Inositol Pyrophosphates Are Required for DNA Hyperrecombination in Protein Kinase C1 Mutant Yeast†

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ABSTRACT: Diphosphoinositol pentakisphosphate ($InsP_7$) and bis-diphosphoinositol tetrakisphosphate ($InsP_8$) contain energetic pyrophosphate groups, occur throughout animal and plant kingdoms, and are synthesized by a recently cloned family of inositol hexakisphosphate kinases ($InsP_6Ks$). We report that these inositol pyrophosphates mediate homologous DNA recombination in yeast *S. cerevisae*. Hyperrecombination, caused by altered protein kinase C1 (PKC1), is lost in yeast with deletion of yeast $InsP_6K$ ($yInsP_6K$) and can be restored selectively by catalytically active yeast or mammalian $InsP_6Ks$. Inositol pyrophosphates are required for two forms of hyperrecombination that differ in mechanism, suggesting some generalities for actions of inositol pyrophosphates in recombination.

Inositol 1,4,5-trisphosphate ($InsP_3$), a major second messenger for multiple intercellular messengers, triggers the release of intracellular calcium (*1*). A variety of higher inositol polyphosphates exist including the recently discovered pyrophosphates diphosphoinositol pentakisphosphate ($InsP_7$)[1] and bis-diphosphoinositol tetrakisphosphate ($InsP_8$) which contain energetic pyrophosphate bonds (*2*−*6*). $InsP_7$ is formed by a family of recently cloned inositol hexakisphosphate ($InsP_6$) kinases (*7*, *8*) including $InsP_6K1$ (*8*), $InsP_6K2$ (*8*, *9*), and $InsP_6K3$ (Saiardi and Snyder, in preparation). $InsP_8$ is synthesized by a distinct $InsP_7$ kinase which has been purified (*10*) but not yet cloned. Inositol polyphosphates can also be synthesized by inositol phosphate multikinase (IPMK), which can form $InsP_3$, $Ins(1,3,4,5)P_4$, $Ins(1,4,5,6)P_4$, $Ins(1,3,4,5,6)P_5$, and diphosphoinositol tetrakisphosphate ($PP\text{-}InsP_4$) but not $InsP_7$ (*11*, *12*).

The higher inositol phosphates have been implicated in regulation of mRNA transport from the nucleus (*13*, *14*) and stimulation of DNA-dependent protein kinase activity (*15*) as well as influencing vesicular dynamics (*16*−*21*). However, physiological functions of inositol pyrophosphates have not been definitively characterized.

Homologous recombination is the principal mode of repairing double-stranded DNA breaks in yeast (*22*, *23*). In a study of underlying mechanisms, Huang and Symington (*24*) identified a yeast mutant with defects in protein kinase C1 (*pkc1-4*) and a 70-fold increase in the rate of recombination. The elevated rate of recombination was completely reversed by mutation of a gene designated Kinase C Suppressor-1 (*KCS1*) (*25*). We recently identified the protein encoded by *KCS1* as the yeast version of inositol hexakisphosphate kinase (*yInsP$_6$K*) (*8*). We wondered whether the $yInsP_6K$ enzyme protein or its inositol pyrophosphate products are directly or indirectly involved in regulating recombination events. In the present study, we demonstrate that the formation of $InsP_7$ and $InsP_8$ by $yInsP_6K$ is required for stimulation of hyperrecombination in yeast *S. cerevisae* containing a mutant protein kinase C, establishing a novel function of inositol pyrophosphates.

MATERIALS AND METHODS

Materials, Strains, and Constructs. NGF, X-Gal, and IPTG were from Boehringer Mannheim (Indianapolis, IN). Molecular cloning reagents were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). Glutathione Sepharose 4B was from Pharmacia (Piscataway, NJ). All other reagents were purchased from Sigma (St. Louis, MO), except as indicated. The yeast strains used in recombination assays were generously provided by Dr. Lorraine Symington.

To express GST-$yInsP_6K$ fusion protein in HEK293 cells, the open reading frame (ORF) of $yInsP_6K$ was generated by a PCR reaction and inserted into a pCMV-GST vector (*21*). pCMV-$yInsP_6K$-K771A and pCMV-$yInsP_6K$-K778A constructs were generated using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, Kingsport, TN) and confirmed by DNA sequencing. To express $yInsP_6K$ in yeast cells, the genomic $yInsP_6K$ (includes promoter and sequences for 5′ UTR and 3′ UTR) was generated by a PCR reaction and inserted into a pRS415 vector. pRS415-$yInsP_6K$-K771A and pRS415-$yInsP_6K$-K778A constructs were also generated using the Stratagene QuikChange Site-Directed Mutagenesis Kit. pRS415-$mInsP_6K1$ and pRS415-$hInsP_6K2$ were generated using a two-step PCR strategy and contain the promoter, 5′ UTR, and 3′ UTR sequences derived from the $yInsP_6K$ genomic sequence.

