [214]. Axin also facilitates the GSK-3β-dependent phosphorylation of APC, and is itself also phosphorylated by GSK-3β [215,216]. The direct binding of axin to APC enhances the phosphorylation of APC by GSK-3β, and the presence of β-catenin within the complex stimulates this reaction [217]. The axin-associated PP2A can directly dephosphorylate GSK-3β-phosphorylated APC and axin [217]. Overexpression of B′/PR61 in mammalian cells [214] or co-injection of B′/PR61ϵ and Dsh in early *Xenopus* embryos [218] reduces the level of β-catenin and inhibits β-catenin-mediated transcription, suggesting a negative role for B′/PR61 in the pathway upstream of or parallel to β-catenin phosphorylation. B′/PR61 may directly inhibit $PP2A_C$-mediated dephosphorylation of GSK-3β-phosphorylated APC, axin or β-catenin, or, alternatively, may negatively affect GSK-3β activity. Another explanation for the B′/PR61 overexpression effect could be the removal of 'active' PP2A dimers or trimers other than AB′C out of the APC–GSK-3β–axin–β-catenin complex due to the artificial rise in B′/PR61. Further, co-injection of $PP2A_C$ and Dsh in *Xenopus* embryos promotes Dsh-mediated signalling, whereas β-catenin stability is not affected [218]. This suggests an additional, positive role for $PP2A_C$, downstream of β-catenin stabilization, which might be exerted at the level of Lef/TCF regulation in the nucleus [218]. The dual role of PP2A in Wnt signalling is summarized in Figure 3.

The interaction of PP2A with viral proteins reveals its role in cell transformation

Cellular transformation by the small DNA tumour viruses simian virus 40 (SV40) and polyoma virus depends on the expression of the so-called tumour antigens, which form multiple complexes with cellular proteins involved in signal transduction and growth control, in order to change their normal functions. For instance, SV40 large T binds to the tumour suppressor proteins p53 [219] and Rb [220], and thereby inactivates their function. Polyoma middle T forms large, multimeric complexes, in which it is associated with $pp60^{c\text{-}src}$ [221], with two other c-Src-like kinases ($pp62^{c\text{-}yes}$ and $pp59^{c\text{-}fyn}$) [222,223] and/or with phosphatidylinositol 3-kinase [224].

PP2A is another important cellular target for these viral antigens. Polyoma small t and middle T, as well as SV40 small t, form stable complexes with $PP2A_D$ by displacing the third subunit [225,226]. PP2A is the only cellular protein known to bind to SV40 small t [225]. Only the free A/PR65 subunit and $PP2A_D$, but not free C, can be complexed with small t, indicating that the PP2A–small-t interaction occurs via the A subunit [227]. The structural elements involved in this interaction have been determined [228]. Intriguingly, small t is able to replace the B/PR55 subunit, but not the B′/PR61 subunit, from a trimeric PP2A complex [227,229,230]. In this respect, it has been shown that B/PR55 and the T antigens interact with overlapping HEAT repeats of A/PR65 [23], suggesting that they may compete for the same binding sites on A/PR65.

The interaction of PP2A with SV40 small t alters the substrate specificity of $PP2A_D$ and inhibits $PP2A_D$ enzyme activity towards some substrates [227]. In contrast, complexes between PP2A and polyoma small t/middle T still display serine/threonine PPase activity [231] and, importantly, exhibit 10-fold elevated tyrosine PPase activity as compared with $PP2A_D$ [232]. This suggests that polyoma virus small t/middle T stabilizes the PTPase activity of PP2A in polyoma-virus-transformed cells in a PTPA-like fashion. A single point mutation in middle T, changing the conserved Cys^{120} to Trp, abolishes interaction with PP2A, $pp60^{c\text{-}src}$ and phosphatidylinositol 3-kinase, and abrogates cellular transformation [233]. Similarly, deletion of the cysteine-rich cluster harbouring Cys^{120} in middle T, or of the corresponding region in small t, abolishes PP2A binding and the transforming ability of middle T [234]. In fact, all mutations that disrupt PP2A binding to middle T also disrupt the association between middle T and c-Src, making it likely that PP2A is required to recruit c-Src into the complex [234,235]. PP2A activity seems not to be required for this recruiting ability, since catalytically inactive $PP2A_C$ mutants can still bind middle T and support complex-formation with $pp60^{c\text{-}src}$ [236].

Apparently the viral antigens, particularly SV40 small t, target PP2A to overcome its negative role in some signalling pathways leading to increased cell proliferation. It is in the interest of the virus to remove this block. When analysed in CV-1 cells, complex-formation of PP2A with SV40 small t resulted in inhibition of

