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Report

The First Green Lineage cdc25 Dual-Specificity Phosphatase

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KEY WORDS

Cdc25, alga, plant, cell division, cell cycle, regulation, control, G_2/M, transition, MPF

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NOTE

Supplemental data can be found online at http://www.landesbioscience.com/journals/cc/khadarooCC3-4-sup.pdf.

ABSTRACT

The Cdc25 protein phosphatase is a key enzyme involved in the regulation of the G_2/M transition in metazoans and yeast. However, no Cdc25 ortholog has so far been identified in plants, although functional studies have shown that an activating dephosphorylation of the CDK-cyclin complex regulates the G_2/M transition. In this paper, the first green lineage Cdc25 ortholog is described in the unicellular alga *Ostreococcus tauri*. It encodes a protein which is able to rescue the yeast *S. pombe cdc25-22* conditional mutant. Furthermore, microinjection of GST-tagged *O. tauri* Cdc25 specifically activates prophase-arrested starfish oocytes. In vitro histone H1 kinase assays and anti-phosphotyrosine Western Blotting confirmed the in vivo activating dephosphorylation of starfish CDK1-cyclinB by recombinant *O. tauri* Cdc25. We propose that there has been coevolution of the regulatory proteins involved in the control of M-phase entry in the metazoan, yeast and green lineages.

INTRODUCTION

Cell cycle regulation is highly conserved throughout evolution and is basically ensured by the kinase activity of the family of cyclin dependent kinases (CDKs) and their regulatory cyclin subunits.[1,2] Regulatory mechanisms present in some lineages have somehow evolved differently in other species; for example, the Rb/E2F/DP pathway which is present in animals and plants is nevertheless absent in yeast.[3] Therefore, comparative studies of the cell cycle among models belonging to different lineages can provide crucial information in distinguishing between the core cell cycle common to all phyla and adaptations specific to a lineage, clade or organism.[4]

Cdc25 is the key enzyme involved in the regulation of the G_2/M transition.[5] Its phosphatase activity activates the CDK-cyclin complex which is inhibited by the kinase activity of Wee1.[6] This regulation pathway is essential for M-phase entry and a deletion of both Wee1 and Mik1 or of Cdc25 in *Schizosaccharomyces pombe* is lethal.[7] The high degree of conservation of the G_2/M transition regulation pathway implies that coevolution of the CDK-cyclin complex, Wee1 and Cdc25 has occurred.[8] This hypothesis predicts that orthologs of all the genes, which were present early during evolution, must be found in the different lineages whose G_2/M transition is regulated by the well-conserved CDK-cyclin complexes.[9] As predicted, CDKs, cyclins and wee1 orthologs are found in all currently sequenced eukaryotic genomes, including metazoans, fungi and plants.[3,10] Also, orthologs of Cdc25 have been isolated in both vertebrate and invertebrate animals and in fungi, but no Cdc25 ortholog has so far been identified in plants, even though the genomes of *Arabidopsis thaliana* and rice have been completely sequenced.[11-14]

Though higher plants lack the cdc25 gene, the inhibitory kinase wee1 gene is well conserved: overexpression of *A. thaliana* Wee1 in fission yeast causes cell cycle arrest—the cells grow but do not divide, thus providing evidence for a functional Wee1 protein in *A. thaliana*.[10,15] In addition, various studies have shown that

1. The Thr14-Tyr15 CDK site which is dephosphorylated by Cdc25 in animals is conserved in plant CDKs,
2. the in vitro dephosphorylation by *S. pombe* Cdc25 is correlated with the activation of the CDK-cyclin complex in tobacco stem cells, and
3. the in vivo overexpression of *S. pombe* Cdc25 phosphatase in tobacco BY2 cells yields smaller cells reminiscent of the wee phenotype.[16,17]

Interestingly, CDK activity is also inhibited by tyrosine-phosphorylation in the *Fucus spiralis* zygote.[18] These arguments suggest that the plant G_2/M transition is switched on by an unknown activating dual-specificity phosphatase and switched off by the inhibitory kinase, Wee1; this unknown green lineage activating phosphatase being very weakly related or unrelated to the Cdc25 phosphatase family.[14,19]

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Ostreococcus tauri is a unicellular alga which diverged very early in the green lineage.[20] It is the smallest free-living eukaryotic cell described to date (diameter 1 μm) and has a minimal cellular organization with a nucleus, only one chloroplast and one mitochondrion, and a nude plasma membrane.[21,22] Its 11.5 Mb genome which is distributed among 18 chromosomes is currently being fully sequenced (Derelle E; personal communication).

In this paper, we describe a functional cdc25 gene in *O. tauri* which is the first cdc25 ortholog found in the green lineage. The *O. tauri* cdc25 gene codes for a protein having a dual specificity phosphatase activity which is able to rescue the *S. pombe* Cdc25 conditional mutant and which specifically activates the CDK1-cyclin B complex in prophase-arrested starfish oocytes in vitro and in vivo.

EXPERIMENTAL PROCEDURES

Materials. The *Ostreococcus tauri* culture used in this study is the strain OTTH0595.[23] Cultures were grown in Keller's medium (Sigma-Aldrich, Saint-Quentin Fallavier, France) dissolved in 0.2 μm filtered sea water (NaCl 38 $g.l^{-1}$). Growth conditions were a temperature of 18°C, a permanent irradiance of 60 μmol $quanta.m^{-2}.s^{-1}$ and mild agitation.[24] Cell population growth was followed by flow cytometric analysis. The starfishes were collected in the Banyuls bay during the breeding season (November to March for *Marthasterias glacialis*, December to May for *Astropecten aranciacus*) and kept in running sea water.[25] Pieces of ovaries were taken by incising the starfish dorsal wall. The temperature sensitive *S. pombe* h^+ *cdc25-22 leu1-32 ura4-218 ade6-M210* mutant strain and the pREP41 vector were kindly provided by Dr. G. Lenaers (Université Paul Sabatier, Toulouse, France).[26,27]

Cloning the *Ostreococcus tauri* cdc25 Gene. The complete open reading frame (ORF) of *O. tauri* Cdc25 gene was obtained by the combined use of the SMART RACE™ cDNA Amplification kit (Clontech, Palo Alto, CA) and the Universal GenomeWalker™ kit (Clontech).