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[1] Abbreviations: PKC, protein kinase C; $InsP_7$, diphosphoinositol pentakisphosphate; $InsP_8$, bis-diphosphoinositol tetrakisphosphate; $InsP_6K$, inositol hexakisphosphate kinase; GST, glutathione-*S*-transferase.

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Expression of GST Fusion Protein in HEK293 Cells. To test protein expression in HEK293 cells, indicated constructs in Figure 1 were cotransfected into the cells using the calcium phosphate method. After 48 h, cells were harvested and lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 50 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 5 μg/mL aprotinin, 1 μg/mL leupeptin, 6 μg/mL chymostatin, 0.7 μg/mL pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Cell lysate was then centrifuged at 20000*g* for 20 min to remove insoluble materials. Equal aliquots of the supernatant were incubated with Glutathione Sepharose 4B beads for 2 h at 4 °C and then washed 4 times with lysis buffer. Bound proteins were analyzed by SDS−PAGE followed by immunoblotting with anti-GST antibody as previously described (*21*).

$InsP_6$ Kinase Assay. $InsP_6$ kinase enzymatic activity was assayed in 10 μL of reaction mixture containing 10 ng of recombinant GST-$InsP_6$K fusion proteins expressed in HEK293 cells, 20 mM HEPES (pH 6.8), 6 mM $MgCl_2$, 1 mM dithiothreitol, 5 mM ATP, 5 mM NaF, 10 mM phosphocreatine, 0.01 mg/mL phosphocreatine kinase (Calbiochem, San Diego, CA), 5 μM $InsP_6$, and 40 nM [^{3}H]$InsP_6$ (NEN, Boston, MA). Samples were incubated at 37 °C for 1 h, and reactions were terminated either by addition of 1 μL of 1 M HCl or by immersion in an ice/water bath. The reaction mixture was spotted onto a PEI-TLC plate and developed in 1.3 M HCI. Signals were visualized using a PhosphorImager machine after the TLC plate was exposed for 5 days.

Yeast Culturing and Radiolabeling with [^{3}H]Inositol. Yeast cultures were grown in CSM media (Bio101, Carlabad, CA) containing 2% glucose, 0.17% yeast nitrogen base, 5% ammonium sulfate, and 100 μCi/mL [^{3}H]inositol. Cell culture (2 mL) was seeded at 10^5 cells/mL and grown at 23 °C for 24 h and then shifted to 30 °C for an additional 1 h. Cells were harvested and washed twice with ice-cold water. Cell pellets were lysed in 0.2 mL of ice-cold lysis buffer (2 M perchloric acid, 0.1 mg/mL $InsP_6$, 2 mM EDTA) with two cycles of vigorous bead-beating (4 mm glass beads presoaked in lysis buffer). Lysates were centrifuged for 5 min at 4 °C and neutralized with K_2CO_3 as described previously (*8*).

Separation of Inositol Phosphates by HPLC. Inositol phosphates were resolved by HPLC using a 4.6 × 125 mm Partisphere SAX column (Whatman Inc., Clifton, NJ) that was eluted with a gradient generated by mixing Buffer A (1 mM Na_2EDTA) and Buffer B [Buffer A plus 1.3 M $(NH_4)_2HPO_4$, pH 3.8, with H_3PO_4] as follows: 0−5 min, 0% B; 5−10 min, 0−30% B; 10−60 min, 30−100% B; 60−75 min, 100% B. Fractions (1 mL) were collected and counted using 5 mL of Ultima-Flo AP LCS-cocktail Packard (Downers Grove, IL). Inositol phosphates were identified by their coelution with the standards of inositol phosphates.

Determination of Recombination Rates. Colony Sectoring assay and other experiments on DNA recombination were performed as described previously by Huang et al. (*24*).

Intracellular Localization of yInsP$_6$K. The GFP-yInsP$_6$K fusion proteins were expressed in yeast cells using a pGFP-C-FUS plasmid (*26*). pGFP-C-FUS plasmid is a 6.3 kb vector containing the URA3 marker and the MET25 promoter. Expression of GFP fusion proteins was induced by growing the transformed yeast cells on SC plates lacking uracil and methionine for 24 h, and the expressed fusion proteins were analyzed by western blot using an anti-GFP antibody. For confocal images, yeast cells were fixed for 45 min in 4% formaldehyde in PBS and washed 3 times in PBS. Nuclei were stained with 100 ng/mL 4′,6-diamidino-2-phenylindole (DAPI) for 10 min. Images of fluorescent cells were obtained on a Zeiss 510 confocal microscope.

