An. R. Acad. Nac. Farm., 2007, 73 (4): 901-925

Revisión

Roles of Protein Phosphatase Type 1 in *Schizosaccharomyces pombe*

Recibido el 4 de octubre de 2007

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ABSTRACT

Protein phosphatases are considered to be global regulators in many biological processes. They are divided in families on the basis of substrate specificity, mechanisms of catalysis and evolutionary relations. Protein Phosphatase Type 1 (PP1) holoenzymes are composed of a small number of catalytic subunits and an array of regulatory, targeting, subunits. The *S. pombe* genome encodes two highly related catalytic subunits, Dis2 and Sds21. We fused enhanced green fluorescence

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Abbreviations: PP, Protein Phosphatase; ***S. pombe,*** *Schizosaccharomyces pombe*; **EGFP,** Enhanced Green Fluorescent Protein; **RNA,** Ribonucleic Acid; **RNAi,** Interfering RNA; **Dis,** Defective in sister chromatid disjoining; **Tyr,** Tyrosine; **PTP,** Protein Tyrosine Phosphatase; **Asp,** Aspartic Acid; **Ser,** Serine; **Thr,** Threonine; ***S. cerevisiae,*** *Saccharomyces cerevisiae*; ***A. thaliana,*** *Arabidopsis thaliana;* ***D. melanogaster,*** *Drosophila melanogaster;* ***C. elegans,*** *Cenorabditis elegans;* **DNA,** Deoxyribonucleic Acid; **BIM,** Bloked in mitosis; **GFP,** Green Fluorescent Protein.

protein (EGFP) coding sequences to the aminus-termini of endogenous *dis2*$^+$ and *sds21*$^+$ genes. We have described that Dis2 and Sds21 localize in different cell compartments and structures as centromeres- kinetochores, nucleoli, a ring at the cell equator in dividing cells, endocytic vesicles and the cell tips. Each of these locations suggests different functions of a single catalytic PP1 subunit mediated by its interaction with different targeting proteins. This converts PP1 into a multifunctional enzyme that acts as a global regulator in many cellular processes.

Key words: Protein phosphatases, mitosis, endocytosis, polarised growth, fission yeast.

RESUMEN

Funciones de Proteína Fosfatasa Tipo 1 en *Schizosaccharomyces pombe*

Las proteína fosfatasas están consideradas reguladores globales en muchos procesos biológicos. Se clasifican en distintas familias según su especificidad de sustrato, sus mecanismos de catálisis y sus relaciones evolutivas. Los holoenzimas de proteína fosfatasas de tipo 1 (PP1) están compuestos por un pequeño número de subunidades catalíticas y un amplio número de subunidades reguladoras. El genoma de *S. pombe* codifica dos subunidades catalíticas muy relacionadas, Dis2 y Sds21. Hemos fusionado la proteína verde fluorescente «mejorada» (EGFP) al extremo amino-terminal de los genes endógenos de *dis2*$^+$ y *sds21*$^+$. Hemos descrito que Dis2 y Sds21 se localizan en distintos compartimentos y estructuras celulares, como los centromeros-kinetocoros, núcleo, un anillo en el ecuador de células en división, vesículas endocíticas y extremos celulares. Cada una de estas localizaciones sugiere diferentes funciones para las subunidades catalíticas de PP1 que interaccionan con distintas proteínas reguladoras. Esto convierte a PP1 en un enzima multifuncional que actúa como un regulador global en muchos procesos celulares.

Palabras clave: Proteína fosfatasas, mitosis, endocitosis, crecimiento polarizado, levadura de fisión.

INTRODUCTION

Protein phosphorylation is a global cell regulatory mechanism that controls many cellular processes. Gene expression, cell shape, metabolism, movement and proliferation, are examples of processes regulated by phosphorylation. Approximately one third of all intracellular proteins are regulated by phosphorylation and this

reflects the diversity and extension of this regulatory mechanism (1, 2). In contrast to initial views of signalling pathways as simple linear arrangements of phosphorylation cascades that function in isolation, it is now clear, that there are multiple signalling networks that function in coordination. The level of phosphorylation of a protein within a signalling pathway is thus a dynamic process that is determined by the concerted action of protein kinases and phosphatases (3).

Protein phosphorylation has also been linked to human diseases and many protein kinases have been characterized (4). It has been reported recently that protein kinases have become the second largest drug targets for the pharmaceutical industry (5). In contrast, protein phosphatases have lagged behind and only more recently have been recognized as partners in the regulation of signalling responses. The distinct but complementary function of these enzymes have found their role in controlling the rate and duration of the cellular response activated by protein kinases (6).

The physiological importance of protein phosphatases was demonstrated initially when they were shown to be the targets of many naturally occurring toxins and they could also function as tumour suppressors. Later they were found to be the target of viral proteins, including the viral oncoprotein E4orf4 and the middle-T and small-T antigens (7, 8). More recently, large-scale loss-of-function RNA interference (RNAi) screens have revealed that a surprising number of phosphatases are promoters of cell survival and cell death as well as having roles in the regulation of cell cycle progression (8, 9).

CLASSIFICATION OF PROTEIN PHOSPHATASES

Protein phosphatases can be classified into three groups on the basis of sequence, structure and catalytic mechanism (Figure 1A). The first group comprises the classic Ser/Thr phosphatases: the large phosphoprotein phosphatase family (PPP family) that includes PP1, PP2A, PP2B, PP4, PP5, PP6 and PP7 and the protein phosphatase, Mg^{2+} or Mn^{2+} dependent (PPM) family (PP2C). The protein Tyr phosphatase (PTP) superfamily forms the second group, and the

third group consists of the Asp-based protein phosphatase with a DXDXT/V catalytic signature (10).

