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Inositol Diphosphate Signaling Regulates Telomere Length*

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Activation of phospholipase C-dependent inositol polyphosphate signaling pathways generates distinct messengers derived from inositol 1,4,5-trisphosphate that control gene expression and mRNA export. Here we report the regulation of telomere length by production of a diphosphorylinositol tetrakisphosphate, PP-IP_4, synthesized by the *KCS1* gene product. Loss of PP-IP_4 production results in lengthening of telomeres, whereas overproduction leads to their shortening. This effect requires the presence of Tel1, the yeast homologue of ATM, the protein mutated in the human disease ataxia telangiectasia. Our data provide *in vivo* evidence of a regulatory link between inositol polyphosphate signaling and the checkpoint kinase family and describe a third nuclear process modulated by phospholipase C activation.

Appropriate cellular responses to environmental changes involve intracellular second messenger systems that transduce the signals from cytoplasm to nucleus, thereby initiating adaptive genetic programs. One well described intracellular messenger system works through receptor-coupled activation of phospholipase C (PLC),[1] which hydrolyzes phosphatidylinositol 4,5-bisphosphate to yield inositol 1,4,5-trisphosphate (IP_3) (reviewed in Refs. 1 and 2). In metazoans, cellular production of IP_3 functions as a signal for calcium release from intracellular stores through allosteric activation of an IP_3 receptor channel. Recent studies indicate that IP_3 also plays an important role as precursor to multiple inositol polyphosphates (IPs), each with potentially unique signaling capability (reviewed in Ref. 3). This signaling potential is not restricted to the cytoplasm, because IPs are known to function in nuclear processes in budding yeast (reviewed in Ref. 4). Activation of yeast phospholipase C (Plc1) produces IP_3, which is rapidly phosphorylated by the dual specificity 6-/3-kinase Ipk2 (initially cloned as ArgRIII/Arg82 (5)) to yield IP_4 and IP_5 (6–8). This phosphorylation step is coupled to transcriptional regulation, possibly through chromatin remodeling (7, 9–11). IP_5 is then phosphorylated by the 2-kinase Ipk1, generating IP_6, which is required for efficient mRNA export from the nucleus (12–14). Both IP_5 and IP_6 can be converted to the diphosphoryl inositols PP-IP_4 and PP-IP_5 through the action of Kcs1, a kinase required for normal vacuolar morphology (15–18). PP-IP_5 has recently been suggested to play a role in chemotaxis in *Dictyostelium* (19). A nuclear role for Kcs1 is implied by its initial cloning as a regulator of mitotic DNA recombination, and inositol kinase activity is required for this regulation, but further understanding of its nuclear activity is lacking (20, 21).

Recent work has provided further linking of IP production and nuclear function through an important family of protein serine/threonine kinases known as phosphatidylinositol 3-kinase related kinases (PIKKs). IP_6 stimulates DNA repair by non-homologous end joining with mammalian proteins *in vitro* (22). Non-homologous end joining can be reconstituted *in vitro* with a limited number of purified proteins, including the Ku heterodimer, which binds DNA ends and IP_6 (23, 24), and the PIKK, DNA-dependent protein kinase (DNA-PK_{cs}) (reviewed in Refs. 25 and 26). Other PIKKs, namely ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) gene products in mammalian cells and Tel1 and Mec1 in yeast, have been shown to have important activities in DNA damage checkpoint and telomere maintenance (27–29).

Telomeres are specialized protein-nucleic acid structures at the ends of linear, eukaryotic chromosomes that preserve genetic information during DNA replication, promote genomic stability, and are important in both cellular senescence and oncogenic transformation (reviewed in Refs. 30–32). Telomeric DNA is maintained through the action of the ribonucleoprotein telomerase, which acts in late S-phase to add template-independent, species-specific TG-rich repeats to the lagging strand, ensuring that chromosome length and coding sequences are maintained. Telomere length is heterogeneous among chromosomes and cells, with the average length established by a dynamic equilibrium between forces that shorten (exonuclease access, low telomerase activity) and those that lengthen telomeric sequences (capping proteins, high telomerase activity). Here we describe a role for the phospholipase C-dependent IP signaling pathway in the regulation of telomere length through production of an inositol diphosphate, PP-IP_4, synthesized by Kcs1. This modulation is dependent on Tel1 providing a functional connection between PIKKs and inositol phosphates *in vivo*.

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[1] The abbreviations used are: PLC, phospholipase C; IP, inositol phosphate; IP_6, inositol hexakisphosphate; IP_3, inositol 1,4,5-trisphosphate; IP_4, inositol tetrakisphosphate; IP_5, inositol pentakisphosphate; PP-IP_4, diphosphorylinositol tetrakisphosphate; PP_2-IP_3, (bis)diphosphorylinositol trisphosphate; PP-IP_5, diphosphorylinositol pentakisphosphate; HPLC, high performance liquid chromatography; kin^-, catalytically inactive kinase; PIKK, phosphatidylinositol 3-kinase-related kinase.