Phylogenetic Analysis of *Ostreococcus tauri* cdc25 Gene. Sequences were aligned with ClustalW and the alignments were manually improved using BioEdit.[28,29] Three different methods were used for the construction of the phylogenetic trees. Maximum likelihood phylogenetic analyses (quartet puzzling, using 25000 puzzling steps and a VT+γ substitution model) were performed using TREE-PUZZLE.[30-32] Neighbor-joining trees were constructed using TreeCon, based on Poisson corrected distances.[33] MrBayes was used for Bayesian inference of phylogenetic trees, using a JTT+γ substitution model.[34,35] Only unambiguously aligned positions were taken into account and in the NJ approach, bootstrap analyses with 500 replicates were performed to test the significance of the nodes.

Production of Recombinant Cdc25 Protein in Bacteria. The ORF of cdc25 was PCR amplified using the Advantage2 Taq DNA polymerase (Clontech) with specific primers containing restriction sites for directional cloning by BamHI and XhoI (forward primer FpgexBam : 5'-gtggatccATGGAGGTGCGGGAGGCGAACAA-GCGCGCG-3' and reverse primer RpgexXho 5'- tagattatactcgag-TCATTCGTTGTCCATGTCGGCCAC-3'). The PCR program used was an initial denaturation step at 95°C for 1 min to hotstart the enzyme, followed by 35 cycles at 94°C for 40 sec, 60°C for 1 min and 68°C for 1.5 min, and a final extension at 68°C for 5 min. The PCR product was gel purified using the Nucleospin® Extract 2 in 1 (Macherey-Nagel, Düren, Germany), and then cloned into the pGEM®-T Easy Vector (Promega, Madison, WI) for sequencing and subcloning. By restriction digesting the recombinant vector with BamHI (Promega) and XhoI (Promega), the cdc25 gene was directionally subcloned into the BamHI-XhoI digested pGex4T1 vector (Amersham Biosciences, Piscataway, NJ) and electroporated in *E. coli* DH5-alpha strain. For production, the recombinant pGex4T1-Cdc25 vector was electroporated in *E. coli* BL21 cells. The bacteria were grown to an A_{600} of 0.5 at RT in LB medium containing 100 $\mu g.mL^{-1}$ ampicillin. Recombinant protein expression was induced by addition of Isopropyl-beta-D-thiogalactoside (IPTG) to the cells for 6 hours at a final concentration of 0,2 mM. The GST-fused protein was affinity purified on glutathione sepharose using a GStrap FF column with an Äktaprime system (Amersham Biosciences, Orsay, France).

In vitro Phosphatase Assays. One arm of *Marthasterias glacialis* starfish was sectioned and the gonads were separated from their follicle cells by artificial calcium-free sea water treatment.[25] Prophase-arrested oocytes were rinsed in normal sea water, aliquoted in IPNP medium (50 $mmol.l^{-1}$ Tris pH 7.5, 150 $mmol.l^{-1}$ NaCl, 50 $mmol.l^{-1}$ NaF, 10 $mmol.l^{-1}$ Na pyrophosphate, 1 $mmol.l^{-1}$ Na_3VO_4, 10 $mmol.l^{-1}$ Phenylphosphate, 0.1 $mg.ml^{-1}$ soybean Trypsin Inhibitor, 0.1 % (V/V) Triton X100), were flash frozen in liquid nitrogen and stored at -80°C for further use. Aliquots of oocytes were thawed and centrifuged at 15000 x g for 10 minutes at 4°C. The extract was incubated at 4°C for 1h with sepharose beads grafted with $p13^{suc1}$ which specifically

Figure 1. Alignment of Cdc25 amino acid sequences. Comparison of the C-terminal end of the *Ostreococcus tauri* (Ot) Cdc25 protein sequence with other Cdc25s from different organisms (*M. musculus* [Mm], *D. melanogaster* [Dm], *S. pombe* [Sp], *P. carinii* [Pc], *S. cerevisiae* [Sc] and *C. elegans* [Ce]). Identical amino acids are boxed, gaps introduced during the alignment process are indicated with dashes. A consensus sequence was made showing the conserved residues among the Cdc25-family. The two bars indicate the CE[Y-F]SXXR motif and the conserved aspartate residue upstream from the (H)CX_5R motif, the typical signature for the Dual-Specificity Phosphatases (DSP),[51] to which Cdc25 belongs. The number of amino acid residues encompassing the regions beyond the conserved domains are given in parentheses. Supplemental Data: Table S1, Part A. List of NCBI Accession Numbers of the sequences used in the alignment represented in Figure 1 can be found online.