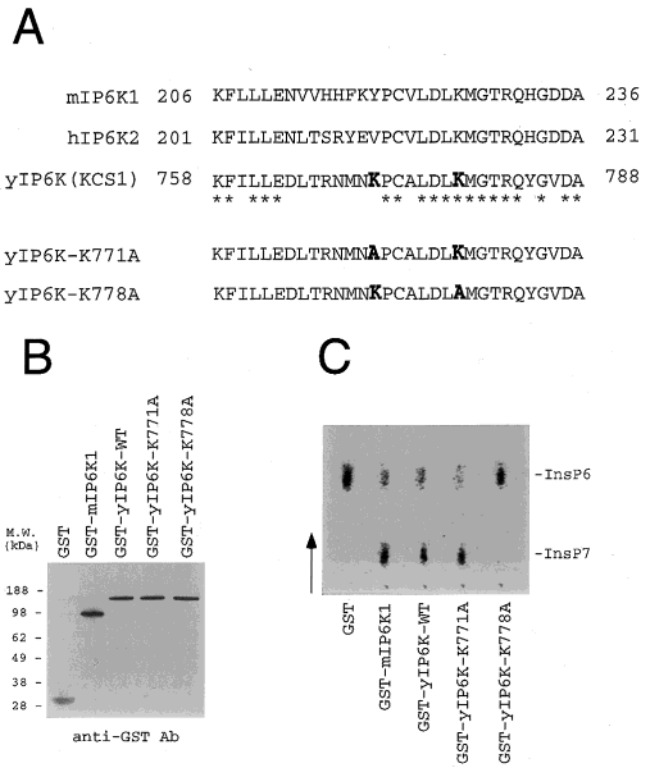

FIGURE 1: Expression and kinase activity assays of yInsP$_6$K in HEK293 cells. (A) Alignment of the inositol phosphate binding motif of different InsP$_6$Ks as well as the two yInsP$_6$K mutants used in this study. Identical amino acids are demarcated by asterisks. Numbers to the right and left of the sequences indicate their positions in the respective complete amino acid sequences; mInsP$_6$K1, mouse InsP$_6$K1; hInsP$_6$K2, human InsP$_6$K2. (B) Expression of GST fusion proteins in HEK293 cells. Expressed proteins were pulled-down with Glutathione Sepharose 4B beads and visualized by immunoblotting with anti-GST antibody (*21*). (C) Enzymatic activity of recombinant GST fusion proteins. Indicated constructs were transfected into HEK293 cells and then purified using a glutathione resin. In each reaction, 10 ng of recombinant protein was used, and $InsP_6$ and $InsP_7$ were separated by TLC (*8*). No activity was observed using GST alone. yInsP$_6$K-K778A was shown to be a catalytically inactive mutant, while yInsP$_6$K-K771A mutant contains the same kinase activity as the wild-type yInsP$_6$K.

RESULTS

Kinase Activity of yInsP$_6$K Is Required for Hyperrecombination in pkc1-4 Yeast. Symington and co-workers (*24*, *25*) observed pronounced hyperrecombination in yeast containing mutant *PKC1*, which was suppressed by an additional mutation designated *KCS1*. When we cloned a family of inositol phosphate kinases that can form inositol pyrophosphates, we showed that *KCS1* encodes the yeast form of InsP$_6$K (*yInsP$_6$K*) (*8*). To establish whether suppression of mutant yeast by yInsP$_6$K1 derives from the catalytic activity or some other property of *yInsP$_6$K*, we have conducted a series of experiments.