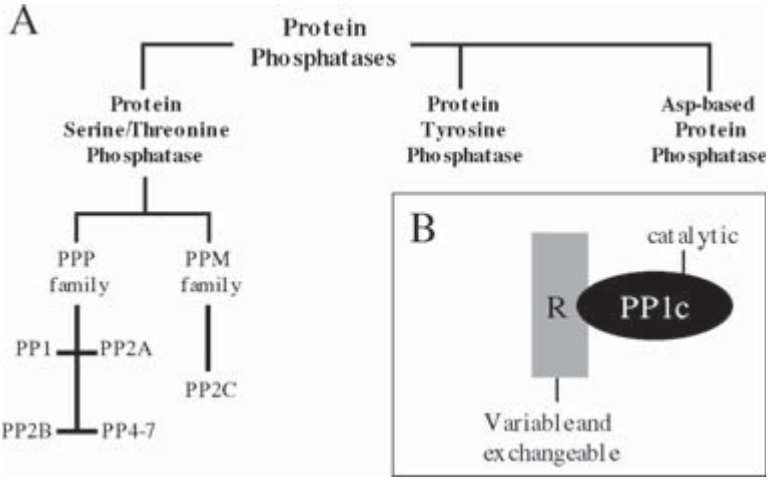

FIGURE 1. ***Classification of protein phosphatases and core configuration of PP1. A)*** *Schematic representation of the different families and subfamilies of protein phosphatases.* ***B)*** *PP1 consists of a catalytic subunit (PP1c) and a variable and exchangeable regulatory subunit (R).*

Genomic studies have provided a catalogue of protein phosphatases for several eukaryotic organisms. Human DNA encodes 518 protein kinases with 428 known or predicted to phosphorylate Ser and Thr residues and 90 belonging to the tyrosine kinase family. By contrast, there are only 147 human protein phosphatase catalytic subunits with 107 belonging to the Tyr phosphatase family (2, 11). Of these 107 catalytic subunits, 81 are predicted to be active protein phosphatases (12). Interestingly, although more than 98% of protein phosphorylation events take place on Ser and Thr side chains, only 40 of the 147 known protein phosphatase are specific for these aminoacids. Of these 40 phosphatases, 18 are PP2C enzymes that do not have additional regulatory subunits. For the remaining Ser/Thr phosphatases, function is defined by additional regulatory subunits or interacting proteins that bind the phosphatase catalytic subunits (and holoenzymes) target them to specific locations and/or substrates and control their activity.

PP1 AND ISOFORMS

In humans, Protein Phosphatases Type 1 are encoded by three highly related genes (PP1α, PP1βδ and PP1γ) and alternative splicing generates the γ1 and γ2 isoforms. With the exception of *S. cerevisiae*, which has one PP1 gene, all eukaryotes have multiple genes with 8 in *A. thaliana*, 4 in *D. melanogaster* and a predicted 30 in *C. elegans* (13).

Although the cytosolic forms of mammalian PP1, such as those targeted to glycogen and myosin, have been studied in greatest detail, a PP1 activity enriched in the nucleus. The significance of the different PP1 isoforms remains unclear; however, *in vivo* data show that they have distinct subcellular localization patterns (14, 15). All isoforms are found in the nucleus in interphase cells, with PP1γ and PP1βδ showing additional accumulations in nucleoli. PP1 isoforms localization patterns are also dynamic and change both through the cell cycle and in response to various cellular perturbations. It is important to note that global patterns of localization that are observed for each isoform represent the sum of many different PP1-targeting subunit complexes, as shown by the immunostaining of several nuclear targeting subunits with overlapping localization patterns. The distinct localization patterns of the PP1 isoforms therefore imply difference in the specificity of interaction with particular targeting subunits, and so preferential incorporation into different signalling complexes.

It is commonly assumed among enzymologists that the substrate specificity of an enzyme is determined primarily by sterochemical complementarity between a substrate and the active site. The multiplicity of localizations and functions in the case of PP1 suggest that the preference for a phosphorylated sequence in the different protein substrates is not very stringent and does not explain *in vivo* specicificity. PP1 has evolved effective catalytic machinery but lacks strong substrate specificity in its active site. PP1 finds its targets via a large number of regulatory subunits, which influence the activity, and cellular localization of the phosphatase (Figure 1B) (16). To date, more than 50 PP1-protein interactions have been identified (6, 17, 18). With few exceptions so far, PP1 associates with its targeting subunits through the small motif RVXF. Co-crystallization of PP1

with a synthetic peptide encompassing the RVSF sequence established the RVXF motif as a conserved binding sequence that associates with a hydrophobic pocket on the surface of the PP1 catalytic subunit (19). Recently a systematic analysis of structural elements that mediate the binding specificity of PP1 interacting proteins proposed a refined consensus motif for high affinity PP1 ligands (20). Application of the results of this study to protein sequence databases searches enabled the authors to predict PP1-interacting partners and mutational analysis have demonstrated that differences in peptide-protein interactions dictate the affinity of PP1 for cellular regulators and control the dynamic physiological regulation of PP1 functions in the cell.

PP1 IN *S. pombe*

While mammalian cells contain four isoforms of the PP1 catalytic subunit (α, β, γ and δ), fission yeast contains two (6, 21). The unicellular lifestyle, sophisticated genetics and systematic approaches to study phosphorylation (22) makes yeasts particularly attractive models to study PP1 function (23).