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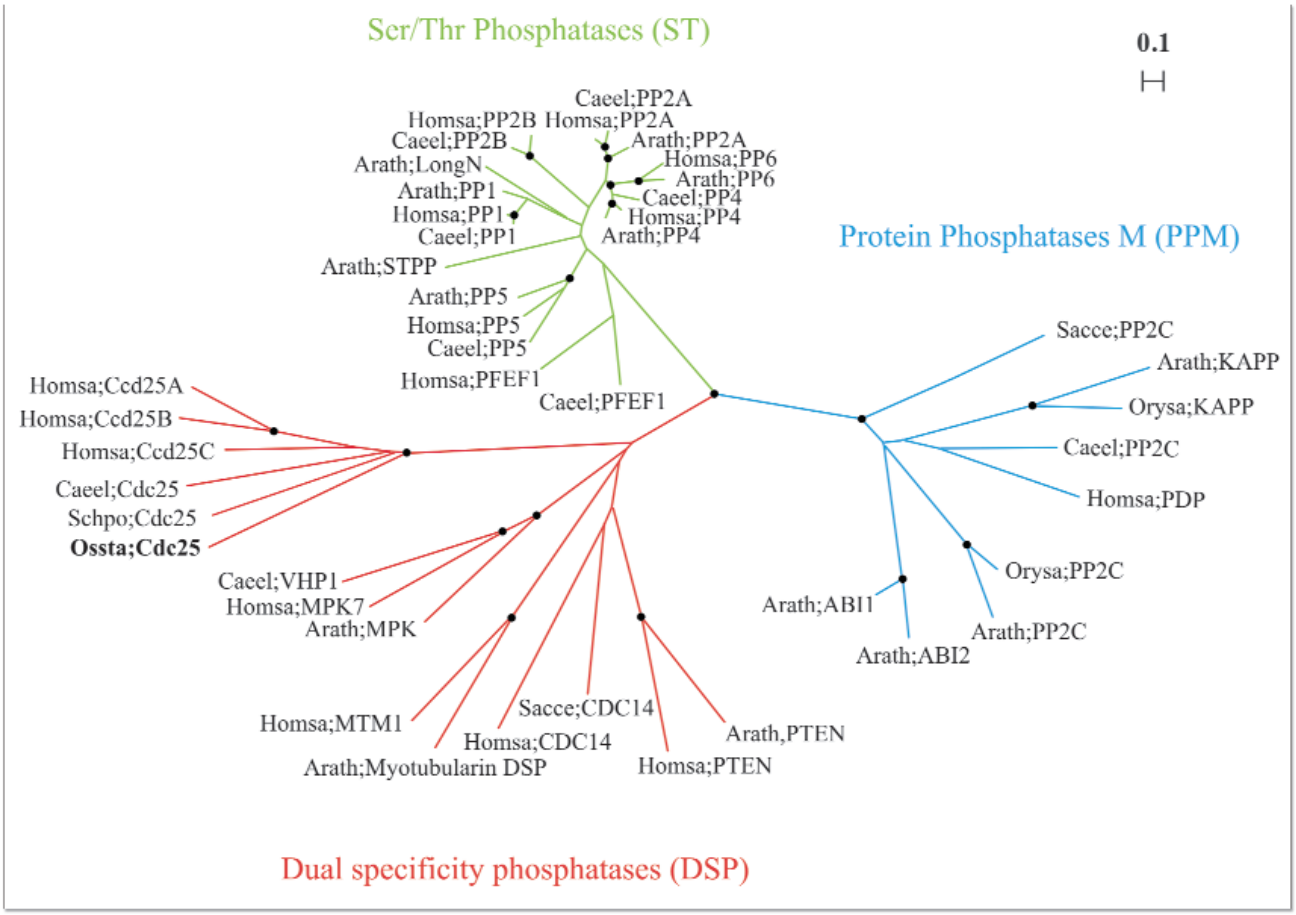

Figure 2. Phylogenetic analysis of Cdc25 genes. Classification of the *Ostreococcus tauri* Cdc25 within the phosphatase superfamily. For each family, representative protein sequences were taken from different organisms (*Saccharomyces cerevisiae* [Sacce], *Homo sapiens* [Homsa], *Arabidopsis thaliana* [Arath], *Caenorhabditis elegans* [Caeel], *Ostreococcus tauri* [Ossta], *Oryza sativa* [Orysa]), in order to perform phylogenetic analyses. Because of their short alignable region compared with the other members of the phosphatase superfamily, the Low Molecular Weight Phosphatases (low-Mr) were not included in the analysis. All tree construction methods (pairwise distance, maximum likelihood and Bayesian inference) gave the same topology. Here, the maximum likelihood tree constructed with TREE-PUZZLE is shown. Dots on the branches indicate a Quartet Puzzling Support Value higher than 70%. Supplemental Data: Table S1, Part B. List of NCBI Accession Numbers of the sequences used in making the phylogenetic tree represented in Figure 2 can be found online.

bind the CDK-cyclin complex. The beads were rinsed three times with TBS/T buffer and then washed with 1X PBS. They were further incubated for different time-lapses with recombinant *O. tauri* GST::Cdc25 at RT and washed three times with TBS/T.

a. Kinase Activity. Histone H1 kinase activity was measured using 5 µl of kinase buffer (8 mM Hepes, 10 mM $MgCl_2$, 0.1 mM ATP, 10 $\mu g.\mu l^{-1}$ histone H1, 1 % (V/V) gamma ^{32}P ATP). The reaction was stopped by adding 20 µl of 1X Laemmli sample buffer and boiled for 3 minutes. After autoradiography of the SDS-PAGE, the histone H1 bands were excised and counted by liquid scintillation.[36]
b. Anti-phosphotyrosine (Anti-Ptyr)Western Blotting. Immunoblotting using the anti-Ptyr antibody[37] was revealed by alkaline phosphatase detection method.[36]

Starfish Oocyte Microinjection. Prophase-arrested *Astropecten aranciacus* oocytes were separated from their surrounding follicle cells by calcium-free sea water treatment, and kept in natural filtered sea water until microinjection following the Hiramoto method.[38]