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EXPERIMENTAL PROCEDURES

Strains, Media, and Genetic Methods—Yeast strains used in this study are isogenic with W303α (*leu2–3,112 his3–11,15 ura3–1 ade2–1 trp1–1 rad5–535 can1–100*) except for changes introduced by transformation as indicated. Yeast were grown either in standard rich (yeast-peptone-dextrose) medium or in complete minimal medium containing 2% glucose or galactose and lacking appropriate nutrients to verify and maintain plasmids and markers. Yeast genetic manipulations were performed using standard procedures. Deletion strains *plc1*::*KANMX, ipk2*::*KANMX, ipk1*::*KANMX, tel1*::*ura3*, and *yku70*::*ura3 ura3*::*HIS3* were generated as described previously (7, 12, 33, 34). Double and triple deletion strains were constructed by mating to isogenic strains of the opposite mating type followed by sporulation of the resulting diploid and dissection of tetrads. Nutrient requirements, inositol labeling, and PCR were used to screen spores for double mutations.

The entire *KCS1* and *DDP1* open reading frames were deleted from the diploid *Saccharomyces cerevisiae* strain W303 and replaced with the selectable marker *HIS3* as described (35). Tetrads were dissected using a Zeiss micromanipulator following sporulation of the heterozygous diploids on 0.3% potassium acetate plates. The *kcs1*::*HIS3* and *ddp1*::*HIS3* spores both segregated 2:2 (not shown) and were identified by growth patterns on medium lacking histidine as well as by PCR analysis.

Gene Identification and Plasmid Construction—To identify candidate mammalian inositol diphosphoryl synthases, human expressed sequence tag data bases were searched with BLASTP, gapped BLASTP, or TBLASTN with appropriate query sequences. The full-length human cDNA 646420 (designated as hIP6K, an orthologue of yeast Kcs1) was obtained from Research Genetics, Inc. as an insert in pBluescript SK(−). The entire open reading frame minus the codons for the first six amino-terminal amino acids was inserted in-frame into the NcoI and XhoI sites of pGEX-KG, derived from pGEX-2T (Amersham Biosciences) and kindly donated by Dr. John Moskow in the laboratory of Dr. Daniel Lew (Duke University, Department of Pharmacology and Cancer Biology), to generate pGEX-hIP6K. The entire open reading frame of the *KCS1* gene was amplified by polymerase chain reaction (PCR) from *S. cerevisiae* genomic DNA and inserted into a pGEX vector to create an amino-terminal glutathione *S*-transferase fusion. Primers were designed to incorporate EcoRI sites in-frame with the initiation sites and to place XhoI sites after the stop codons. Catalytically inactive Kcs1 was generated by a PCR strategy converting both Asp^{791} and Lys^{793} to alanine. The presence of the desired mutations was confirmed by sequencing the resulting construct termed pGEX-kcs1kin$^-$. Complementation analyses in haploid *kcs1*Δ strains were performed by expressing wild type and catalytically inactive forms of Ksc1 and hIP6K using the episomal plasmid pRS426 containing a galactose-inducible promoter. In each case, the insert was removed from the pGEX vector with EcoRI and XhoI and ligated into the EcoRI-XhoI sites of pRS426GAL. These constructs were termed pRS-KCS1, pRS-kcs1kin$^-$, and pRS-hIP6K.

The construct for *PLC1* overexpression was based on pYEX4T, modified by removal of the *LEU2* sequence. The entire *PLC1* coding sequence was removed in a BamHI-NotI fragment from the p*GAL1–10*-PLC1 vector described previously and provided by Dr. Jeremy Thorner (6). This fragment was inserted into the modified multiple cloning site of the pYEX4T vector behind the CUP1 promoter with the resulting plasmid pYEX-PLC1.

Inositol Radiolabeling of Yeast—Approximately 10^5 cells were inoculated into 1 ml of appropriately modified complete minimal medium containing *myo*-[^{3}H]inositol (American Radiolabel Corporation, St. Louis, MO) and were labeled for 24 h with shaking at 30 °C. Soluble inositols were harvested as described (35). Water-soluble inositol isomers were frozen quickly in a dry ice-isopropanol bath and stored at −80 °C prior to HPLC analysis. To analyze inositol isomers, samples were thawed, equilibrated to 10 mM ammonium phosphate, pH 3.5, and resolved on a 4.6 × 125-mm Partisphere SAX-HPLC strong anion exchange column (Whatman). Samples were eluted with the following gradient: 10 mM ammonium phosphate increased linearly to 1.7 M ammonium phosphate over 25 min followed by an isocratic flow at 1.7 M ammonium phosphate for 50 min. Radioactivity was measured using a BetaRAM™ in-line detector (INUS Systems, Tampa, FL). Individual IP isomers were assigned in the chromatograms as described (12). PP-IP_4 and PP-IP_5 were assigned based on published elution profiles and sensitivity to hydrolysis by mammalian diphosphoryl inositol pyrophosphatase (36).