The *S. pombe* genome encodes two highly related PP1 catalytic subunits Dis2 and Sds21. PP1 localisation was determined by fusing sequences encoding EGFP to the N-termini of Dis2 and Sds21. In order to maximise the likelihood that the tagged proteins were expressed at their normal physiological levels, the «marker switch approach» (24) to integrate the manipulated versions of *dis2* and *sds21* genes at their native loci was used. Either gene can be individually deleted, however, simultaneous deletion of both is lethal (21, 25). Importantly, both N-terminally EGFP-tagged proteins were shown to be functional as *Δsds21* cells expressing a Dis2.NEGFP at the *dis2*$^{+}$ locus and *Δdis2* cells expressing Sds21.NEGFP at the *sds21*$^{+}$ locus were viable and did not show any apparent defect in morphology, generation time and the actin and microtubule cytoskeletons.

PP1 has been localised to the nucleus in all organisms studied to date, such as *S. cerevisiae*, *S. pombe*, *Aspergillus nidulans*, and mammalian cells. Consistent with these previous studies, Dis2.

NEGFP and Sds21.NEGFP were greatly enriched in the nucleus, being Sds21.NEGFP concentrated in the nucleolus (Figure 2A, B). Intriguingly, in addition to the anticipated nuclear localisation, new locations for Dis2.NEGFP, but not for Sds21.NEGFP, were also observed. Dis2.NEGFP appeared as a bright dot at the nuclear periphery, at the cell tips, endocytotic vesicles and in a ring at the cell equator in early anaphase (Figure 2A, inset). Deletion of *dis2*$^{+}$ from *sds21.NEGFP* cells led to an increase in Sds21.EGFPN protein levels and the incorporation of Sds21.NEGFP into all of the locations normally occupied by Dis2, with the exception of chromatin and the staining around the cell equator during division (Figure 2C).

The bright nuclear dot of Dis2.NEGFP colocalised with a centromeric marker, Cnp1.Cherry, and chromatin immunoprecipitation showed that Dis2.Npk associated with centromeric sequences of the central core non-repetitice domains of the centromeres (Figure 2D).

The cytoplasmic Dis2.NEGFP foci could be divided into two distint subpopulations. One appearing at the cell cortex and moving from it into the cytoplasm for a short distance and another one associated with the cell tips (Figure 3A). The internalisation was dependent on a functional F-actin cytoskeleton and on the endocytotic factor Sla2. The recruitment to cell tips was dependent on the *S. pombe* Bud14 homologue, Wsh3/Tea4 (26, 27) (Figure 3B), and the Kelch domain protein Tea1 (Figure 3C). Wsh3/Tea4 co-localised with Dis2 and physically associated with it (Figure 3D, E). This association relied upon the conserved PP1 binding consensus site in Wsh3/Tea4 ($RV^{223}XF^{225}$) (Figure 3E). Mutation of this sequence blocked the recruitment of Dis2 to cell tips, but neither Tea1 nor Wsh3/Tea4 itself. Moreover, mutation of the PP1 binding site of Wsh3/Tea4 compromised the control over the establishment and choice of polarised tip growth that accompanies cell cycle progression of unperturbed cultures and led to a major deficiency in re-establishing polarised growth from existing tips following osmotic stress. This function may be due, in part, to the impact of Wsh3/Tea4 upon actin polymerisation as the ability of excess Wsh3/Tea4 to induce excessive F-actin cables was abolished by mutation of the PP1 binding sites (28).