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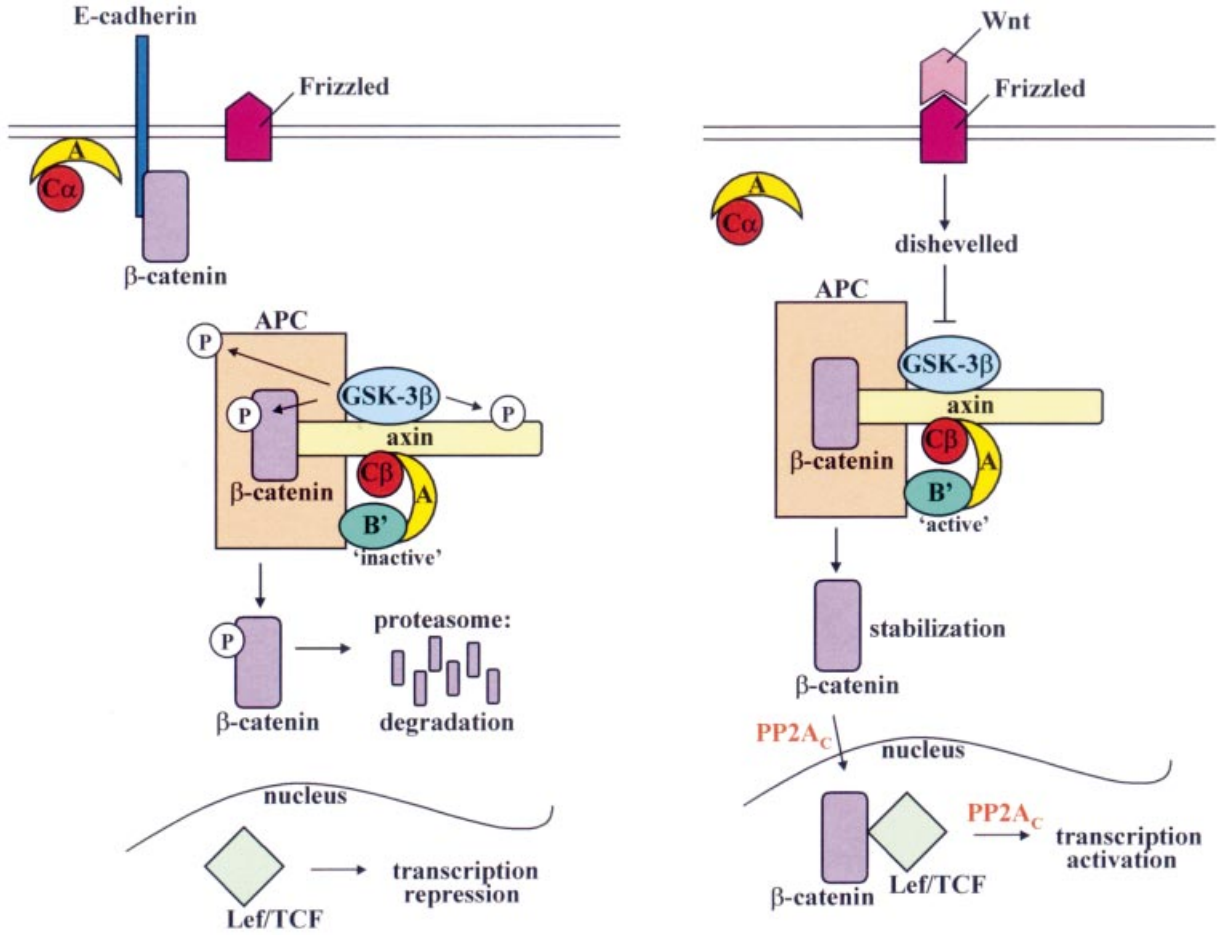

Figure 3 Role of PP2A in Wnt signalling

In the absence of a Wnt signal (left panel), β-catenin is present in two distinct complexes. One complex is located at the plasma membrane, where PP2A$_{C\alpha}$ stabilizes the β-catenin–E-cadherin complex, which itself mediates interactions with the actin cytoskeleton. The other complex is located in the cytoplasm and contains axin, APC, GSK-3β and PP2A (AB′Cβ). Within this complex GSK-3β is thought to be constitutively active, resulting in the phosphorylation of β-catenin, APC and axin. In this case, the associated PP2A activity may not be high enough to counteract GSK-3β-mediated phosphorylation. This may be achieved by negative regulation of PP2A$_C$ activity by B′/PR61 – hence the PP2A AB′C trimer is denoted 'inactive'. Phosphorylated β-catenin is unstable, becomes ubiquitinated and is eventually degraded by proteasomes. In the presence of a Wnt ligand (right panel), GSK-3β activity in the APC–β-catenin–axin–GSK-3β–PP2A complex is blocked by Dishevelled, resulting in the accumulation of unphosphorylated axin, APC and β-catenin. PP2A may contribute to this state by directly dephosphorylating APC and axin, and possibly β-catenin. This implies that PP2A should be activated – or, alternatively, that the B′/PR61-mediated inhibition of PP2A$_C$ activity should be relieved. How exactly this is achieved is not clear. Unphosphorylated axin will be degraded specifically, leading to dissociation of unphosphorylated β-catenin from the complex and accumulation in the cytosol. After translocation to the nucleus, it can transactivate specific target genes. There is evidence that PP2A$_C$ may be involved in this part of the pathway as well, either in the translocation of β-catenin to the nucleus or in the regulation of Lef/TCF transcriptional activity by β-catenin.

PP2A-mediated dephosphorylation of MEK and ERK, and thus in receptor-independent activation of these two kinases. Importantly, small t stimulation of ERK and MEK activity is totally dependent on binding to PP2A [229]. The effects of small t on MAPK signalling depend on the cell type examined, since in REF52 cells no effect was observed on MAPK-induced AP-1 activity upon small t overexpression, unless small t was co-expressed with either ERK or MEK [237]. Another mechanism by which SV40 small t promotes cell growth and transformation is by stimulating PKCζ activity, resulting in MEK activation and nuclear factor-κB (NF-κB)-dependent transactivation. Following inhibition of PP2A, PKCζ and NF-κB apparently become constitutively active [238]. A third transcriptional target of small t (in addition to AP-1 and NF-κB) is CREB (cAMP regulatory element binding protein). Small t inhibits dephosphorylation of PKA-phosphorylated CREB and thereby stimulates CREB-dependent transactivation [239]. Also, the ability of SV40 small t to activate AP-1- and CREB-regulated promoters (e.g. that of the cyclin D1 gene) has been genetically linked to its PP2A binding [240]. Finally, small t also induces transactivation of Sp1-responsive promoters through inhibition of PP2A activity [241].