Southern Analysis of Telomere Length—Details of this analysis are as published previously (37). Genomic DNA was prepared from 5-ml cultures grown to saturation and digested with PstI before electrophoresis on a 1% Tris borate-EDTA-agarose gel. The fragments were transferred to Hybond N+ nylon membranes (Amersham Biosciences) and hybridized at 65 °C to a probe derived from pYT14 containing telomeric DNA and a portion of the Y′ subtelomeric repeat. The probe was labeled with [α-^{32}P]dCTP using Ready-To-Go labeling beads (Amersham Biosciences). Note that the Southern blot strategies are the accepted methods in the field; however, the effect appears small because the PstI site used for cleaving is about 800 bp from the chromosome end. Thus less than half of the fragment is composed of telomeric sequences. The bottom smear represents the terminal fragment resulting from cutting at the last Y′ elements (one of the subtelomeric repeats) on the chromosome. The bands near the top of the gel reflect hybridization to fragments generated by cutting tandem Y′ elements of two different sizes. These bands vary in intensity, even in wild-type strains, probably as a consequence of a high rate of unequal recombination. Although we cannot exclude the possibility that there is a somewhat higher rate of this type of recombination in one or more of the mutants, none of them has the dramatic phenotype seen in yeast strains undergoing alternative lengthening of telomere recombination, where the upper bands become extremely prominent. For example, see Fig. 3 in Ritchie *et al.* (37).

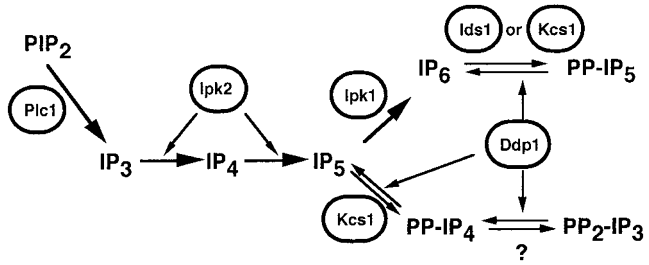

FIG. 1. **Schema of phospholipase C-dependent inositol polyphosphate metabolism in *S. cerevisiae*.** A single phospholipase C (*Plc1*) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to release IP_3 from the membrane. IP_3 serves as substrate for the inositol kinase Ipk2, which phosphorylates both the D-6 and D-3 position hydroxyl groups to produce Ins(1,3,4,5,6)P_5. IP_5 is then phosphorylated by Ipk1 at the 2-hydroxyl to generate IP_6. IP_6 can be further phosphorylated to PP-IP_5 by one of two inositol diphosphoryl synthases in this strain, Kcs1, or a second kinase, Ids1. Kcs1 also phosphorylates IP_5 to make PP-IP_4. The kinase(s) responsible for synthesis of PP_2-IP_3 is currently not defined, although it is likely that Kcs1 or Ids1 functions at this step. The inositol diphosphates PP-IP_4, PP_2-IP_3, and PP-IP_5 are dephosphorylated to IP_5 and IP_6 by an inositol diphosphoryl phosphatase Ddp1. Note that the ring position phosphates that Kcs1 and Ids1 phosphorylate are undetermined; thus it is possible that the PP-IP_5 products of each kinase are distinct isomers (which are omitted from the diagram only for the sake of simplicity).

RESULTS

Genetics of Inositol Polyphosphate Synthesis in S. cerevisiae—A graphic summary of the metabolism of soluble inositol phosphates in budding yeast is depicted in Fig. 1. A single phospholipase C (Plc1) and three inositol polyphosphate kinases, Ipk2, Ipk1, and Kcs1, function to generate over seven species of water-soluble IP messengers. Plc1 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to release IP_3 from the membrane (6). IP_3 serves as substrate for the nuclear inositol kinase Ipk2 (also known as Arg82), which phosphorylates both the D-6 and D-3 position hydroxyl groups to produce IP_5 (7, 38). IP_5 is phosphorylated at the 2-hydroxyl by Ipk1 to generate IP_6 (12). Kcs1 has been reported to function as a inositol diphosphoryl synthase to produce PP-IP_4 and PP-IP_5 from IP_5 and IP_6 substrates, respectively (17). Dephosphorylation of inositol diphosphates occurs through a phosphatase Ddp1 (36).