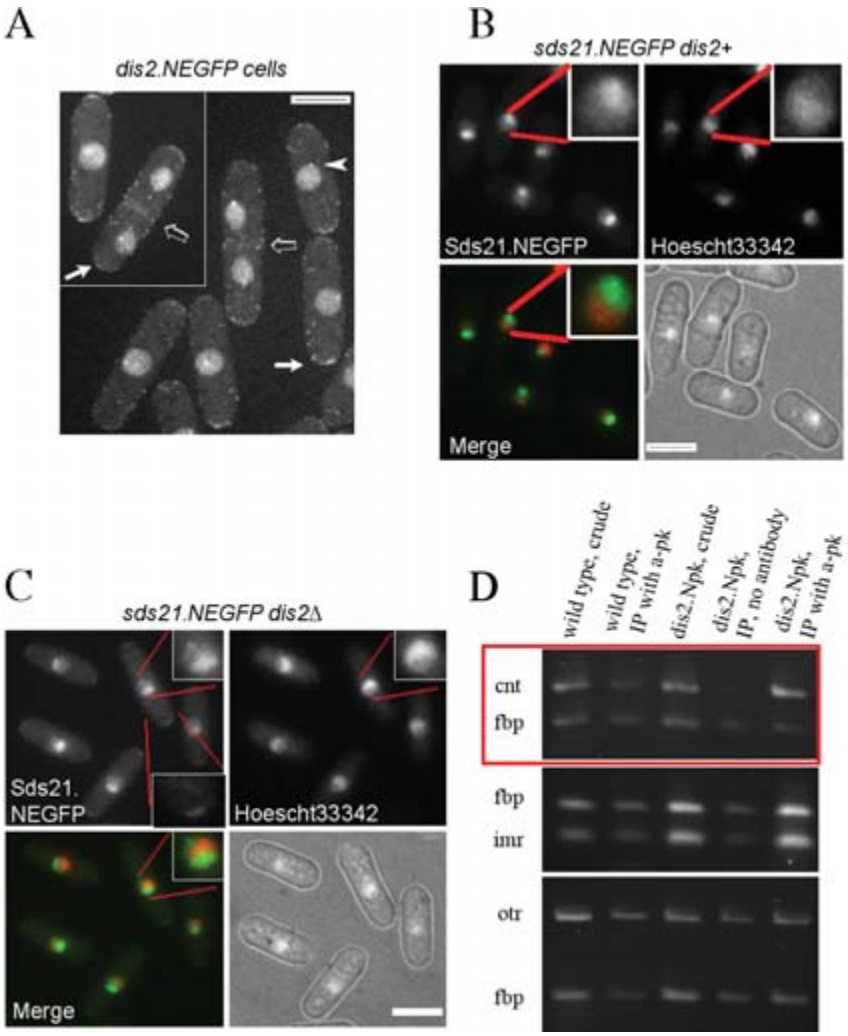

FIGURE 2.- ***Subcellular localisation of Dis2.NEGFP and Sds21.NEGFP.*** ***A)*** *Dis2.NEGFP localized in the nucleus and diffusely in the cytoplasm. It also associated with a bright nuclear dot (➤), the cell tips (➙), cell equator (⇨) and endocytic vesicles.* ***B)*** *Sds21.NEGFP localized in the nucleolus, not stained with Hoescht 33342 which stains the chromatin rich region.* ***C)*** *Sds21.NEGFP in dis2Δ cells localized in the nucleolus, the bright nuclear dot and the cell tips (insets).* ***D)*** *ChIP analysis showed a clear enrichment of the signal arising from PCR with primers to the central but no other sequences in immunoprecipitates from dis2.Npk cells (lane 5), but not when antibodies were not conjugated to the beads (lane 4) or the strain did not contain the tagged dis2⁺ gene (lane 2). Bar, 5 μm (A, B, C).*

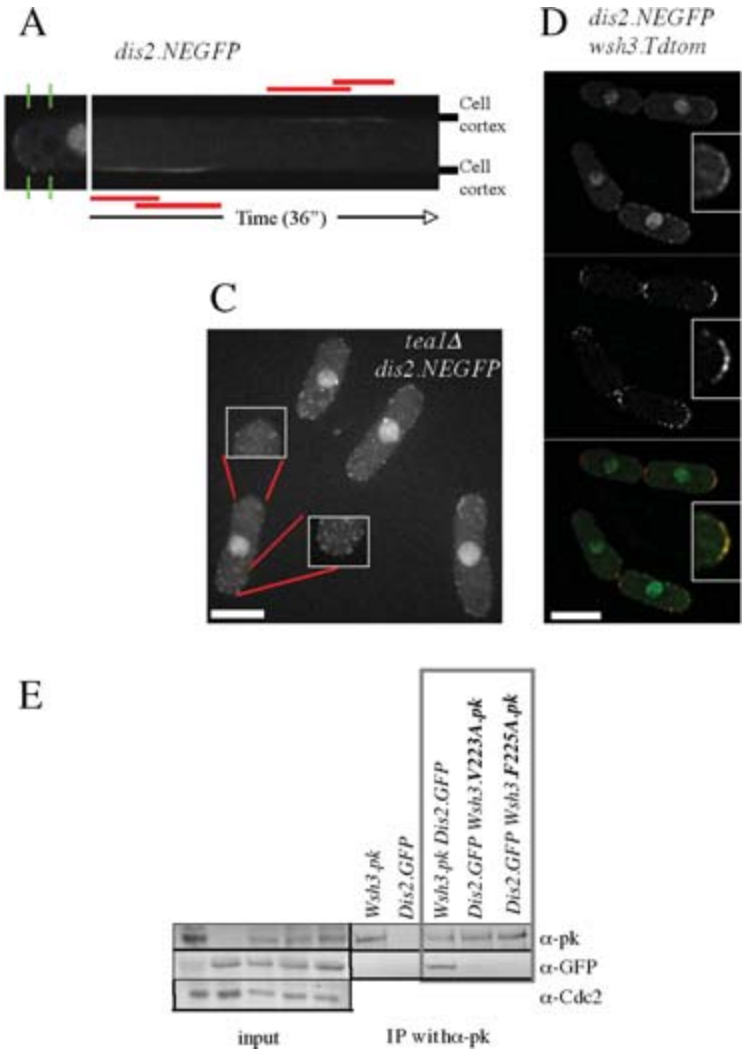

FIGURE 3.- ***Dis2 internalisation and recruitment to the cell tips. A)*** *Kymograph of the area between the green lines shows the internalisation movement of Dis2.NEGFP.* ***B, C)*** *In wsh3Δ* ***(B)*** *and tea1Δ* ***(C)*** *cells the Dis2.NEGFP cap structure at the cell tips was absent.* ***D)*** *Consecutive green and red images of dis2.NEGFP wsh3.tdTom cells showed the colocalisation of Dis2.NEGFP and Wsh3.tdTom at cell tips.* ***E)*** *Extracts were prepared and Pk immunoprecipiates were isolated and blotted with antibodies to recognise the Pk or GFP epitopes. Dis2 and Wsh3 co-immunoprecipitated. Mutations of the PP1 binding site of Wsh3 (V223A or F225A) abolished Dis2 and Wsh3 interaction. Bar, 5 μm (B, C, D).*