The role of polyoma small t and middle T in signalling is less clear. In any case, they appear to use PP2A differently: small t promotes cell cycle progression in a manner dependent on its binding to PP2A, whereas a middle T mutant that still binds PP2A is unable to promote cell proliferation [242]. This could indicate that, in the multimeric middle T complex, PP2A serves other functions (such as its formerly described tyrosine kinase-recruiting function).

In addition to its role in cell transformation mediated by the small DNA tumour viruses, overexpression of PP2A$_c$ has been shown to reduce HA-*ras*-induced cellular transformation [250]. Disturbance of PP2A has also been implicated in other virus-

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related phenomena. By increasing the ratio of $PP2A_D$ over holoenzyme, HIV-1 transcription and virus production was inhibited [243]. The HIV-1-encoded protein vpr has been shown to mediate a G_2 arrest through a specific interaction with B/PR55, targeting the complex to the nucleus and leading to dephosphorylation of Cdc25 [245]. The complex of vpr with the HIV-1-encoded nucleocapsid protein (NCp7) stimulated PP2A *in vitro*, more than did both proteins separately, probably acting as polycations [244]. The adenovirus type 5 seems to induce apoptosis through a direct and specific interaction of the adenovirus E4orf4 protein with B/PR55α [246–249].

PP2A and (viral) DNA replication

One of the essential functions of the SV40 large T antigen is the initiation of viral DNA replication. To achieve this goal, it possesses site-specific DNA binding, ATPase and DNA unwinding (or helicase) activities. Large T is phosphorylated at multiple Ser and Thr sites (reviewed in [251]), which play a role in its regulation. Inhibition of large T dephosphorylation by addition of low concentrations of OA substantially inhibits SV40 DNA replication *in vitro* [252]. Addition of purified $PP2A_C$ to a cell-free SV40 replication system stimulates DNA replication by direct dephosphorylation of the inhibitory Ser^{120}, Ser^{123}, Ser^{767} and Ser^{679} sites of large T [253,254]. SV40 small t inhibits the PP2A-mediated dephosphorylation of the large T inhibitory sites, suggesting that it maintains large T in an inactive state until its activation at an appropriate point in the cycle [255]. In addition to free $PP2A_C$, the trimeric ACB″/PR72 [63] and AB′C complexes [48] can also dephosphorylate the inhibitory Ser^{120} and Ser^{123} residues and stimulate large-T-dependent origin unwinding. In contrast, $PP2A_D$ and trimeric ABC actively inhibit large T function by dephosphorylating the stimulatory $p34^{cdc2}$ target site, Thr^{124} [63]. As such, dephosphorylation of large T by PP2A constitutes a nice example of the substrate specificity of different PP2A holoenzyme complexes.

More recently, a role was also described for PP2A in the replication of chromosomal DNA. Immunodepletion of PP2A from a cell-free *Xenopus* egg extract resulted in a strong inhibition of DNA replication, due to the inhibition of DNA replication initiation, but not elongation [256]. The newly discovered B″/PR48 subunit may mediate these effects of PP2A, since it interacts with Cdc6, a protein required for the initiation of DNA replication [56].

PP2A and signal transduction

Eukaryotic cells can adjust their metabolism, growth and differentiation in response to extracellular signals via complex networks of reversible phosphorylation. In classical models of regulation by reversible phosphorylation, PPases reverse the effects of protein kinases by dephosphorylating the substrates of these kinases. However, a lot of data have emerged indicating that one of the major classes of phosphatase substrates is in fact the kinases themselves, the activity of which – in many cases – is negatively regulated by dephosphorylation. Conversely, the activity of the PPases can be regulated by kinases as well. In the case of PP2A, these phosphorylations affect its activity negatively.

PP2A as a kinase phosphatase

PP2A can modulate the activities of several kinases *in vitro* and *in vivo*, in particular phosphorylase kinase [257], the ERK/MAPKs, the calmodulin-dependent kinases, PKA, protein kinase B (PKB), PKC, $p70^{S6}$ kinase, the IκB kinases (where IκB is inhibitor of nuclear factor-κB) and the Cdks (reviewed in [258]). The major PKA PPase activity in cell extracts is PP2A-like [259]. PKB is inactivated *in vitro* by PP2A and is stimulated in cells upon treatment with OA [260] and calyculin A [261]. PP2A also mediates the dephosphorylation and inactivation of PKBα promoted by hyperosmotic stress [261]. A PP2A trimer containing B/PR55 inactivates PKCα *in vitro* [262], and in cell extracts the PPase responsible for PKCα dephosphorylation was identified as a membrane-bound, B/PR55-containing PP2A trimer [263]. *In vitro*, PP2A can dephosphorylate and inactivate MEK1 and ERK-family kinases [264–266], and both kinases are activated after treatment of cells with OA [267,268]. As mentioned above, SV40 small t activates MEK1 and ERK [229], and genetic evidence implicates PP2A in the Ras/MAPK pathway during photoreceptor development in *Drosophila* [205] and during vulval induction in *Caenorhabditis elegans* [269]. A major ERK PPase in extracts from PC12 cells is attributable to PP2A and an as yet unidentified PTPase [270]. IκB, an inhibitory subunit of NF-κB that is proteolytically degraded upon phosphorylation, becomes phosphorylated by a cytokine-regulated IκB kinase complex that contains two kinases, IKKα and IKKβ. IKKα is activated upon exposure of cells to OA and is inactivated by PP2A *in vitro* [271].