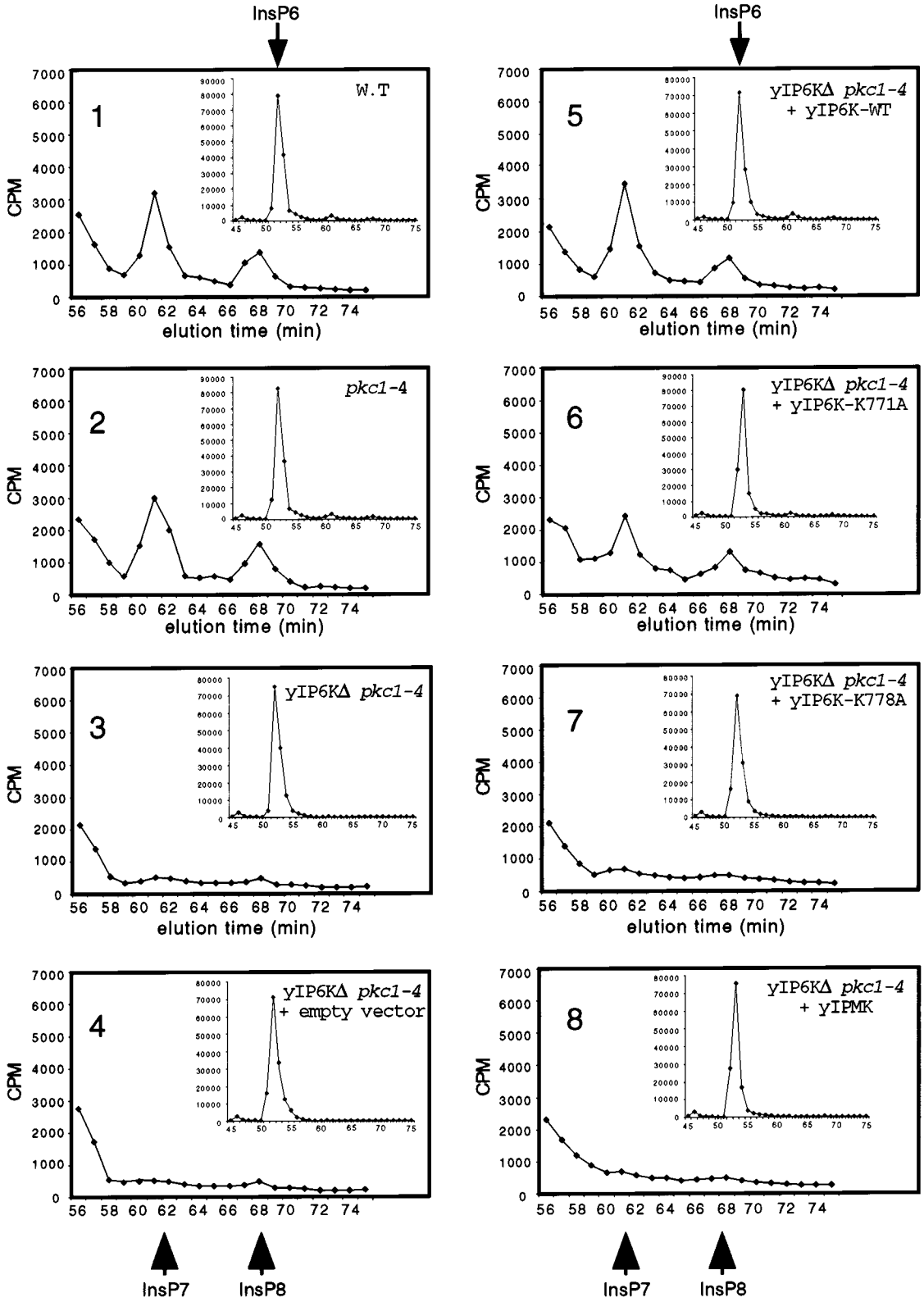

FIGURE 2: Partisphere SAX HPLC analysis of inositol phosphates in yeast cells. 1, Wild-type strain (YKH12a) (*24*); 2, *pkc1-4* mutant strain (YKH29a); 3, *yInsP$_6$KΔ/pkc1-4* double mutant strain (YKH114a); 4−8, *yInsP$_6$KΔ/pkc1-4* double mutants transformed with the indicated genes which were constructed into a pRS415 vector (*LEU2 CEN ARS*) and expressed under the control of *yInsP$_6$K* promoter. Inositol phosphates were extracted and resolved by HPLC. Fractions (1 mL) were collected and counted. The insets to each panel show the same data using a different *y*-axis scale. The positions of individual inositol phosphates were assigned from their elution times matching those of corresponding, authentic ^{3}H-labeled standards, which were separately determined in parallel HPLC runs. Data are presented as mean values from three independent experiments whose results varied less than 5%.

We utilized the yeast *pkc1-4* mutant strain in which hyperrecombination is rescued by deletion of *yInsP$_6$K*. We transformed these yeast with wild-type *yInsP$_6$K* or mutants in which lysine-778 was replaced by alanine (K778A), which

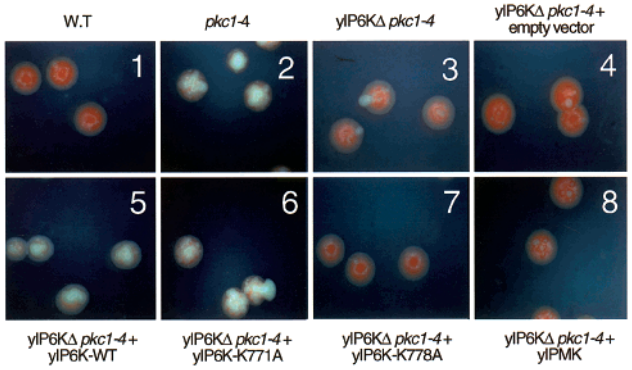

FIGURE 3: DNA hyperrecombination in yeast *pkc1-4* strain requires the kinase activity of yInsP_6K. The wild-type stain used in this study forms red colonies as a result of the presence of mutated *ade2* gene (*24*). Recombination between the two mutant ade2 alleles can produce a wild-type *ADE2* gene, resulting in the formation of a white sector in the red colony. The rate of sectoring corresponds to the rate of DNA recombination in these strains. Yeast cells were transformed using the lithium acetate method. Seven colonies were picked from each plate, diluted in H_2O, and then replated on the SC-Leu plate (2% glucose, 0.67% yeast nitrogen base, 2% agar). The untransformed yeast cells were plated on complete SC plates directly. Pictures were taken after growing 3 days at 30 °C.