POSSIBLE FUNCTIONS OF DIS2 AND SDS21 IN THE NUCLEUS

While PP1 has been found in the nucleus of all organisms, there are differences between the localisation of specific isoforms and cell cycle stages between organisms. In mammalian cells, fluorescence microscopy revealed specific and distinct *in vivo* localisation patterns for PP1α-GFP, PP1β/δ-GFP and PP1γ-GFP (14). These localisations agreed with immunolocalisation data shown for endogenous isoforms using anti-peptide antibodies specific for each isoform (29). In *S. cerevisiae*, GFP-Glc7 localises in the nucleus throughout the mitotic cell cycle, and is enriched in the nucleolus (30). Finally, BIMG-GFP, in *Aspergillus nidulans*, also localises to the nucleus. However, its localisation is dependent upon nutritional conditions; on a poor growth medium, BIMG-GFP shows a greater affinity for the nucleolus than the nucleoplasm, whereas on rich medium, it is more evenly distributed between the two nuclear regions (31). PP1 also changes its distribution in response to nutritional conditions in mammalian tissue cultures, where PP1 is located primarily in the cytoplasm in quiescent cells, but accumulates in the nucleus upon addition of serum and entry into the mitotic cell cycle (32). Likewise, in budding yeast, GFP-Glc7 is uniformly dispersed throughout the cell in stationary phase (33). In the case of PP1 in *S. pombe*, changes in Dis2 and Sds21 nuclear localisation have not been observed in response to different nutritional conditions.

Not much is known about the possible roles for PP1 in the nucleolus. An *Aspergillus nidulans* PP1 mutant, *bimG11*, has hyperphosphorylated nucleolar proteins, supporting the idea that substrates for PP1 are found in the nucleolus (34). In contrast to the uncertainty about the possible role of PP1 in the nucleolus, many studies have linked PP1 function to key nuclear processes such as cell-cycle progression, replication, transcription and RNA processing.

One of the most supported roles for chromatin-associated PP1 is the reversal of signalling by protein kinases of the Aurora family. One of the mitotic substrates of Aurora kinases is histone H3, which is phosphorylated on serine 10 (Ser 10) by the unique Aurora protein kinase in budding yeast, IplI (35) and in fission yeast, Ark1 (36), and Aurora B protein kinase in *Drosophila* (37). Various studies have

reported a correlation between the phosphorylation of histone H3 along chromosomes in G2 and chromosome condensation (35, 37, 38) and also between chromosome decondensation in telophase and PP1 activity or histone H3 dephosphorylation (39, 40). Accordingly, in fission yeast and in *Drosophila*, phosphorylation of histone H3 by Ark1 and Aurora B, respectively, is involved in the recruitment of condensin complex to mitotic chromatin, and chromosome condensation (36, 37, 41). Moreover, it has been published that histone H3 is an *in vivo* mitotic substrate of PP1 in budding yeast (35) and an *in vitro* one in *Xenopus* (42). These observations have led to the hypothesis that chromosome (de)condensation requires histone H3 (de)phosphorylation. However, differences seem to exist between species since mutation of Ser 10 of histone H3 to alanine did not cause any observable growth defect in budding yeast (35) and neither Ser 10 nor the entire N-terminal tail of *Xenopus* histone H3 is essential for chromosome condensation (43). The histone H3 kinase activity of *Xenopus* Aurora-B depends on its phosphorylation by an unknown kinase, which may well be Aurora-B itself, as its budding yeast counterpart, Ipl1, is known to undergo autophosphorylation (42-44). Interestingly, in *Xenopus* this Aurora-B activation has been shown to be antagonized by PP1 (42).

Although the antagonistic relationship between Aurora and PP1 seems to be conserved from yeast to human, the possible interaction in fission yeast between Ark1 and Dis2/Sds21 and the involvement of Dis2/Sds21 in the dephosphorylation of H3 have not been studied yet. Interestingly, *ark1*$^+$ overexpression greatly inhibited cell growth of the cold sensitive conditional *dis2.11* mutant at the permissive temperature, and exacerbated the mitotic defects of *dis2.11* resulting in a complete absence of colony formation at the restrictive temperature (our unpublished results). Wild type cells tolerate *ark1*$^+$ overexpression, and only display a minor phenotype, as 3% of the population prematurely commit to mitosis (36). Thus, the effect of *ark1*$^+$ overexpression in *dis2.11* could suggest a genetic interaction between *dis2*$^+$ and *ark1*$^+$ and antagonising relationship between these proteins in *S. pombe*.

Recently it has been suggested that Dis2 controls the release of cells from the G2 DNA damage checkpoint (45, 46). Dis2 overexpression sensitises cells to DNA damage so that they are unable

to arrest in G2 phase after DNA damage. Instead they go into mitosis and cells undergo a catastrophic mitosis. Consistent with a role for Dis2 in release from G2 DNA damage checkpoint arrest, *dis2Δ* and *dis2.11* cells have a prolonged delay in response to DNA damage in the G2 phase. However, Dis2 is unlikely to be the only protein responsible for cell cycle re-entry following DNA damage checkpoints arrest because the majority of *dis2Δ* cells do eventually recover, albeit several hours after wild type cells. Moreover, the study by Elzen and O'Connell also showed that Dis2, but not Sds21, was able to dephosphorylate and inactivate Chk1 kinase *in vitro* and *in vivo*. However, whether this dephosphorylation is responsible for the release of the checkpoint arrest remains to be determined.

POSSIBLE FUNCTIONS OF DIS2 AT THE CENTROMERES

Multiple studies carried out in budding yeast and animals suggest that PP1 may be counteracting Aurora kinase at the kinetochore. In *S. cerevisiae*, temperature sensitive *ipl1* mutants missegregate chromosomes severely and die at elevated temperatures (47). The ts^- growth phenotype of such mutants can be partially suppressed by mutations of *S. cerevisiae* PP1, $GLC7^+$ (48). Furthermore, increasing the dosage of *GLC7* results in chromosome missegregation in wild-type cells and lethality in *ipl1* mutant cells, presumably due to exacerbation of the *ipl1* mutant phenotype. These observations indicate that a reduction in Ipl1 protein kinase activity can be compensated for by a reduction in PP1 activity, and exacerbated by an increase in PP1 activity. This suggests that PP1 opposites Ipl1 protein kinase in regulating yeast chromosome segregation.