Direct interaction of PP2A with protein kinases: the concept of signalling modules

Very recent evidence has demonstrated the existence of another molecular device for the feedback regulation of signalling pathways, the so-called signalling modules. In these supramolecular structures, a kinase, which itself is regulated by phosphorylation, interacts directly with a phosphatase, for which it can become a substrate (or vice versa). As such, kinases and phosphatases can regulate their own activities (feedback) within a self-correcting signalling complex. For instance, protein kinase CKIIα (formerly known as casein kinase IIα) interacts with $PP2A_D$ via the C subunit in quiescent cells, and stimulates $PP2A_C$ activity towards Raf-phosphorylated MEK1 [272]. Expression of activated Raf results in disruption of the CKIIα–PP2A association [273], which may be a necessary step for maximal activation of the MAPK pathway by Raf. Another signalling complex was identified in T lymphocytes between Ca^{2+}/calmodulin-dependent kinase IV and PP2A [274]. PP2A dephosphorylates and inactivates this kinase, as measured by decreased transcriptional activity of CREB, even in the presence of high Ca^{2+} concentrations. In rat brain extracts, PP2A was found in complexes with $p70^{S6}$ kinase [275,276] and with the p21-activated kinases PAK1 and PAK3 [275]. *In vitro*, $p70^{S6}$ kinase is inactivated by purified PP2A [277]. A sixth PP2A-interacting kinase is the Janus kinase JAK2, which associates transiently with PP2A upon interleukin-11 stimulation of adipocytes [278]. In L6 muscle cells, PP2A is associated with JAK2 in the basal state and upon insulin stimulation of the cells. Apparently, insulin inhibits PP2A activity by increasing tyrosine phosphorylation of $PP2A_C$ via JAK2 [75].

Implication of PP2A in other signalling complexes

In the sarcoplasmic reticulum of cardiac muscle cells, PP2A has been found in a macromolecular complex together with the ryanodine receptor calcium release channel, the FK506 binding protein FKBP12.6, PKA, PP-1 and the anchoring protein muscle A-kinase anchor protein (AKAP), where it is probably involved in the regulation of channel activity [279]. PP2A ($PP2A_C$, PR65 and probably a B′/PR61 subunit) is also present in immunoprecipitates of rat forebrain class C L-type calcium channels, where it is able to reverse channel phosphorylation by AKAP-anchored PKA [280]. Another scaffolding protein, termed CG-NAP (centrosome- and Golgi-localized PKN-associated

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protein) was found to bind $PP2A_C$ through the B″/PR130 subunit [281]. PP-1_C, the regulatory R_{II} subunit of PKA and the PKC-like PKN kinase were identified as other members of the complex. CG-NAP is localized to centrosomes throughout the cell cycle and to the Golgi apparatus during interphase, suggesting that it may serve a targeting function for the associated kinases and phosphatases. Finally, in quiescent macrophages as well as those treated with colony-stimulating factor-1, $PP2A_C$ and PR65 were found in a large complex with the Raf-1 kinase, where they apparently serve to facilitate Raf-1 activation [282].

The identification of other PP2A-interacting proteins defines roles for PP2A in translation, apoptosis and stress responses

PP2A and the initiation of translation: Tap42/α4 and the TOR pathway

The *TAP*42 (two A phosphatase-associated protein) gene of *Sacch. cerevisiae* was isolated as a multi-copy suppressor of *SIT*4 deficiency, and encodes a dimerization partner of both SIT4 (suppressor of *His4* transcription 4) and PPH21/22 [283]. Interestingly, $PP2A_C$ can bind Tap42 directly, independently of the A and B subunits. Tap42-associated $PP2A_C$ accounts for about 2% of the total cellular PP2A. The Tap42–PP2A and Tap42–SIT4 complexes are disrupted upon nutrient deprivation or treatment of the cells with the immunosuppressant rapamycin, leading to inhibition of the TOR pathway [283]. In *Sacch. cerevisiae*, TOR, a protein kinase related to phosphatidylinositol 3-kinase and DNA-dependent protein kinase, functions in a pathway that connects nutrient stimulation to the initiation of protein synthesis. Rapamycin binds to the cyclophilin FKBP (FK506 binding protein), and the resulting complex specifically inhibits TOR function. The effect of rapamycin and starvation on the Tap42–PPase complexes therefore suggests that formation of these complexes necessitates TOR activity (or vice versa), and thus implicates Tap42, SIT4 and PP2A in the TOR pathway [283].