abolishes catalytic activity, or in which lysine-771 is replaced by alanine (K771A) with retention of catalytic activity (Figure 1). The *yInsP_6KΔ/pkc1-4* double mutant strain fails to generate InsP_7 or InsP_8 following labeling of inositol phosphate pools with [^{3}H]inositol, while transformation with wild-type (WT) *yInsP_6K* restores inositol pyrophosphate generation as does transfection with *yInsP_6K*-K771A. By contrast, the kinase-dead *yInsP_6K*-K778A fails to augment formation of InsP_7 and InsP_8 (Figure 2).

The amount of InsP_7 or InsP_8 is the same in the *pkc1-4* mutant strain and the wild-type strain (Figure 2), indicating that InsP_6K kinase activity is not regulated by DNA hyperrecombination.

The technique we have employed to monitor recombination, as developed by Symington and associates (*24*), uses two heteroalleles of the adenine-2 gene which occur in a direct repeat orientation. *Ade2* mutations lead to red colonies, while white sectors are formed following recombination to provide a wild-type *Ade2* gene. As reported previously (*25*), the recombination rate is stimulated 40−50-fold in the *pkc1-4* mutant with reversal of this increase by deletion of *yInsP_6K* (Figure 3; Table 1). Transformation of the *yInsP_6KΔ/pkc1-4* double mutant with *yInsP_6K* or catalytically active *yInsP_6K*-K771A stimulates the recombination rate 50-fold, while no stimulation is evident with the catalytically inactive *yInsP_6K*-K778A or a yeast inositol phosphate multikinase (*yIPMK*) (also known as ArgRIII or Arg 82), which generates inositol polyphosphates, but not InsP_7 or InsP_8 (*11*, *12*). This indicates that inositol pyrophosphates, rather than the InsP_6K protein itself or non-pyrophosphate inositol phosphates, are required for recombination.

Mammalian InsP_6K Stimulates Hyperrecombination. To obtain more rigorous evidence that inositol pyrophosphate formation is crucial for recombination, unrelated to specific yeast proteins, we transformed the double mutant yeast with mouse InsP_6K1 (*mInsP_6KI*) and human InsP_6K2 (*hInsP_6K2*), whose amino acid sequences differ about 70% from yInsP_6K.

Table 1: Regulation of Hyperrecombination by Inositol Pyrophosphate Biosynthesis[a]

strain genotype	plasmid transformed	rate/cell/generation	relative rate
WT	/	2.33×10^{-5}	1
yIP6KΔ	/	2.79×10^{-5}	1.2
pkc1-4	/	1.18×10^{-3}	51
yIP6KΔ*pkc1-4*	/	3.26×10^{-5}	1.4
	yIP6K Promoter (pRS415 Vector)		
yIP6KΔ*pkc1-4*	empty vector	3.97×10^{-5}	1.7
	yIP6K-WT	1.07×10^{-3}	46
	yIP6K-K771A	9.08×10^{-4}	39
	yIP6K-K778A	4.19×10^{-5}	1.8
	yIPMK	4.90×10^{-5}	2.1
	ADH Promoter (pPC97 Vector)		
yIP6KΔ*pkc1-4*	empty vector	3.31×10^{-5}	1.4
	mIP6K1	9.55×10^{-4}	41
	hIP6K2	1.09×10^{-3}	47
	yIP6K-WT	1.15×10^{-3}	49
	yIP6K-K771A	9.35×10^{-4}	42
	yIP6K-K778A	3.72×10^{-5}	1.6

[a] Rates of recombination were evaluated at the ADE2 locus. Yeast cells were grown at 30 °C on SC (for untransformed cells) or SC-Leu plates (for transformed cells) for 3 days. Seven colonies were picked from each plate, diluted in H_2O, and then replated on plates lacking adenine to determine the number of Ade+ cells and on YEPD plates to determine the total number of cells. The median frequency of Ade+ cells was used to calculate the rate of recombination per cell per generation (*24*). Data are presented as mean ± SEM from three independent experiments.

Moreover, *mInsP_6K1* and *hInsP_6K2* differ substantially from each other in sequence (*8*). Transformation with *mInsP_6K1* and *hInsP_6K2* markedly increases the formation of InsP_7 and InsP_8 (Figure 4). This transformation increases recombination about 40-fold, establishing an obligatory role for inositol pyrophosphates in the hyperrecombination of the *pkc1-4* strain (Figure 5; Table 1).