PP1 and Aurora may be counteracting each other in the control of the phosphorylation state of kinetochore proteins. And the phosphorylation state of kinetochore proteins may affect their binding to centromeric DNA, their competence to bind microtubules and/or their association with other kinetochore proteins.

In budding yeast, the phosphorylation state of Ndc10, a component of the kinetochore complex CBF3, affects the microtubule-binding activity of kinetochores reconstituted in cell extracts. Interestingly, Ndc10 is hyperphosphorylated in *glc7.10* mutant cell

extracts, which display low microtubule-binding activity of kinetochore complexes *in vitro* and exhibit a high frequency of chromosome loss *in vivo* (49) Thus, a role for Glc7 in the regulation of kinetochore-microtubule attachment by phosphorylation can be inferred. However, Ndc10 has not yet been shown to be a substrate of Glc7. Moreover, even when Biggins and colleagues demonstrated that Ndc10 was phosphorylated by Ipl1 *in vitro* (44), its phosphorylation status *in vivo* has not been studied yet.

Recent studies in *S. cerevisiae* have generated insight into the molecular mechanisms of kinetochore bipolar microtubule attachment (chromosome bi-orientation). One of the key players in establishing and monitoring bi-orientation is the chromosome passenger complex Aurora B/survivin (Ipl1/Sli15) (50, 51). The complex Ipl1-Sli15 has been proposed to facilitate the re-orientation of kinetochore-spindle pole connections until tension is exerted on sister kinetochores by bipolar microtubule attachment (51). Sli15 associates with Ipl1 and stimulates its kinase activity (52, 53). Interestingly, in *S. cerevisiae* the temperature-sensitive mitotic defects of *sli15* mutants are attenuated by overexpression of a dominant-negative truncated version of Glc7 (53) Moreover, it seems that Ipl1 is also required to activate the spindle checkpoint when tension is not exerted on kinetochores (44).

Furthermore, it has been found that Ipl1 phosphorylates Dam1 *in vitro* and that Dam1 phosphorylation is dependent on Ipl1 *in vivo* (52). Dam1 is a component of the multimeric spindle-associated DASH complex that is required for bi-orientation of sister kinetochores and for mitotic spindle integrity (54, 55). Interestingly, *dam1* mutants that might mimic constitutive phosphorylation of Dam1 are able to partially suppress the defects of *ipl1.2* but are synthetically lethal with *glc7.10*. Furthermore, overexpression of Glc7 exacerbates the temperature-sensitive growth defect of *dam1* mutant cells, indicating that Dam1 may also be a substrate of PP1 (52). Recently, Cheeseman and co-workers have shown that Dam1 phosphorylation does not reduce DASH complex binding to microtubules *in vitro* or change the composition of the complex (52). Instead, they suggest that Dam1 phosphorylation weakens/abolishes the association between the DASH complex and the rest of the kinetochore (56, 57). Interestingly, this hypothesis has been recently

supported by the work of Shang and colleagues (58). They have shown that the interactions between Dam1 and Spc34 (a subunit of the Dam1 complex) and Ndc80 (a subunit of the Ndc80 complex), are weakened by mutations mimicking phosphorylation at Ipl1 sites (58). A model has been proposed in which phosphorylation of essential kinetochore components, such as Dam1 and Ndc80 complexes, weakens their physical interactions and therefore results in the detachment of the kinetochore from microtubules. Dephosphorylation by Glc7 enables the re-association of the DASH complex with the kinetochore. This process of dissociation and reassociation may facilitate the re-orientation of kinetochores and may continue until bi-orientation is eventually established (Figure 4).

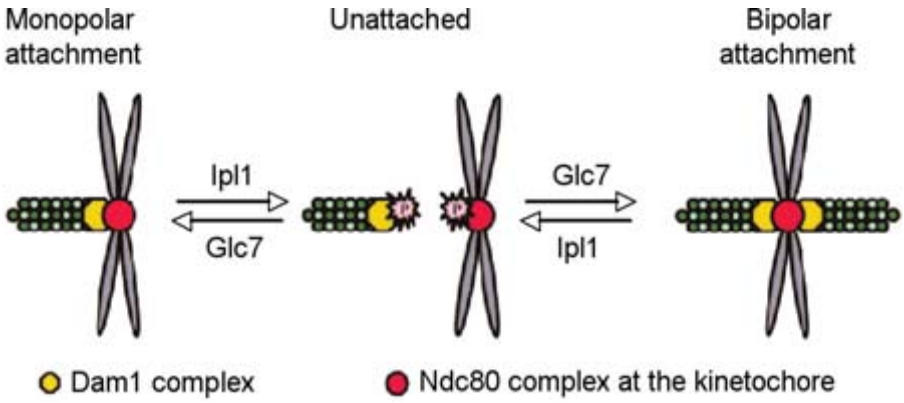

FIGURE 4. ***Model for phosphoregulation at the yeast kinetochore.*** *Interactions between the Dam1 complex and the Ndc80 complex are proposed to be weakened when Dam1 and Ndc80 are phosphorylated by Ipl1. The weakening of this association facilitates the subsequent establishment of new kinetochore-microtubule attachments and the establishment of bipolar attachment. Glc7 might be counteracting Ipl1. Model adapted from (56) and (58).*

POSSIBLE FUNCTIONS OF DIS 2 IN ENDOCYTOSIS

The extensive characterisation of endocytosis in *S. cerevisiae* provides a framework within which to view Dis2 association with

endocytic vesicles. Dis2.EGFPN dots were moving from the cell cortex towards the interior of the cell for a short distance. This pattern of movement was very similar to that recently described in budding yeast for proteins involved in endocytic internalisation. In basis of the analysis of the motility of fusions between endocytotic proteins and GFP a model for the early endocytotic pathway has been proposed (59-61) (Figure 5).