To further study the role of these kinases and phosphatases in inositol metabolism we analyzed a series of yeast mutant strains using isotopic equilibrium radiolabeling. Steady state metabolic radiolabeling of wild-type yeast cells with [^{3}H]inositol indicates that the major soluble IP is IP_6, representing ~2% of the recovered radioactivity from soluble cellular extracts (Fig. 2). Other IPs, including PP-IP_5, are present at low levels

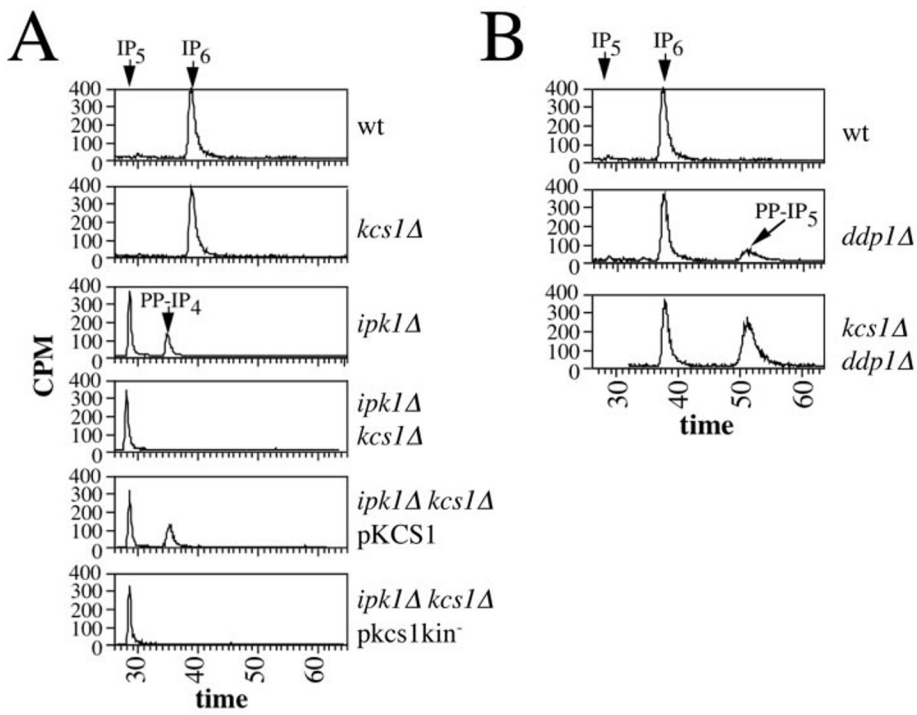

FIG. 2. **Steady state analysis of inositol polyphosphates in mutant strains.** Haploid yeast strains were labeled with [^{3}H]inositol to steady state in overnight cultures, and then soluble inositols were isolated and analyzed by HPLC. IP standards are indicated as *arrows*. Strains in *A* are, *top* to *bottom*, wild type (*wt*), *kcs1*Δ, *ipk1*Δ, *ipk1*Δ *kcs1*Δ, *ipk1*Δ *kcs1*Δ transformed with pRS-KCS1 (*pKCS1*), and *ipk1*Δ *kcs1*Δ transformed with pRS-kcs1kin$^-$ (*pkcs1kin*$^-$). Strains in *B* are, *top* to *bottom*, wild type, *ddp1*Δ, and *ddp1*Δ *kcs1*Δ.

but are not observed under these labeling conditions (therefore HPLC traces only show relevant times of elution). A significant branch point in the IP pathway is exposed in metabolically labeled cells upon deletion of the IP_5 2-kinase (*ipk1*Δ) cells, which accumulate IP_5 and a diphosphoryl inositol, PP-IP_4 (Fig. 2*A*) (12). Deletion of *KCS1* in the *ipk1*Δ strain abrogates the peak of PP-IP_4, which can be complemented with active Kcs1; thus production of PP-IP_4 is primarily dependent on Kcs1 *in vivo* (Fig. 2*A*) (17). Disruption of *KCS1* alone does not alter IP_6 levels, demonstrating that PP-IP_4 is not required for synthesis of IP_6 (Fig. 2*A*). Disruption of the *DDP1* gene exposes a peak of PP-IP_5, which is not seen at appreciable levels in wild-type cells (Fig. 2*B*) (36). Elimination of *KCS1* in the *ddp1*Δ strain (*kcs1*Δ *ddp1*Δ) leads to an increase rather than an ablation of PP-IP_5 production (Fig. 2*B*), demonstrating the existence of a second inositol diphosphoryl synthase (designated Ids1), which phosphorylates IP_6 to a PP-IP_5 isomer (see Fig. 1 schematic). This is surprising in light of the published work of others that Kcs1 is the major IP_6 kinase activity in yeast (17). Cellular extracts prepared from *kcs1*Δ *ddp1*Δ mutants have significant IP_6 kinase activity, further indicating the presence of a second kinase (data not shown). Additionally, it does not appear that Ids1 is encoded by *IPK2*, as extracts from *ipk2*Δ *kcs1*Δ mutant cells harbor IP_6 kinase activity, and we were not able to detect this activity using recombinant Ipk2 under a range of conditions (data not shown).