The mouse homologue of Tap42, α4, was originally discovered by its association with Ig-α, a component of the B-cell receptor complex IgR [284]. Upon stimulation of the receptor, α4 becomes phosphorylated, suggesting that it may be implicated in receptor-initiated signalling [285]. Like Tap42, α4 was found to bind to $PP2A_C$, independently of the A and B subunits [286,287]. The $PP2A_C$–α4 complex showed increased activity towards phosphorylase *a*, MAPK-phosphorylated myelin basic protein and histone H1. Again, rapamycin led to disruption of the complex, suggesting the involvement of mTOR, the mammalian homologue of yeast TOR [286,287]. mTOR functions in a mitogen-inducible pathway and causes phosphorylation of the translation inhibitor 4E-BP1 (eukaryotic initiation factor 4E binding protein 1) and of the serine/threonine $p70^{s6}$ kinase, leading eventually to phosphorylation of the 40 S ribosomal protein S6 and the initiation of translation (reviewed in [288]). Interestingly, $PP2A_C$ can dephosphorylate mTOR-phosphorylated 4E-BP1 *in vitro*, whereas the $PP2A_C$–α4 complex cannot [289]. In this case, addition of rapamycin fails to dissociate the complex *in vitro* and cannot restore PP2A activity [289]. This is in line with data from another group, describing a constitutive and rapamycin-insensitive association between α4 and $PP2A_C$, and between α4 and two PP2A-related PPases, PP4 and PP6 (the SIT4 homologue), in mammalian cells [290]. This association was shown subsequently to be inhibitory for the catalyic activity of all three PPases towards *p*-nitrophenyl phosphate as a substrate [291].

The determinants specifying the interaction between $PP2A_C$ and Tap42/α4 have been investigated further. First, it was shown in yeast that inactivation of Cdc55 or Tpd3 resulted in rapamycin resistance, correlating with an increased association between Tap42 and Pph21/22 [292]. Moreover, these authors showed that TOR can phosphorylate Tap42 *in vitro* and *in vivo*, and that inactivation of Cdc55 or Tpd3 enhances the phosphorylation of Tap42 *in vivo*. Thus TOR-phosphorylated Tap42 seems to compete effectively with Cdc55/Tpd3 for binding to $PP2A_C$. Furthermore, Cdc55 and Tpd3 promote the direct dephosphorylation of Tap42, indicating that they inhibit association of Tap42 with $PP2A_C$ not only by direct competition with Tap42, but also by direct dephosphorylation of Tap42. Further, it was shown that mutation of both Tyr^{307} and Leu^{309} of $PP2A_C$ favours the association with α4, whereas the single mutations preferentially result in complex-formation with A/PR65 and/or B/PR55 [293]. These data could indicate that modification of the $PP2A_C$ C-terminus by phosphorylation or methylation influences its interaction with subunits A and B and with α4. Moreover, these authors found that overexpression of α4 resulted in decreased phosphorylation of eukaryotic elongation factor 2, but did not affect phosphorylation of $p70^{s6}$ kinase or of 4E-BP1, suggesting that elongation factor 2 may be a direct target of α4–$PP2A_C$. A model integrating the previous observations and the involvement of PP2A in the TOR pathway is illustrated in Figure 4.

Recently, an α4-related protein, termed α4-b and showing 66% identity with α4, was identified [294]. α4-b is expressed selectively in brain and testis, and also binds to $PP2A_C$. As such, α4 and α4-b may be members of a completely new PP2A subunit family that does not require the presence of the A subunit to bind to $PP2A_C$.

PP2A and the termination of translation

In addition to its role in translation initiation, PP2A is also likely to be involved in the termination of translation through the interaction of $PP2A_C$ with a translation termination factor, eRF1 (eukaryotic release factor 1) [295]. eRF1, or its yeast homologue SUP45, co-operates with eRF3 (SUP35) to terminate protein synthesis in ribosomes by acting as polypeptide chain release factors [296–299]. Interaction with eRF1 occurs via the $PP2A_C$ N-terminus, but no dramatic effects were observed on PP2A activity (or vice versa). However, upon transient overexpression of eRF1 in COS cells, the amount of PP2A associated with the polysomes increases significantly, suggesting that eRF1 recruits PP2A into polysomes and brings it in close contact with putative substrates among the components of the translational apparatus [295].

PP2A and apoptosis

A role for PP2A in apoptosis (programmed cell death) is suggested by its interaction with caspase-3 [300], Bcl2 [103,301] and adenovirus E4orf4 protein [246–249]. Together with caspase-1, caspase-3 is one of the key executioners of apoptosis, being responsible for the cleavage of some key enzymes involved in DNA repair, such as poly(ADP-ribose) polymerase and DNA-dependent protein kinase. Another substrate seems to be A/PR65, which was identified as an interaction partner of caspase-3 in a yeast two-hybrid assay [300]. In Jurkat cells induced to undergo apoptosis, caspase-3 is activated and cleaves A/PR65, thereby increasing the activity of $PP2A_C$, as measured by decreased phosphorylation of MAPK [300]. The activity of Bcl2, a potent anti-apoptotic protein, is regulated by phosphorylation on Ser^{70}. This phosphorylation is required for its apoptosis-suppressing ability and can be reversed by an OA-sensitive PPase, which was identified as a form of PP2A [301]. Moreover, interleukin-3- or bryostatin 1-induced phosphorylation of Bcl2 is followed rapidly by increased association between Bcl2 and $PP2A_C$, prior to dephosphorylation of Bcl2 [301].