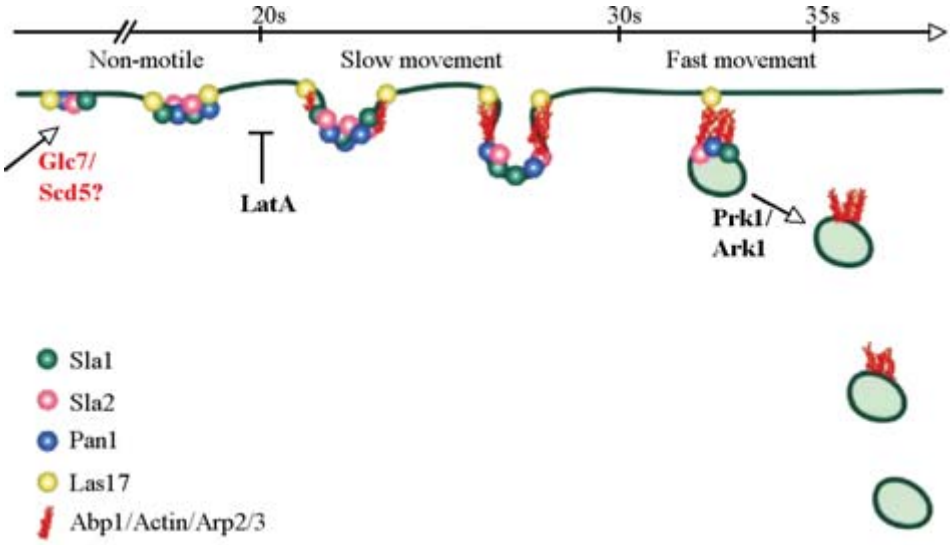

FIGURE 5. ***Pathway for the association of receptors, adaptors and actin during endocytic internalisation.*** *First, Sla1, Sla2, Pan1 and Las17 assemble in a nonmotile complex at the plasma membrane. Then, actin, Abp1, and the Arp2/3 complex are recruited, whereupon the complex containing Sla1, Sla2 and Pan1 slowly moves away from the plasma membrane, towards the interior of the cell. The early patch components are disassembled during this slow movement phase. The patches containing only late components change then to a fast movement phase during which the late components are disassembled. Actin polymerisation is required for the movement of the presumed endocytic vesicles and its associated protein complex, and for subsequent disassembly of the complex. The early components of the patches (Sla1, Sla2 and Pan1) are likely to be disassembled as a response to phosphorylation by the Ark1 and Prk1 kinases. This phosphorylation might disrupt the association of Sla1 with Pan1. If Pan1 and Sla1 are phosphorylated and then lost from the complex, dephosphorylation might allow their re-incorporation into cortical complexes. This dephosphoryaltion might be carry out by the Glc7-Scd5 complex. Adapted from Kaksonen et al. (59).*

The site of endocytosis is determined in a non-motile phase by the co-operative action of molecules such as clathrin, Sla1, Sla2 and Pan1. The association of these proteins with the emerging vesicle persists through the next, slow motile, phase during which the recruitment of myosin 5 and polymerisation of actin accompanies the formation of the clathrin pit. The vesicle then enters a rapid motile phase following the cessation of further actin polymerisation, the departure of myosin 5 and the scission of the pit to form the vesicle. Actin polymerisation is accompanied by the recruitment of homologues of the higher eukaryotic proteins N-WASP and the Arp2/3 complex which harness actin polymerisation to promote scission and the eventual long range, fast motility of the free vesicle in the final phase (60, 61). The early components of the patches (Sla1, Sla2 and Pan1) are likely to be disassembled as a response to phosphorylation by the Ser/Thr kinases Ark1 and Prk1 (note that *S. cerevisiae* Ark1 is not related to the fission yeast aurora-related kinase Ark1). Ark1 and Prk1 localise to actin patches in an Abp1-dependent manner (62) and are thus likely to be components of late patches. Recent data support a model in which, following the association of actin with the endocytic machinery, Ark1 and Prk1 can phosphorylate Sla1 and Pan1 and this Prk1-dependent phosphorylation disrupts the association of Sla1 with Pan1 (63). If Pan1 and Sla1 are phosphorylated and then lost from the complex, dephosphorylation might allow their re-incorporation into cortical complexes. This remains to be shown, as the role of phosphatases in endocytosis has not been extensively studied. However, interactions between Sla1 and the yeast homologue of PP1, Glc7 (64, 65), as well as between Pan1 and Glc7 have been reported (66). Interestingly, the *glc7* conditional mutant *glc7.10* has been shown to be defective in vacuolar fusion and in secretory and endocytic vesicular transport (67). Similar phenotypes are observed after the functional disruption of proteins involved in endocytosis such as Scd5 or Sla2 (67, 68).Scd5 has been identified as a PP1-binding protein in various screens (65, 69, 70). Scd5 binds to Glc7 via a classic PP1 binding motif and the disruption of the RVXF motif of Scd5 severely compromised endocytosis, actin structure and confers temperature sensitive lethality. Sla2 interacts physically and genetically with Scd5 (71, 72), and Scd5 is required in conjunction with clathrin for the association of Sla2 with cortical actin (73). A possible scenario could be that

Scd5 targets PP1 to actin patches and vesicles, where the Glc7/Scd5 holoenzyme counteracts Prk1 phosphorylation being potential substrates of Glc7/Scd5 the phosphoproteins Sla2, Sla1 and Pan1 (73, 74). As Prk1 phosphorylation acts at multiple levels to regulate actin dynamics and the association of endocytic proteins a dynamic interplay between Scd5/Glc7 and Prk1 is likely to be critical to control endocytosis.