The experiments transforming yeast with *yInsP_6K* had employed the *yInsP_6K* promoter (Figures 2 and 3). This promoter, however, was not strong enough to stimulate InsP_7 generation in yeast transformed with the mammalian forms of *InsP_6Ks* (data not shown). We utilized a strong constitutive alcohol dehydrogenase promoter (*ADH*) to drive the expression of mammalian *InsP_6Ks* (Figures 4 and 5). We have replicated the experiments with *yInsP_6K* utilizing the *ADH* promoter and show that, with this strong promoter, transformed *yInsP_6K* elicits even greater formation of InsP_7 than transformation utilizing the *yInsP_6K* endogenous promoter (Figure 4). Utilizing this strong promoter, we confirm that the catalytically inactive *yInsP_6K*-K778A fails to augment InsP_7 and InsP_8 formation and also fails to stimulate recombination, while the catalytically active *yInsP_6K*-K771A stimulates both inositol pyrophosphate formation and DNA recombination (Figure 5).

Inositol Pyrophosphates Are Required for both Gene Conversion and Pop-out Events in the pkc1-4 Strain. In the system we used, a *URA3* gene was inserted between the two mutant *ade2* alleles. The recombination between *ade-2* repeats can involve two principal mechanisms. One of these, designated gene conversion, occurs with the duplication remaining intact and can employ a double-crossover event, recombination between sister chromatids, or intrachromosomal interaction. Alternatively, recombination can lead to the loss of one of the *ade2* repeats and the *URA3* marker