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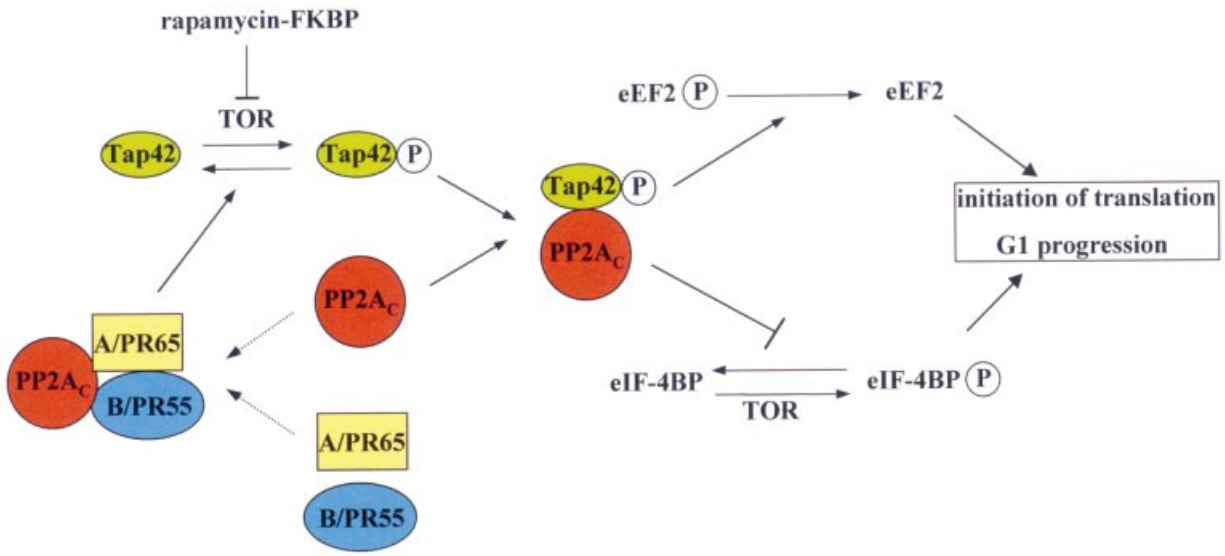

Figure 4 Model implicating PP2A in the TOR pathway

Upstream activators of TOR in mammalian cells are not indicated, but include phosphatidylinositol 3-kinase, phosphoinositide-dependent kinase 1 and protein kinase B [288]. An additional downstream target of TOR is $p70^{S6}$ kinase, which, upon phosphorylation, becomes activated towards the ribosomal S6 protein, and as such contributes to the initiation of translation. It is noteworthy that Tap42/α4 can bind directly to $PP2A_C$, independently of the A and B-type subunits. Abbreviations: FKBP, FK506 binding protein; eEF, eukaryotic elongation factor; eIF-4BP, eukaryotic initiation factor 4E binding protein.

Similar data were obtained in experiments using ceramide as the apoptotic inducer [103]. The adenovirus E4orf4 protein has many biological functions, including the induction of apoptosis in transformed cells. E4orf4 has been shown to interact with PP2A [246], either through Bα or through some of the B′ subunits [247,248]. However, for the induction of apoptosis, only the interaction with Bα is essential [248,249].

PP2A and the heat-shock response

One of the members of the family of heat-shock transcription factors, HSF2, which regulate the expression of the heat-shock protein (*hsp*) genes, has been shown to interact with A/PR65 [302]. This interaction blocks the association of A/PR65 with $PP2A_C$ and stimulates PP2A activity in cells. The ability of HSF2 to interfere with $PP2A_C$ binding to A/PR65 is due to its interaction with the critical Lys^{416} residue [34] localized within HEAT repeat 11 of A/PR65 [303]. Further evidence for a role for PP2A in the heat-shock response comes from the observation that PP2A can dephosphorylate hsp27 (heat-shock protein 27) *in vitro* and in interleukin-1β- or TNFα-treated MRC-5 cells *in vivo* [304]. Tyrosine phosphorylation of $PP2A_C$ by $pp60^{c\text{-}src}$ abolished PP2A activity towards phosphorylated hsp27, and may provide a mechanism for attenuation of the signal.

PP2A and the DNA-damage response

Two-hybrid assays have indicated that the B′/PR61α and B′/PR61δ subunits of PP2A can associate with cyclin G [305]. Cyclin G is a p53-responsive gene product, which is induced upon activation of p53 by DNA-damaging agents [306], and complex-formation between cyclin G and B′/PR61 occurs only after induction of p53 [305]. The precise function of cyclin G has not yet been established, but it is known that it contributes to G_2/M arrest of cells in response to DNA damage [307], and in some cells it enhances the apoptotic response [308]. Immunolocalization studies in rat brain have indicated that the regional and subcellular localization of B′/PR61α and B′/PR61δ and of cyclin G are very similar at postnatal stages, but their developmental regulation differs [309].

Overview

In summary, many proteins have been identified that interact with PP2A, each exhibiting its own specific effect. Some of them affect PP2A activity (such as I_1^{PP2A}, I_2^{PP2A}, PTPA, Tap42/α4, SV40 small t, polyoma middle T/small t, HIV-1 NCp7:Vpr, adenovirus E4orf4, CKIIα, Hox11 and PKR), some are PP2A substrates (such as Bcl2, $p70^{S6}$ kinase, Ca^{2+}/calmodulin-dependent kinase IV, vimentin, paxillin and SCR), for some PP2A itself is a substrate (such as caspase-3, JAK2, PME-1 and PKR) and some act as targeting proteins (such as eRF1, axin, APC and CG-NAP). An overview is given in Figure 5.