It is noteworthy that the chemical nature of the PP-IP_5 isomers generated by Kcs1 and Ids1 are not known; thus it is plausible that these kinases phosphorylate different positions, thereby generating distinct PP-IP_5 products that are not resolved by our HPLC analysis. The observed elevation of PP-IP_5 in the *kcs1*Δ *ddp1*Δ strain suggests either that: 1) Ids1 activity is negatively regulated by Kcs1 and/or one of its products; or 2) Kcs1 converts the product of Ids1 to PP_2-IP_4; thus when Kcs1 is deleted, PP-IP_5 accumulates. Ids1 activity may also function as a minor kinase relative to Kcs1 in the synthesis of PP-IP_4 from Ins(1,3,4,5,6)P_5 as analysis of *ipk1*Δ *kcs1*Δ*ddp1*Δ mutant cells reveals a small but significant peak of PP-IP_4 (not shown). Furthermore, PP_2-IP_3 derivatives are observed when *DDP1* is eliminated in *ipk1*Δ strains; however, it is not known whether Kcs1 or Ids1 activity is responsible for generating this product(s). Collectively, our new results along with previously published data lead to the schematic pathway depicted in Fig. 1.

Inositol Phosphates and Telomere Length in Vivo—Evidence implicating IPs in the regulation of nuclear function and DNA repair processes in mammalian and yeast systems provided the impetus to examine these pathways in budding yeast. Examination of non-homologous end joining repair in *plc1, ipk2*, *ipk1*, or *kcs1* mutant yeast revealed that loss of inositol signaling pathways does not result in detectable disruption of non-homologous end joining in living yeast (data not shown). It is of interest that yeast possess two PIKKs, Tel1 and Mec1, which are orthologues of ATM and ATR that play an important evolutionarily conserved role in the regulation of telomere length. We therefore continued our examination of PIKK-related function by determining the length of telomeres in yeast defective in inositol metabolism. We find that strains with the genotypes *plc1*Δ, *ipk2*Δ, or *kcs1*Δ have similarly elongated telomeres, averaging 375 bp as compared with the average of 325 bp observed in wild-type cells (Fig. 3*A*). In contrast, *ipk1*Δ cells have telomeres slightly shorter than wild type (Fig. 3*A*). The change in IP profile shared among the first three mutant strains is the inability to synthesize the diphosphoryl inositols PP-IP_4 (and downstream higher phosphorylated forms such as PP_2-IP_3) and PP-IP_5 (Fig. 1). The *ipk1*Δ cells have a telomere shift in the opposite direction, fail to synthesize IP_6 and PP-IP_5, and have increased PP-IP_4 levels (Fig. 2). This finding clearly demonstrates that loss of PP-IP_5 production is not responsible for the lengthened telomeres. Instead, these data implicate PP-IP_4 production or a downstream metabolite as a negative regulator of telomere length. Importantly, *kcs1*Δ cells do not universally display the defects in gene expression or mRNA export observed in *plc1*Δ, *ipk2*Δ, or *ipk1*Δ cells, suggesting that the observed telomere phenotype is distinct from the previously reported nuclear activities of IP_4, IP_5, and/or IP_6. Furthermore, the *kcs1*Δ *ddp1*Δ strain, which accumulates large amounts of PP-IP_5, has long telomeres identical to *kcs1*Δ alone (not shown). Wild-type telomere length is observed in *ddp1*Δ strains, and double and triple mutants for *ddp1*Δ, *ipk1*Δ, and *kcs1*Δ are consistent with loss of Kcs1 being a dominant phe-

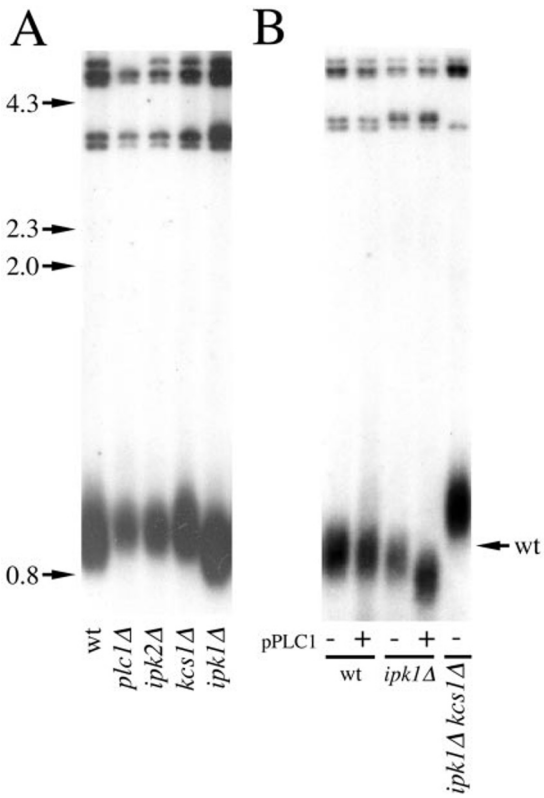

FIG. 3. **Telomere analysis of inositol-signaling mutants.** *A*, loss of function mutants. The strains indicated were grown in yeast-peptone-dextrose medium, and genomic DNA was prepared and probed for telomeric sequences. Strain is indicated below each *lane*. From *left*, these are wild type (*wt*), *plc1*Δ, *ipk2*Δ, *kcs1*Δ, and *ipk1*Δ. Molecular weight standards are indicated by the *arrows* to the *left*. *B*, gain of function mutants. Wild type, *ipk1*Δ, and *ipk1*Δ *kcs1*Δ strains were transformed with pYEX vector control (−) or pYEX-PLC1 (+), grown in complete minimal medium-Ura, and probed for telomere length as in *A*. Strains are indicated below each lane. From *left*, these are wild type + pYEX, wild type + pYEX-PLC1, *ipk1*Δ + pYEX, *ipk1*Δ + pYEX, and *ipk1*Δ *kcs1*Δ + pYEX. *Vec*, vector.

notype. Collectively, these results implicate phospholipase C-dependent PP-IP_4 production through Kcs1 activity as a negative regulator of telomere length.