Rescue of the *S. pombe cdc25-22* Conditional Mutant. Cloning into the NdeI-BamHI digested pREP41 yeast expression vector was carried out as for the pGEX4T1 construction, except that:

a. pREP41 specific primers (forward primer: FprepNde : 5'-gtggattcat-ATGGAGGTGCGGGAGGCGAACAAGCGCGCG-3' and reverse primer RrepBam : 5'-actcgaggatccTCATTCGTTGTCCATGTCGGCC-ACTTCGTC-3') were used, and
b. the NdeI (Promega) and BamHI (Promega) restriction enzymes were used.[39]

The recombinant pREP41 vector was then electroporated into *E. coli* DH5-alpha cells. The resulting construct which contains the *O. tauri* cdc25 gene under control of thiamine-repressible nmt promoter, was transformed into the *S. pombe* h^+ *cdc25-22 leu1-32 ura4-218 ade6-M210* strain by electroporation and selected on leucine- and thiamine-deficient minimal medium.40 Positive clones growing on leucine-deficient medium at the permissive temperature (30°C) were tested at the restrictive temperature (37°C).41 Expression of the *O. tauri* Cdc25 was repressed by the addition of 25 µM thiamine.

RESULTS

An Ortholog of cdc25 is Present in *Ostreococcus tauri*. During the sequencing of the complete genome of *O. tauri*, a clone showing significant similarity to the C-terminal part of the cdc25 gene family was identified. The complete gene was obtained by using a RACE-PCR approach and was later confirmed by the contig assembly of the genome sequencing project. This yielded a complete open reading frame (ORF) of 1188 bp without any intron (accession number AY330645) encoding a 395 residue protein. *O. tauri* cdc25 ORF has a GC content of 62% which is much higher than the GC content range of 40–45% found in animal and yeast cdc25 orthologs. Blast search analysis of the *O. tauri* Cdc25 protein sequence revealed a typical

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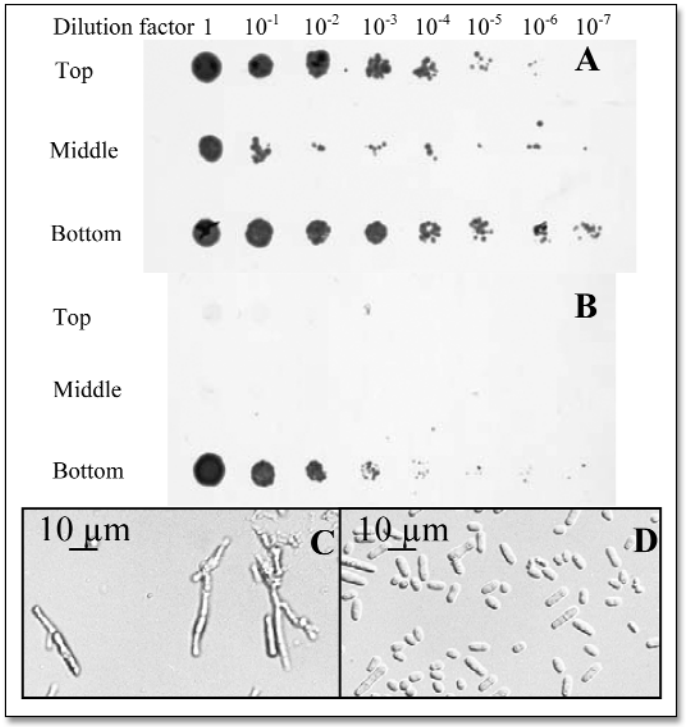

Figure 3. Rescue of *S. pombe cdc25-22* conditional mutant strain. Petri dishes containing 10-fold serial dilutions of (top) nontransformed (middle) pRep41 transformed and (bottom) *O. tauri* Cdc25 transformed *S. pombe cdc25-22* strains grown at 30°C (A) and 37°C (B) in EMM + 225 mg.l^{-1} leucine medium. Only *O. tauri* Cdc25 transformed cells grow under restrictive condition. (C and D) Photomicrographs of liquid cultured cells of recombinant *S. pombe cdc 25-22* at restrictive temperature: longer cells are observed for the pRep41 transformed cells (C) whereas *O. tauri* Cdc25 transformed cells (D) show small round cells reminiscent of the wee phenotype and rod-shaped cells corresponding to the phenotype of the cells growing normally at 30°C.

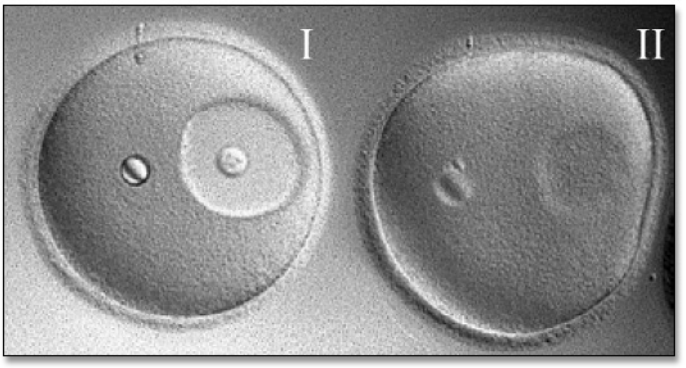

Figure 4. Microinjection of *O. tauri* Cdc25 in prophase arrested starfish oocyte. The recombinant *O. tauri* GST::Cdc25 was microinjected in starfish oocytes. (I) Control oocyte microinjected with buffer solution 1X PBS. The small sphere above the germinal vesicle is a mineral oil drop microinjected together with the solution. (II) Oocyte microinjected with *O. tauri* Cdc25 phosphatase: the oocyte matures into a Germinal Vesicle BreakDown (GVBD) state; the nuclear envelope fades and eventually disappears. The maturation of the oocyte observed shows that the CDK-cyclin complex has been activated by the *O. tauri* Cdc25.