Although endocytosis is less extensively characterised in fission yeast, where it has been studied, it mirrors that of budding yeast. The fission yeast Sla2/End4 gene product localises in the identical manner to its budding yeast orthologue and is similarly required for endocytosis (75). By analogy with the three phases in budding yeast, we found that Dis2 associates with vesicles during the non-motile phase and leaves just prior to the highly motile one. It was recruited to the patches after Sla2/End4. As has been previously reported for Sla2/End4, de-polymerisation of actin with LatA did not block recruitment of Dis2 to the cell cortex but did block Dis2.NEGFP foci at the cell cortex before internalisation (76-78).

POSSIBLE FUNCTIONS OF DIS 2 IN POLARIZED CELL GROWTH

The recruitment of a second population of Dis2.NEGFP foci to cell tips by Wsh3/Tea4 in a manner that is dependent upon the Kelch domain protein Tea1 is highly reminiscent of budding yeast, where Bud14 protein relies upon the Kelch domain proteins Kel1 and Kel2 to recruit Glc7 to the bud tip to control dynein activity during spindle orientation. The *S. pombe* Bud14 homologue, Wsh3/Tea4 (26, 27) both co-localised with Dis2 and physically associated with it. The binding of PP1 to Wsh3/Tea4 is critically important for the role played by Wsh3/Tea4 in maintaining polarised tip growth as cells transit the cell cycle or recover from osmotic stress. This function is likely to involve modulation of the F-actin cytoskeleton as the ability of Wsh3/Tea4 to promote F-actin polymerisation is compromised when it cannot bind PP1 (Figure 6).

Thus, like its *A. nidulans* homologue BimG, Dis2 function is required for tip growth. The requirement for PP1 function during

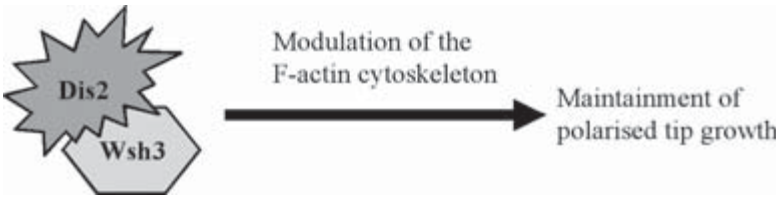

FIGURE 6. ***Involvement of Dis2 and Wsh3 in polarised tip growth.*** *Recruitment of Dis2 to the cell tips by Wsh3 is required for the maintainment of polarised tip growth, most likely due to the modulation of the F-actin cytoskeleton.*

recovery from stress echoes the requirement for phosphorylation of the polo kinase Plo1 on serine 402 to promote tip growth during recovery from centrifugation and heat stress (79). However, it remains to be established whether Dis2 counteracts the action of polo kinase or any of the other protein kinases, such as Pom1, Kin1, Pck2 and Pck3 that modulate cell morphogenesis in fission yeast (80-82). Alternatively it could play a critical role in modulating the activity of the kinases of the stress response pathway that associates with Wsh3/Tea4 (27). In this scenario the impact on morphogenesis would be mediated by stress signalling to either halt growth in the initial response to the stress, or to re-initiate it once cell composition has been changed to deal with this altered state.

Although Bud14/Glc7 is required for spindle orientation rather than cell morphogenesis the conservation of Bud14 related molecules across species indicates that the identification of the substrates of the Dis2-Wsh3/Tea4 holoenzyme and clarifying its relationship with the cell polarity/morphogenesis kinases will shed much light on the control of the microtubule cytoskeleton and cell morphogenesis by protein phosphorylation.

FUTURE PERSPECTIVES

The field of protein phosphorylation research has entered a new era for several reasons. First, because of what is a nearly complete list of catalytic subunits in several organisms, it is now possible to do functional genomic screens for the role of phosphatases in specific biological processes. Second, as more phosphatase-targeting subunits

are identified and characterized, it is increasingly clear that they have a fundamental role in phosphatase function and that a comprehensive catalogue of these proteins is crucial to our understanding of the varied intracellular functions of their associated catalytic subunits (83).

Targeting these regulatory subunits or disrupting their interaction with the catalytic subunit might also represent a feasible means to treat specific diseases. The potential of this idea was typified in the large-scale loss of function screen done by MacKeigan and colleagues (84) This group found that 32% of the phosphatases screened promoted cell survival, whereas 10 protein phosphatases were classifies as «cell death phosphatases» (that is, the reduction of their cellular levels caused resistance to apoptosis-inducing agents), and can therefore be considered as putative tumour suppressors.

Mass spectrometry-based proteomics has become a powerful tool to identity the components of multiprotein complexes, and more recently, several techniques have exploited the use of heavy isotope tags to compare and quantitate relative protein levels under different biological conditions. Clearly, mass spectrometry-based approaches and large-scale functional genomics will be driving forces in this new era in which protein phosphatases will sit on centre stage with their protein kinase counterparts.

ACKNOWLEDGMENTS

This work was supported by Cancer Research UK (CRUK), Direccion General de Investigacion Cientifica y Tecnica (DYGYCYT) and the Basque Government.

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