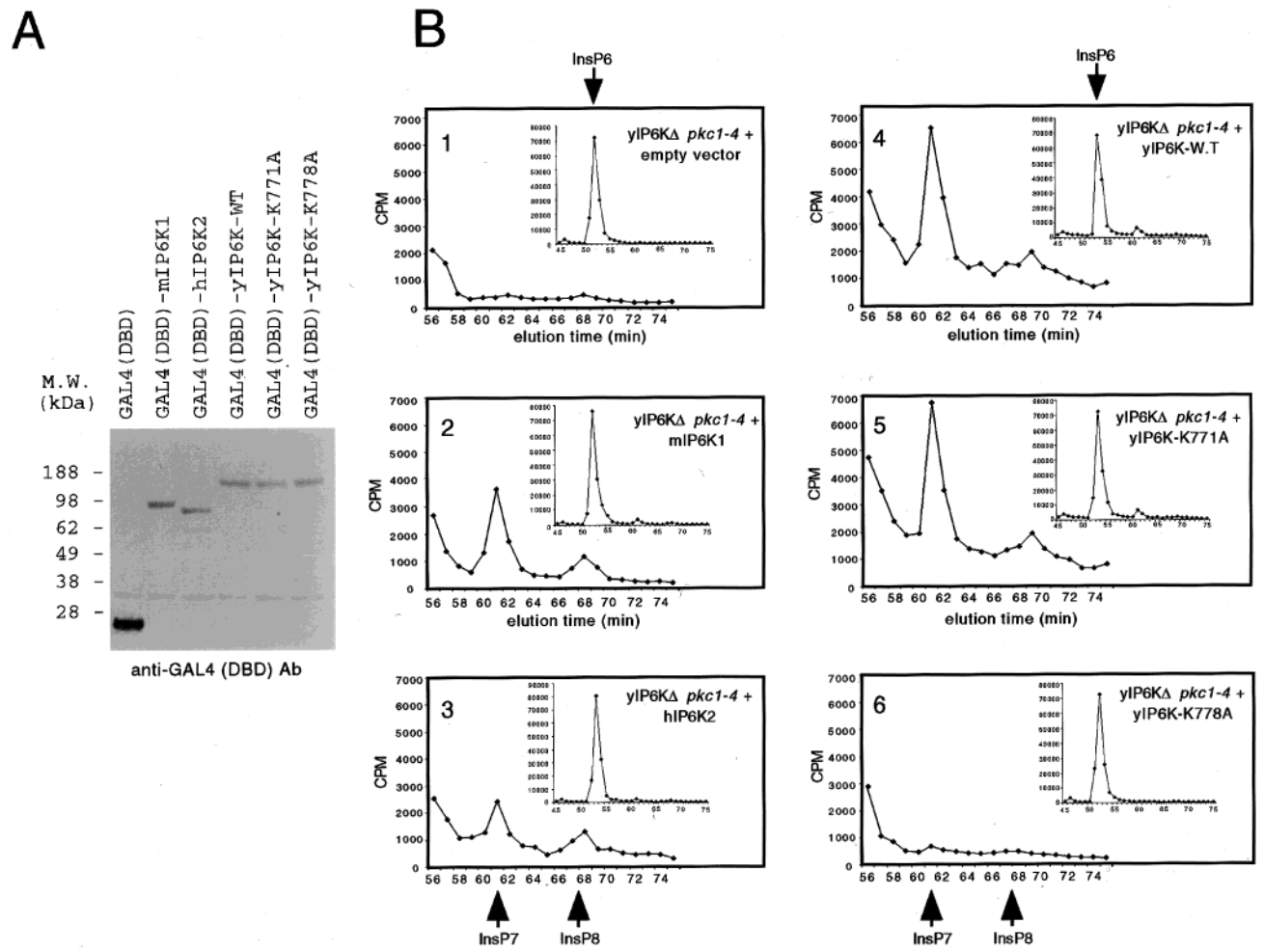

FIGURE 4: Mammalian $InsP_6K1$ and $InsP_6K2$ can substitute for $yInsP_6K$ to generate inositol pyrophosphates. (A) Expression of Gal4 [DNA binding domain (DBD)] fusion proteins in yeast cells. Indicated constructs were transformed into *yInsP$_6$KΔ/pkc1-4* strain, and the expressed fusion proteins were visualized by immunoblotting with anti-GAL (DBD) antibody. (B) Inositol phosphates generated in indicated yeast strains were analyzed by HPLC as described previously. 1, *yInsP$_6$KΔ/pkc1-4* double mutant; 2−6, *yInsP$_6$KΔ/pkc1-4* double mutants transformed with the indicated genes which were constructed into a pPC97 vector (*CEN ARS*) and expressed under the control of an *ADH* promoter. Mouse $InsP_6K1$ as well as human $InsP_6K2$ can produce both $InsP_7$ and $InsP_8$ in yeast cells.

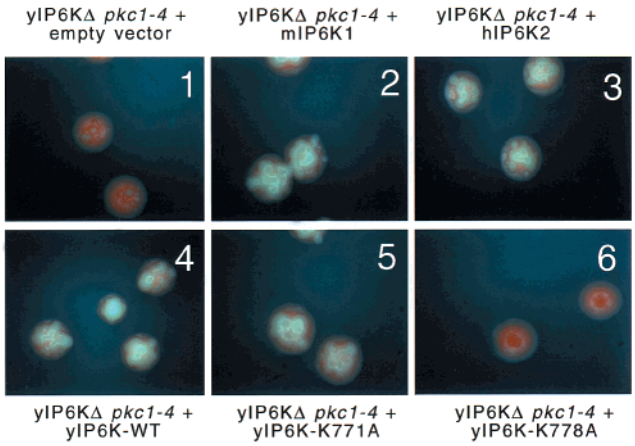

FIGURE 5: Mouse $InsP_6K1$ and human $InsP_6K2$ can substitute for $yInsP_6K$ to support the elevated rate of recombination in *pkc1-4* yeast. Indicated constructs were transformed into *yInsP$_6$KΔ/pkc1-4* strain, and the colony sectoring assays were performed as described in Figure 3.

and so is designated "pop-out". The pop-out mechanism can employ replication mispairing, single-strand annealing, unequal sister chromatid conversion, unequal sister chromatid exchange, or intrachromosomal crossing over.

In this system, pop-out and gene conversion forms of hyperrecombination can be distinguished, because yeast in which pop-out takes place will be *URA3*-negative while those with gene conversion will be *URA3*-positive. After selection on plates lacking adenine for recombination events, we monitored the percentage of *URA3*-positive colonies in the *pkc1-4* strain with or without deletion of *yInsP$_6$K* as well as in the *yInsP$_6$KΔ/pkc1-4* double mutants with various transformations by mammalian or yeast *InsP$_6$K* with or without catalytic activity (Table 2). Results are similar whether we employ the weak promoter (pRS415 backbone) or the strong *ADH* promoter (pPC97 backbone). Approximately 50% of yeast are *URA3*-positive under all of these conditions. Thus, as reported previously (*24*), pop-out and gene conversion mechanisms occur in the *pkc1-4* yeast to a similar extent, and the percentage is unaltered by various manipulations of *yInsP$_6$K*.

We wanted to monitor levels of recombination separately for pop-out and gene conversion mechanisms. Because of the selection system employed, it is not feasible to monitor