Cdc25 signature encompassing the 180 amino acids of the C-terminal domain containing the conserved active site, a rhodanese fold with the CE[Y-F]SXXR motif and the aspartate residue of the essential DCR acceptor motif (Fig. 1).[42,43] No domain homology could be found in the databases for the N-terminal part of the protein, as is usually observed in animal and yeast Cdc25 N-terminal moieties. *O. tauri* cdc25 hosts a consensus 14-3-3 binding site, 2 destruction box (RXXL) motifs but no KEN motif.[44-46] Finally, 15-serine, 8-threonine and 6-tyrosine potent phosphorylation sites are also counted, among which one putative MAP kinase phosphorylation site on threonine-183 and more importantly 4 putative CDK A phosphorylation sites.[47]

The expression of *O. tauri* cdc25 was analysed by northern blotting of *O. tauri* total RNA isolated from non synchronized cultures. A band corresponding to approximately 1.6 kb was obtained (data not shown), indicating that the *O. tauri* cdc25 gene is expressed. This size is compatible with the estimated size of the gene obtained from the complete genome sequencing project. This expression has also been confirmed by specific PCR amplification of a fragment of *O. tauri* cdc25 using a cDNA library and lastly from total RNA by the Titan one step RT-PCR kit (data not shown).

A True cdc25 Dual-specificity Phosphatase in *Ostreococcus tauri.* Alignments of the superfamily of protein phosphatases dataset, including the divergent family of low molecular weight phosphatases, showed unambiguously that the *O. tauri* sequence belongs to the cdc25 dual-specificity phosphatase subfamily (Fig. 2). Within this cdc25 subfamily, *O. tauri* branched off first, being clearly different from metazoan and yeast sequences (Fig. 2). Since the systematic and careful searches for orthologs of cdc25 genes in plants by using animal or yeast sequences have been unsuccessful, the availability of *O. tauri* cdc25 sequence originating from the green lineage prompted us to look again for any homology with its conserved C-terminal part. Again, no clear ortholog could be found in the two completely sequenced streptophyta genomes *Arabidopsis thaliana* and rice. Furthermore, no ortholog was found in the very large unpublished EST dataset of the moss *Physcomitrella patens* (Ralf Reski, Freiburg University, Germany, personal communication). Interestingly, a putative Cdc25-like phosphatase sequence containing the HCX_5R motif but a divergent DVR acceptor motif was identified in *Chlamydomonas reinhardtii* (ChlamyDB[§] Accession No. 833010A05.y1; http://www.biology.duke.edu/chlamy_genome). This new cdc25-like candidate sequence is highly divergent and only functional characterization will ensure that it is an ortholog of the Cdc25 phosphatase. These results indicate that a true cdc25 gene was present at the base of the green lineage as an ortholog is found in *O. tauri* and a putative one in *C. reinhardtii*. However, no ortholog has been identified in higher plants.

***Ostreococcus tauri* Cdc25 Rescues *S. pombe cdc25-22* Temperature Sensitive Mutants**. Since there are no genetic systems yet available in *O. tauri*, the rescuing of a *Schizosaccharomyces pombe cdc25-22* conditional mutant by recombinant *O. tauri* Cdc25 was tested. This strain which grows normally at the permissive temperature of 30°C arrests at the G_2/M transition at the restrictive temperature of 37°C due to inactivation of the thermo-labile endogenous cdc25. The complete ORF of the *O. tauri* gene was subcloned in the *S. pombe* expression vector pREP41 and transformed in the *S. pombe cdc25-22* mutant. After selection on leucine-deficient minimal medium plates, only colonies transformed by the pREP41 containing *O. tauri* cdc25 gene grew at restrictive temperature (37°C), whereas the colonies transfected with the vector alone (control) grew very poorly (Fig. 3A and 3B). Furthermore, microscopic examination showed that the colonies transfected with *O. tauri* cdc25 have a similar morphology (small rod-shaped cells) to the wild type strains or to the colonies growing at permissive temperature (Fig. 3D). Small and round cells corresponding to the wee phenotype are also observed. In contrast, the cells in the few colonies obtained at restrictive temperature and not rescued by *O. tauri* cdc25 appeared elongated with a phenotype typical of cdc25 cell cycle-arrested mutant cells (Fig. 3C).

***Ostreococcus tauri* Cdc25 is an Active Phosphatase**. To investigate further the phosphatase activity of *O. tauri* cdc25 on the CDK-cyclin complex, starfish oocytes were microinjected with purified recombinant *O. tauri* GST::Cdc25 protein.[48] These oocytes are arrested in the prophase of the first meiotic division (germinal vesicle {GV} stage) and contain an inactive Thr-14 Tyr-15 phosphorylated CDK.[49] Oocytes microinjected with recombinant *O. tauri* Cdc25 phosphatase underwent M-phase entry as shown by