Other putative PP2A substrates: the Rb and p53 tumour suppressor proteins

Phosphorylation is one of the major mechanisms regulating p53 function in response to DNA-damaging agents. p53 is phosphorylated on multiple serine/threonine sites *in vitro* and *in vivo* (reviewed in [310]). Some of these sites are important for tumour suppression, DNA binding or transactivation, whereas the function of others is currently unknown. *In vitro*, p53 can be dephosphorylated by both PP-1 [311] and PP2A [255,311,312]. The latter is inhibited *in vitro* by SV40 small t [255]. Treatment of cells with PP-1- and PP2A-specific inhibitors, including OA, results in the accumulation of hyperphosphorylated p53 [312–315]. Transient expression of SV40 small t enhances p53 phosphorylation, DNA binding and transactivation activity, whereas OA additionally causes p53-dependent apoptosis [315]. On the other hand, there is some cross-talk between PP2A and p53, in the sense that p53 may also affect PP2A regulation

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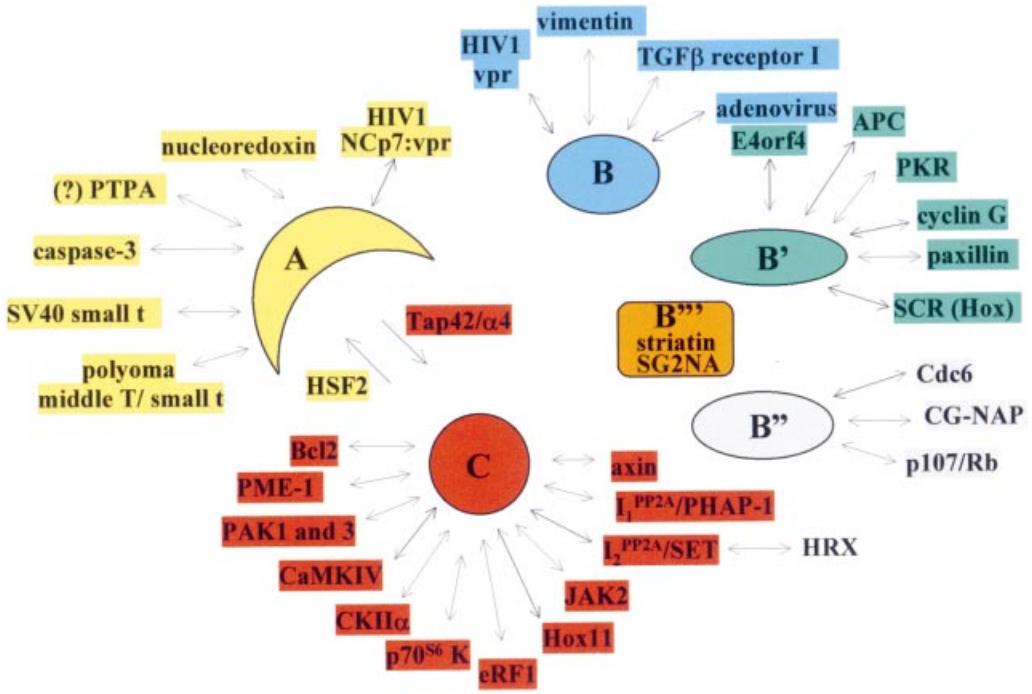

Figure 5 PP2A interacts with a variety of viral and cellular proteins through its A, B/B′/B″/B‴ or C subunits

These interacting partners serve as PP2A substrates (such as vimentin), act as targeting proteins (such as eRF1) or affect PP2A activity (such as the viral antigens). For PME-1, JAK2, PKR and caspase-3, PP2A itself is a substrate. Note that HSF2 and Tap42/α4 can interfere with $PP2A_C$ binding to A/PR65 by binding to the A and C subunits respectively. The still growing list of interacting proteins adds quite a level of complexity to the overall regulation of PP2A. Abbreviation: CaMKIV, Ca^{2+}/calmodulin-dependent kinase IV.

through its effect on the expression of PTPA [132] and cyclin G [305,306].

Rb is phosphorylated in a cell-cycle-dependent manner: in G_0 or early G_1, it is present as a hypophosphorylated protein, whereas in cells progressing towards S-phase it becomes hyperphosphorylated. At the end of M-phase, Rb is quickly dephosphorylated again. PP-1 is the major Rb phosphatase *in vivo* [316–318]. However, specific inhibition of PP2A activity exhibits an indirect effect on Rb phosphorylation [319]. In this case, decreased Rb phosphorylation results from the suppression of G_1 Cdks (including Cdk2, Cdk4 and Cdk6), that normally phosphorylate Rb during G_1. In other words, PP2A activity is crucial for the activation or maintenance of G_1 Cdk activity, implying a positive role for PP2A in the G_1/S transition. On the other hand, the Rb-related p107 protein seems to be dephosphorylated directly by a PP2A PPase in response to UV irradiation [320]. Probably this effect is mediated by the B″/PR59 subunit, which interacts directly with p107 [55].

PP2A and pathologies

Tau and Alzheimer's disease

The high levels of expression of both $PP2A_C$ isoforms in brain [9] and the brain-specific expression of some members of the B/PR55 [35,37,39] and B′/PR61 [44,45] subunit families suggests that PP2A has unique functions in neuronal cells (reviewed in [321]).