To test whether increases in PP-IP_4 production would lead to further telomere shortening, we overexpressed *PLC1* in several mutant strains, which (based on our previous studies) significantly elevates IP production in cells (12). Wild-type strains overexpressing *PLC1* have increased IP_6 levels (20-fold), no detectable increase in PP-IP_4 levels, and normal telomere length (Fig. 3*B*). In contrast, *ipk1*Δ cells overexpressing *PLC1* have significantly elevated levels of PP-IP_4 (not shown) and undergo further shortening of telomeres to an average length of 265 bp from the 325 bp observed in wild type (Fig. 3*B*). When Kcs1 is eliminated from this strain, the cells again have a long telomere phenotype (Fig. 3*B*). These data are consistent with a gain of function effect mediated by PP-IP_4. Comparing the telomere lengthening in the absence of inositol diphosphates and the shortening seen with increases in Plc1- and Kcs1-mediated production of PP-IP_4, we see a range of modulation of telomere length of over 120 bp, accounting for roughly one-third of the total wild-type telomere length (Fig. 3*B*).

To confirm that inositol kinase activity is required for normal telomere length, we examined telomeres in *kcs1*Δ strains complemented with either wild-type Kcs1, kinase-inactivated Kcs1 (kcs1kin$^-$), or a human inositol diphosphoryl synthase (hIP6K) (Fig. 4). Both Kcs1 and hIP6K expression complement telomere length in the *kcs1*Δ strain, whereas Kcs1 kinase-inactive expression does not (Fig. 4), demonstrating that kinase activity is required. The ability of Kcs1 to complement in dextrose indicated that the *GAL* promoter was not "off" and may be leaky in these strains. We therefore performed complementation analysis in the *ipk1*Δ *kcs1*Δ strains, which enable detection and quantification of PP-IP_4. Indeed, the Kcs1 strains produced significant levels of PP-IP_4 even in dextrose, confirming the leaky expression. Furthermore, growth of cells in galactose, which is a strong inducer of *GAL* promoters, only modestly elevated PP-IP_4 levels as compared with dextrose (Fig. 4). Analysis of *ipk1*Δ *kcs1*Δ complemented strains confirm that expression of *KCS1* restores telomere length equivalent to the *ipk1*Δ strain alone (Fig. 4). Intriguingly, expression of inactive kinase in this strain not only fails to complement telomere length but also further lengthens telomeres, suggestive of a direct kinase-effector protein interaction at the telomere (Fig. 4). Consistent with this idea, hIP6K only partially restores telomere length to the length observed in *ipk1*Δ cells in the *ipk1*Δ *kcs1*Δ mutant cells, despite PP-IP_4 synthesis equivalent to exogenous Kcs1 (HPLC data not shown). These results indicate a requirement for kinase activity and PP-IP_4 production, as well as a possible role for a non-catalytic protein component, such as a determinant that mediates proper localization or regulation.

Kcs1 Regulation of Telomeres Requires Tel1—With this evidence for PP-IP_4 as a regulator of telomere length, we used genetics to identify other components of the novel PP-IP_4 pathway. Because of the initial report that IP_6 interacts with DNA-dependent protein kinase, we considered Tel1 and Ku70/80 to be reasonable effector candidates, because both have important yet independent roles in maintenance of the yeast telomere (34). We predicted that the effect of PP-IP_4 would be absent in cells missing a critical component of the IP effector pathway.

Analysis of *yku70* mutant cells indicates an average telomere length of ~150 bp (Fig. 5*A*), consistent with published reports. Double (*ipk1*Δ *yku70*Δ and *kcs1*Δ *yku70*Δ) and triple (*ipk1*Δ *kcs1*Δ *yku70*Δ) mutant strains were constructed and tested for telomere length (Fig. 5*A*). In a *yku70*Δ background, the loss of Kcs1 function again lengthens telomeres about 50 bp (average length of 200 bp) consistent with Ku and Kcs1 functioning in independent pathways. To probe the gain of the PP-IP_4 effect on shortening telomeres in the absence of Ku, we examined telomere length in both *yku70*Δ *ipk1*Δ (Fig. 5*A*) and *yku70*Δ *ipk1*Δ overexpressing Plc1 (not shown). Neither condition resulted in further shortening beyond the 150-bp length. Furthermore, we did not find evidence of recombination or senescence to suggest telomeres have reached a critical length, indicating either the gain of function effect cannot be reproduced in the setting of short telomeres or is dependent on the Ku pathway.