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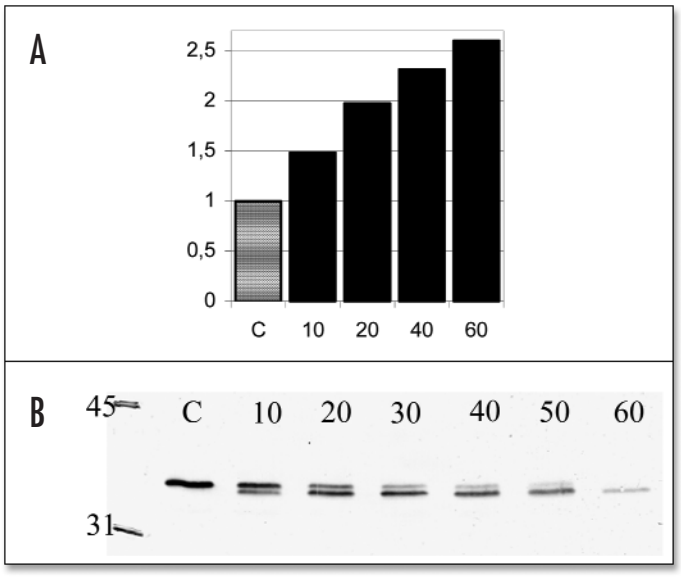

Figure 5. In vitro Phosphatase activity of *O. tauri* Cdc25 in starfish oocyte extracts. After purification of the starfish oocyte inactive CDK-cyclin complex by the use of sepharose beads grafted with $p13^{suc1}$, recombinant *O. tauri* GST::Cdc25 was incubated at RT for different time-lapses. (A) Shows the normalized radioactivity count of the resulting kinase assay of the CDK1-cyclin B complex on its specific substrate histone H1. The kinase activity increases in *O. tauri* Cdc25 incubated samples as compared to the control which is incubated in PBS 1X buffer solution. Therefore in vitro *O. tauri* Cdc25 dephosphorylation activates the CDK1-cyclin B complex. (B) Western Blotting using anti-Ptyr antibody shows that there is a band shift followed by a uniform decrease in tyrosine phosphorylation of the starfish CDK1; *O. tauri* Cdc25 is a dual specificity phosphatase which dephosphorylates the Thr residue before the Tyr residue. These results confirm the in vitro dephosphorylating activity of *O. tauri* Cdc25. C: 60′ PBS1X; 10: 10′ *ot*Cdc25; 20: 20′ *ot*Cdc25; 30: 30′ *ot*Cdc25; 40: 40′ *ot*Cdc25; 50: 50′ *ot*Cdc25; 60: 60′ *ot*Cdc25.

germinal vesicle breakdown (GVBD) (Fig. 4). We moreover checked that the GVBD oocytes contained active histone H1 kinase activity showing that the microinjected recombinant *O. tauri* GST::Cdc25 readily activated endogenous CDK-cyclin complex (data not shown). On the other hand, GVBD was not observed in control oocytes microinjected with buffer alone (Fig. 4).

We also tested the in vitro activity of *O. tauri* Cdc25 phosphatase. For this, the histone H1 kinase activity of the CDK1-cyclin B complex was measured after addition of recombinant *O. tauri* GST::Cdc25 phosphatase to GV-stage inactive CDK which were affinity-purified from the oocyte protein crude extract by $p13^{suc1}$ grafted sepharose beads. This activity increased by a factor of 2.6 following the addition of recombinant *O. tauri* GST::Cdc25 (Fig. 5A) for 1 hour as compared to the control and this was proportional to the time-lapse of *O. tauri* Cdc25 addition to the extracts. To ensure that the *O. tauri* Cdc25 phosphatase specifically activated the CDK1-cyclin B complex, a western-blot revealed by anti-phosphotyrosine antibody was carried out on the purified starfish CDK1-cyclin B complex and incubated with *O. tauri* Cdc25 for various times. The resulting blot clearly shows that the band corresponding to the tyrosine-phosphorylated form of CDK1 decreased significantly on addition of *O. tauri* Cdc25 (Fig. 5B). More precisely, the tyrosine-phosphorylated band showed an intermediate band shift before a more than 5-fold decrease in intensity indicating a more rapid threonine than tyrosine dephosphorylation. This dephosphorylation was dose and time dependent and confirmed the activation of the CDK1-cyclin B complex by dual-specificity dephosphorylation. These results clearly show that the recombinant *O. tauri* Cdc25 protein is functional and has a dual specificity phosphatase activity assayed both in vitro and in vivo.

DISCUSSION

Once a cell is committed to division, the proper succession of the different phases is regulated by checkpoints which block the cell cycle progression until the previous phase is successfully completed.[11] These checkpoints seem to be present in all eukaryotes and the increasing amount of available genomic data from organisms belonging to different phyla shows that most of the proteins involved in these regulations are conserved. In turn, this conservation led to the concept of an ancestral core cell division machinery, at least common to metazoans, fungi and plants.[14] Despite this in silico global similarity, the plant cell cycle is nevertheless poorly functionally characterized. Little is known about the plant cell cycle in planta and only an extensive functional analysis will clearly discriminate the core cell cycle machinery from the important specific adaptations in the models. Among these putative specific adaptations was the persistent absence of a cdc25 ortholog in plants although this essential regulation pathway seems to be functionally conserved. This absence is puzzling because orthologs of other regulation partners of the G_2/M transition such as CDKs, cyclins and Wee1 are present in plants. This is all the more surprising as most of the proteins known to interact with Cdc25 in metazoans and yeast have also been found in plants. Among these proteins are Pin1 and 14-3-3 and their presence indicates that the DNA damage and DNA replication checkpoints may also be evolutionarily conserved from the ancestral machinery.