Within neurons, PP2A seems to be targeted to specific intracellular locations, such as the neurofilaments. Neurofilament-associated PP2A dephosphorylates neurofilaments NF-M and NF-L [322,323]. As phosphorylation controls assembly, neurofilament-associated PP2A probably regulates the stability of neurofilaments and/or their interactions with other components of the neuronal cytoskeleton.

Another potential function of PP2A in the brain is regulation of the phosphorylation state of microtubule-associated proteins. Neuronal-specific microtubule-associated proteins, including tau and MAP-2, bind to microtubules and regulate microtubule stability. They are phosphorylated at multiple sites by a variety of protein kinases, resulting in the dissociation of microtubule-associated proteins from microtubules and loss of stability (reviewed in [324]). Interestingly, the accumulation of hyperphosphorylated tau in neurofibrillary tangles is a pathological hallmark of Alzheimer's disease, and this hyperphosphorylation of tau has been proposed as a mechanism leading to neuronal degeneration, characteristic of this disease [325,326]. As mentioned above, a pool of PP2A, composed mainly of ABαC, is associated with microtubules [183]. In a more recent report, it was shown that only trimeric PP2A forms containing B/PR55α or B/PR55β, but not B/PR55γ or B′/PR61, associate with neuronal microtubules, and that this interaction depends on an as yet unidentified anchoring factor [327]. Moreover, PP2A can dephosphorylate specific sites of (hyper)phosphorylated tau *in vitro* and *in situ* [328–333]. Co-expression of SV40 small t leads to hyperphosphorylation of tau, accompanied by dissociation of tau from the microtubules and loss of microtubule bundles [334]. Similar effects are observed upon treatment of neuronal cells with PP2A-selective concentrations of OA [335]. In addition, PP2A interacts directly with soluble tau and targets it for dephosphorylation [334]. The identification of the structural interactions between tau, PP2A and microtubules has revealed that PP2A binds tau and microtubules through distinct sites, and

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therefore may be able to anchor tau to the microtubules [336]. Moreover, these authors suggest that PP2A activity towards tau can be modulated by microtubule dynamics. Thus disruption of the normal interactions among PP2A, tau and the microtubules may contribute to the development of tauopathies, such as Alzheimer's disease.

PP2A and carcinogenesis

Initial evidence for a negative role for PP2A in carcinogenesis came from the observation that the tumour promoter OA [148] is a potent inhibitor of PP2A [136]. More recently, cytostatin, an inhibitor of cell adhesion and a powerful anti-metastatic drug, was also demonstrated to inhibit PP2A selectively [337]. Additionally, the PR65α and PR65β subunits have been identified as tumour suppressors, their genes being mutated in melanomas, lung and breast carcinomas for PR65α [29], and in 15% of primary lung and colon tumour-derived cell lines for PR65β [27]. Moreover, de-regulation (overexpression) of PR61γ in malignant melanoma, as compared with normal epidermal melanocytes, reveals a role for PP2A in melanoma tumour progression [338]. More recently, a retrotransposon insertion in the gene encoding PR61γ1, causing the expression of an N-terminally truncated form of PR61γ1, was associated with a higher metastatic state of melanoma cells [339]. This increase in cell migration was linked to increased paxillin phosphorylation at the focal adhesions. Apparently, PR61γ1 interacts specifically with paxillin and thereby targets PP2A to the focal adhesions, where it then acts to dephosphorylate paxillin. The N-terminally truncated PR61γ1 did not lose this targeting ability, but the resulting PP2A trimer failed to dephosphorylate paxillin, resulting in enhanced cell spreading [339]. Also, in some human leukaemias, normal PP2A regulation is disturbed, due to certain chromosomal translocations resulting in the formation of SET–CAN and HRX fusion proteins (see above) [112,113]. In addition, PP2A inhibits nuclear telomerase activity in human breast cancer cells [340]. In normal somatic cells, telomerase activity is below detectable levels, but in most primary human malignancies it is elevated by an as yet unknown mechanism, suggesting that *de novo* synthesis of telomeres is crucial for unlimited cell division. By inhibiting this enhanced telomerase activity in cancer cells, PP2A can therefore counteract uncontrolled cell growth.

CONCLUSIONS AND PERSPECTIVES

The pivotal role of PP2A in such a variety of cellular processes simply necessitates proper regulation of enzyme activity and localization. We now have an outline of how these regulatory mechanisms operate, but many challenges remain. One of the central quests in the field is elucidating the dynamics of all observed PP2A interactions, and how they react to extracellular and internal signals that contribute to the overall response of a particular cell. While the role of $PP2A_C$ carboxymethylation appears to be vital for proper PP2A functioning *in vivo*, the regulation of methylesterase and methyltransferase activities remains elusive. Also, the role of PTPA as a putative $PP2A_C$ 'chaperone' should be clarified further. Additional data on the specific functions of the B-type subunits and the cellular processes in which they are involved may emerge from knock-out studies in mice, or, given the many genome projects at hand, from genetic studies in other eukaryotes such as *Drosophila* and *Caenorhabditis*. It may be hoped that these studies may eventually lead to the discovery of therapeutic agents that can counteract PP2A de-regulation in some cancers or virally transformed cells.

J. G.'s research group is supported by grants from the F. W. O.-Vlaanderen, the 'Geconcerteerde OnderzoeksActies' of the Flemish Community, the 'InterUniversitaire AttractiePool', the Human Frontiers Science Program and the E. C. Biomed2 Cancer Research Program. V. J. is a post-doctoral fellow of the F. W. O.-Vlaanderen.

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