In contrast, all effects of PP-IP_4 at the telomere are abolished in the absence of Tel1. Both the double (*ipk1*Δ *tel1*Δ and kcs1Δ *tel1*Δ) and triple (*ipk1*Δ *kcs1*Δ *tel1*Δ) mutant strains have stable, shortened telomeres indistinguishable from *tel1*Δ cells (Fig. 5*B*). This result indicates that the IPs function in the same pathway of telomere regulation as this ATM homologue. Because loss of Tel1 function leads to short telomeres, the data are consistent with Kcs1 production of PP-IP_4 acting as a negative regulator of Tel1 function.

DISCUSSION

Here we report a novel role of a phospholipase C-dependent signaling pathway in the control of telomere length. Individual deletion of each of the three enzymes in the synthetic pathway to PP-IP_4 causes an identical lengthening of telomeres. The long telomere phenotype is seen in yeast lacking all soluble inositol phosphates (*plc1*-null) as well as in those lacking only

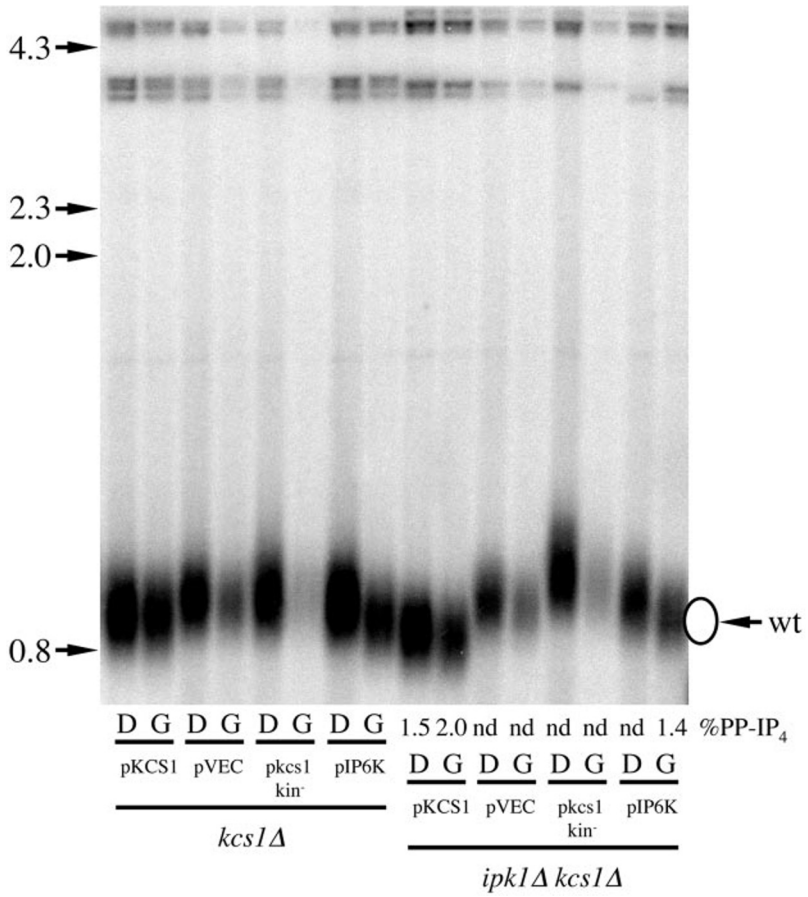

FIG. 4. **Complementation of telomere phenotype requires Kcs1 kinase activity.** *ipk1*Δ or *ipk1*Δ *kcs1*Δ cells were transformed with plasmids encoding various inositol diphosphoryl synthases behind a *GAL10* promoter. Cultures were grown in complete minimal medium-Ura with either dextrose (*D*) or galactose (*G*) as carbon source, and telomeric sequences were probed as described under "Experimental Procedures." The plasmids, from *left* to *right*, are pKCS1 (encoding wild-type Kcs1), pVEC (empty vector control), pkcs1kin$^-$ (encoding an active site point mutant of Kcs1), and pIP6K (encoding a human inositol diphosphoryl synthase). The length of wild type (*wt*) telomeres is indicated by the *oval* and *arrow* on *right*. *nd*, not detected.

Kcs1 kinase activity, which do synthesize PP-IP_5 by way of Ids1 activity. Because *ipk1*-null cells with no PP-IP_5 have the opposite phenotype, loss of PP-IP_5 cannot explain the longer telomeres observed. Therefore the kinase activity of Kcs1 must be producing something else, proximal to IP_6 on the IP pathway, which is needed for normal telomere length. Because IP_5 is a known substrate for Kcs1, the simplest interpretation is that PP-IP_4 loss leads to longer telomeres. We cannot formally exclude PP_2-IP_3 and other molecules downstream of PP-IP_4 or Kcs1 phosphorylation of another inositol substrate such as IP_4 as the molecules acting at the telomere. Importantly, we also report that further increases in PP-IP_4 synthesis lead to further shortening of telomeres. The opposing effects of gain and loss of PP-IP_4 demonstrate that this signaling molecule regulates the equilibrium of telomere length. These dual criteria also distinguish Kcs1 and its products from a number of reported genes involved in the maintenance of telomeres in yeast, most of which have only been reported to either lengthen or shorten telomeres but not both. Such regulation by a signaling-activated intracellular messenger pathway puts telomere maintenance under the potential control of extracellular stimuli, providing a mechanism to alter the average telomere length based on growth conditions or other environmental inputs.