The absence of Cdc25 in plants raises the evolutionary question of the presence or absence of this key enzyme at the base of the green lineage and its possible subsequent evolution as a weakly conserved sequence or its loss and substitution by a yet uncharacterised dual specificity phosphatase which mimics the activity of Cdc25. The ongoing sequencing project of the genome of the unicellular alga *Ostreococcus tauri* revealed for the first time the presence of a cdc25 gene at the base of the green lineage. We have shown that this gene encodes a functional Cdc25 protein. *O. tauri* Cdc25 phosphatase is active in the heterologous models starfish and yeast, thereby confirming its functional conservation across evolution, as can be observed with Cdc25 phosphatases from other organisms.[41] By using the *O. tauri* cdc25 sequence, a putative candidate was found in *C. reinhardtii*, whose genome sequencing has recently been completed. This Cdc25 candidate contains the conserved HCX_5R and the essential aspartic acid of the DCR acceptor motif but does not have neither the consensus Cdc25 motif CE[Y-F]SXXR, nor the cysteine of the DCR motif which is necessary for reversing deleterious oxidation.[50] We, therefore, propose to annotate this *C. reinhardtii* sequence as a cdc25-like gene based on its partial sequence similarity to the *O. tauri* cdc25 gene. We further looked for orthologs of this new *C. reinhardtii* cdc25-like gene and found putative orthologs in the terrestrial plants *A. thaliana* and *O. sativa*. However, these cdc25-like sequences are highly divergent from the known cdc25 family and their identification as a putative Cdc25 phosphatase remains to be functionally confirmed. Further characterisation of such Cdc25 phosphatase orthologs in streptophyta will require a comparative approach including evolutionarily intermediate organisms such as the charophyta, but genomic data for these organisms are not yet available.

In conclusion, the conservation of the actors of the cell cycle regulation pathway suggests that the interacting partners must have coevolved so that any divergent change in one partner is adapted by the other.[8] However, to confirm this hypothesis orthologs of all the genes present early during evolution must be found in the different lineages having conserved this regulatory pathway.[9] The absence of

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cdc25 in plants, in contrast to CDKs, cyclins and wee1, is contradictory to the hypothesis of coevolution and would imply that cdc25 phosphatase activity is a more recent evolutionary acquisition, occurring only in ophistokonts (metazoans and fungi) after their separation from the green lineage. But the formal identification of a functional Cdc25 ortholog at the base of the green lineage strengthens the hypothesis of the coevolution of the regulatory pathway of the M-phase entry and rules out the hypothesis of the cdc25 gene as being a recent evolutionary acquisition specific to the ophistokont lineage. It is now clear that Cdc25 phosphatase appeared earlier in evolution before the divergence between the ophistokont (metazoan and yeast) and the green lineages and was probably present in the ancestral eukaryotic cell.