Because neither the loss nor gain of PP-IP_4 has any impact on telomere length in the absence of Tel1, our work also provides a novel functional interaction between IPs and the activity of a PIKK family member, linking two important nuclear signaling pathways. Given that loss of Tel1 and increase in PP-IP_4 both lead to shortened telomeres, the simplest explanation is that PP-IP_4 inhibits Tel1 kinase function, either through direct or indirect kinase inhibition, consistent with Tel1 being a downstream component of this IP pathway. Assigning Kcs1 a

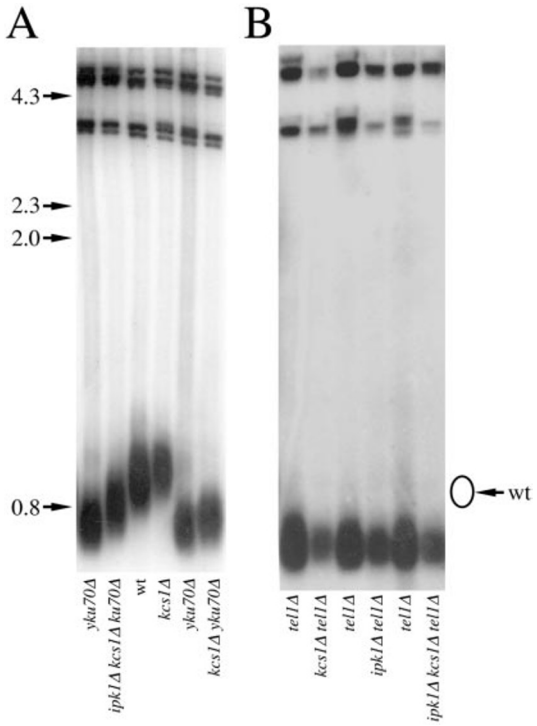

FIG. 5. **Regulation of telomere length by Kcs1 is independent of Ku but dependent on Tel1.** *A*, epistasis analysis of *KCS1, IPK1*, and *YKU70* at the telomere. *B*, epistasis analysis of *KCS1*, *IPK1*, and *TEL1* at the telomere. For *both panels*, single, double, and triple mutant strains were generated from spores grown for at least 100 doublings prior to the Southern analysis shown. Genotypes are indicated *below* each *lane*. The length of wild-type (*wt*) telomeres is indicated by the *oval* and *arrow* on *right*.

role as a negative regulator of Tel1 activity is consistent with earlier reports of the initial cloning of *KCS1* as a second site suppressor of a mitotic recombination phenotype (20) because loss of Tel1 function increases recombination rates (33). Tel1 is not the only potential target of IP regulation as other candidate effectors in this pathway of telomere regulation include the Mre11·Rad50·Xrs2 protein complex components, but their precise biochemical roles at the telomere remain elusive. Because the Mre11·Rad50·Xrs2 complex as well as Tel1 favors lengthening telomeres, they must somehow favor telomerase access to the telomere end, and it is likely that the activity of Kcs1 opposes that access, thus changing the equilibrium to favor average loss of telomere sequence. Given the reported roles for Kcs1 and inositol diphosphates in cellular signaling events, we cannot rule out at this point that other processes are involved. Other important regulators may be gleaned from recent studies describing a genome-wide screen for deletion mutants with a telomere phenotype that yielded many mutants with length alterations similar to the *plc1* and *kcs1* phenotypes (39). The components of the PP-IP_4 pathway were not identified in this laborious screen; however, the authors concede they failed to identify one-third of the described previously deletion strains with telomere phenotypes. The existence of mammalian homologues for both the inositol diphosphoryl synthases and Tel1 indicates that such an interaction may have important implications for human disease.

Our data expand the role of IPs in nuclear function and provide new insights into the complexity of the described metabolic pathways of soluble IPs in yeast. Activation of phospholipase C produces several unique IP messengers, which have been found to modulate nuclear processes including transcriptional control, chromatin remodeling, and mRNA export. We see that the loss of Ipk1 activity uncovers a branch point in the IP synthetic pathway that, despite its low abundance at steady state, plays a significant regulatory role in the cell. Our evidence for a regulatory role of the diphosphoryl inositol PP-IP_4 in determining the telomere length set point reinforces the hypothesis that a primordial role of phospholipase C activation is to generate an ensemble of IP messengers to control several nuclear events.

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