References

1. Stals H, Inze D. When plant cells decide to divide. Trends Plant Sci 2001; 6:359-64.
2. Mironov V, De Veylder L, Van Montagu M, Inze D. Cyclin-dependent kinases and cell division in plants—the nexus. Plant Cell 1999; 11:509-22.
3. Rubin GM, Yandell MD, Wortman JR, Gabor Miklos GL, Nelson CR, Hariharan IK, et al. Comparative Genomics of the Eukaryotes. Science 2000; 287:2204-15.
4. Vandepoele K, Raes J, De Veylder L, Rouze P, Rombauts S, Inze D. Genome-wide analysis of core cell cycle genes in arabidopsis. Plant Cell 2002; 14:903-16.
5. Nilsson I, Hoffmann I. Cell cycle regulation by the Cdc25 phosphatase family. Prog Cell Cycle Res 2000; 4:107-14.
6. Berry LD, Gould KL. Regulation of Cdc2 activity by phosphorylation at T14/Y15. Prog Cell Cycle Res 1996; 2:99-105.
7. Lundgren K, Walworth N, Booher R, Dembski M, Kirschner M, Beach D. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell 1991; 64:1111-22.
8. Goh C-S, Bogan AA, Joachimiak M, Walther D, Cohen FE. Coevolution of proteins with their interaction partners. J Mol Biol 2000; 299:283-93.
9. Pellegrini M, Marcotte EM, Thompson MJ, Eisenberg D, Yeates TO. Assigning protein functions by comparative genome analysis: Protein phylogenetic profiles. Proc Natl Acad Sci USA 1999; 96:4285-8.
10. Sorrell DA, Marchbank A, McMahon K, Dickinson JR, Rogers HJ,Francis D. A WEE1 homologue from Arabidopsis thaliana. Planta 2002; 215:518-22.
11. Smits VAJ, Medema RH. Checking out the G_2/M transition. Biochim Biophys Acta 2001; 1519:1-12.
12. Russell P, Nurse P. cdc25+ functions as an inducer in the mitotic control of fission yeast. Cell 1986; 45:145-53.
13. Criqui MC, Genschik P. Mitosis in plants: How far we have come at the molecular level? Curr Opin Plant Biol 2002; 5:487-93.
14. Dewitte W, Murray JAH. The plant cell cycle. Annu Rev Plant Biol 2003; 54:235-64.
15. Sun Y, Dilkes BP, Zhang C, Dante RA, Carneiro NP, Lowe KS, et al. Characterization of maize (Zea mays L.) Wee1 and its activity in developing endosperm. Proc Natl Acad Sci USA 1999; 96:4180-5.
16. Zhang K, Letham DS, John PC. Cytokinin controls the cell cycle at mitosis by stimulating the tyrosine dephosphorylation and activation of p34cdc2-like H1 histone kinase. Planta 1996; 200:2-12.
17. McKibbin RS, Halford NG, Francis D. Expression of fission yeast cdc25 alters the frequency of lateral root formation in transgenic tobacco. Plant Mol Biol 1998; 36:601-12.
18. Corellou F, Brownlee C, Detivaud L, Kloareg B, Bouget F-Y. Cell cycle in the fucus zygote parallels a somatic cell cycle but displays a unique translational regulation of cyclin-dependent kinases. Plant Cell 2001; 13:585-98.
19. Kraft C. Mitotic entry: Tipping the balance. Curr Biol 2003; 13:R445-R6.
20. Bhattacharya D, Medlin L. Algal phylogeny and the origin of land plants. Plant Physiol 1998; 116:9-15.
21. Courties C, Vaquer A, Troussellier M, Lautier J, Chrétiennot-Dinet MJ, Neveux J, et al. Smallest eukaryotic organism. Nature 1994; 370:255.
22. Chrétiennot-Dinet MJ, Courties C, Vaquer A, Neveux J, Claustre H, Lautier J, et al. A new marine picoeukaryote : *Ostreococcus tauri* gene sp. Nov. (Chlorophyta, Prasinophyceae). Phycologia 1995; 4:285-92.
23. Courties C, Perasso R, Chrétiennot-Dinet MJ, Gouy M, Guillou L, Troussellier M. Phylogenetic analysis and genome size of Ostreococcus tauri (Chlorophyta, Prasinophyceae). J Phycol 1998; 34:844-9.
24. Derelle E, Ferraz C, Lagoda P, Eychenié S, Cooke R, Regad F, et al. DNA libraries for sequencing the genome of *Ostreococcus tauri* (chlorophyta, prasinophyceae) : The smallest free-living eukaryotic cell. J Phycol 2002; 38:1150-6.
25. Vee S, Lafanechere L, Fisher D, Wehland J, Job D, Picard A. Evidence for a role of the α-tubulin C terminus in the regulation of cyclin B synthesis in developing oocytes. J Cell Sci 2001; 114:887-98.
26. Jimenez J, Alphey L, Nurse P, Glover DM. Complementation of fission yeast cdc2ts and cdc25ts mutants identifies two cell cycle genes from *Drosophila*: A cdc2 homologue and string. EMBO J 1990; 9:3565-71.
27. Forsburg SL. Comparison of *Schizosaccharomyces pombe* expression systems. Nucl Acids Res 1993; 21:2955-6.
28. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 1994; 22:4673-80.
29. Hall TA. BioEdit program. Nucleic Acids Symposium Series 1999; 41.
30. Muller T, Vingron M. Modeling amino acid replacement. J Comput Biol 2000; 7:761-76.
31. Uzzell T, Corbin KW. Fitting discrete probability distributions to evolutionary events. Science 1971; 172:1089-96.
32. Schmidt HA, Strimmer K, Vingron M, von Haeseler A. TREE-PUZZLE: Maximum likelihood phylogenetic analysis using quartets and parallel computing. Bioinformatics 2002; 18:502-4.
33. Van de Peer Y, De Wachter R. Construction of evolutionary distance trees with TREECON for Windows: Accounting for variation in nucleotide substitution rate among sites. Comput Appl Biosci 1997; 13:227-30.
34. Huelsenbeck JP, Ronquist F. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics 2001; 17:754-5.
35. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci 1992; 8:275-82.
36. Picard A, Peaucellier G. Behavior of cyclin B and cyclin B-dependent kinase during starfish oocyte meiosis reinitiation: Evidence for nonidentity with MPF. Biol Cell 1998; 90:487-96.
37. Picard A, Galas S, Peaucellier G, Doree M. Newly assembled cyclin B-cdc2 kinase is required to suppress DNA replication between meiosis I and meiosis II in starfish oocytes. EMBO J 1996; 15:3590-8.
38. Hiramoto Y. A method of microinjection. Experimental cell research 1974; 87:403-6.
39. Maundrell K. nmt1 of fission yeast. A highly transcribed gene completely repressed by thiamine. J Biol Chem 1990; 265:10857-64.
40. Prentice HL, Kingston RE. Mammalian promoter element function in the fission yeast Schizosaccharomyces pombe. Nucl Acids Res 1992; 20:3383-90.
41. Gustafson MP, Thomas Jr CF, Rusnak F, Limper AH, Leof EB. Differential regulation of growth and checkpoint control mediated by a Cdc25 mitotic phosphatase from pneumocystis carinii. J Biol Chem 2001; 276:835-43.
42. Altschul S, Madden T, Schaffer A, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucl Acids Res 1997; 25:3389-402.
43. Bordo DBP. The rhodanese/Cdc25 phosphatase superfamily. EMBO Rep 2002; 3:741-6.
44. Tzivion G, Avruch J. 14-3-3 Proteins: Active cofactors in cellular regulation by serine/threonine phosphorylation. J Biol Chem 2002; 277:3061-4.
45. Zur A, Brandeis M. Timing of APC/C substrate degradation is determined by fzy/fzr specificity of destruction boxes. EMBO J 2002; 21:4500-10.
46. Pfleger CM, Kirschner MW. The KEN box: An APC recognition signal distinct from the D box targeted by Cdh1. Genes Dev 2000; 14:655-65.
47. Blom N, Gammeltoft S, Brunak S. Sequence and structurebased prediction of eukaryotic protein phosphorylation sites. J Mol Biol 1999; 294:1351-62.
48. Picard A, Karsenti E, Dabauvalle MC, Doree M. Release of mature starfish oocytes from interphase arrest by microinjection of human centrosomes. Nature 1987; 327:170-2.
49. Borgne A, Ostvold AC, Flament S, Meijer L. Intra-M Phase-promoting factor phosphorylation of cyclin B at the prophase/metaphase transition. J Biol Chem 1999; 274:11977-86.
50. Sohn J, Rudolph J. Catalytic and chemical competence of regulation of cdc25 phosphatase by oxidation/reduction. Biochemistry 2003; 42:10060-70.
51. Shi L, Potts M, Kennelly PJ. The serine, threonine, and/or tyrosine-specific protein kinases and protein phosphatases of prokaryotic organisms: A family portrait. FEMS Microbiol Rev 1998; 22:229-53.

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