Table 2: Inositol Pyrophosphates Are Required for the Hyperrecombinations Generated by both Pop-out and Gene Conversion[a]

strain genotype	plasmid transformed	% Ura+ of Ade+ events	5-FOA^r rate/cell/ generation ($\times 10^{-4}$)	5-FOA^r relative rate
WT	/	45	1.43 ± 0.08	1
yIP6KΔ	/	40	2.00 ± 0.12	1.4
pkc1-4	/	60	18.6 ± 0.98	13
yIP6KΔ*pkc1-4*	/	55	1.98 ± 0.15	1.4
	yIP6K Promoter (pRS415 Vector)			
yIP6KΔ*pkc1-4*	empty vector	50	1.96 ± 0.17	1.4
	yIP6K-WT	35	22.1 ± 1.03	15
	yIP6K-K771A	45	15.8 ± 1.36	11
	yIP6K-K778A	45	2.57 ± 0.11	1.8
	yIPMK	65	2.73 ± 0.15	1.9
	ADH Promoter (pPC97 Vector)			
yIP6KΔ*pkc1-4*	empty vector	55	2.29 ± 0.10	1.6
	mIP6K1	60	17.4 ± 2.01	12
	hIP6K2	50	21.0 ± 1.32	14
	yIP6K-WT	45	24.7 ± 1.98	17
	yIP6K-K771A	65	18.6 ± 1.03	13
	yIP6K-K778A	50	2.16 ± 0.18	1.5

[a] In the system we used, a *URA3* gene was inserted between the two mutant *ade2* alleles. Recombination between the two mutant *ade2* alleles can occur by a pop-out mechanism, resulting in loss of the intervening *URA3* gene, or by gene conversion, in which the *URA3* gene is retained. The percentage of URA+ event was determined by growing cells on SC-uracil plates, while the percentage of URA− event was determined by growing cells on 5-FOA plates (*24*). Data are presented as mean ± SEM from three independent experiments.

recombination by yeast employing the gene conversion mechanism. However, we were able to monitor recombination in yeast employing the pop-out mechanism by monitoring *URA3*-negative cells that survive the 5-FOA selection (Table 2). Recombination is substantially increased in the *pkc1-4* yeast and suppressed by deletion of *yInsP$_6$K*. Rescue takes place with catalytically active *yInsP$_6$K*-K778A but not the catalytically inactive *yInsP$_6$K*-K771A. Moreover, both *mInsP$_6$K1* and *hInsP$_6$K2* restore the high survival rate of the *yInsP$_6$K*Δ/*pkc1-4* double mutant on 5-FOA plates, indicating that the enhanced pop-out rate is caused by the inositol pyrophosphates. The level of recombination in *pkc1-4* yeast utilizing pop-out is increased 13-fold compared to wild type. This contrasts with the 50-fold increase that occurs when both pop-out and gene conversion forms of recombination are utilized by the yeast. From these results, we can imply that at least 50% and possibly more of the augmented recombination in *pkc1-4* yeast is attributable to gene conversion.

Nuclear Localization of yInsP$_6$K Protein. DNA recombination is a nuclear event. If inositol pyrophosphates regulate recombination, *yInsP$_6$K* should occur in the nucleus. We transformed yeast with green fluorescent protein (GFP) linked to WT *yInsP$_6$K*, the catalytically active *yInsP$_6$K*-K771A, and the catalytically inactive *yInsP$_6$K*-K778A (Figure 6). GFP alone is localized to both the nucleus and the cytoplasm. *yInsP$_6$K* also occurs in the nucleus as well as the cytoplasm, which fits with *yInsP$_6$K*'s dual roles in regulating vesicular trafficking and nuclear functions. Wild-type *yInsP$_6$K*, catalytically active *yInsP$_6$K*-K778A and catalytically inactive *yInsP$_6$K*-K771A display a similar localization, indicating that *yInsP$_6$*'s localization does not depend

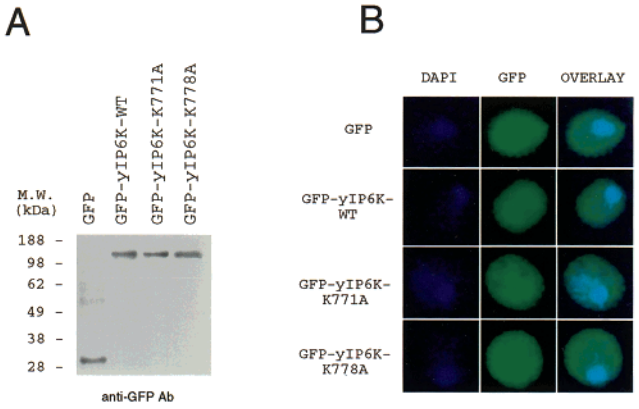

FIGURE 6: yInsP$_6$K protein localizes in both nucleus and cytoplasm. (A) Western blot of expressed GFP fusion proteins. The indicated fusion proteins were expressed in yeast cells using a pGFP-C-FUS plasmid (*26*) and were analyzed by western blot using an anti-GFP antibody. (B) Confocal images of yeast cells transformed with indicated GFP constructs. Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI). Images of fluorescent cells were obtained on a Zeiss 510 confocal microscope. The left panels show DAPI staining. The middle panels show the same fields processed for GFP images. The right panels are merged images.

on InsP$_6$K kinase activity. X-ray irradiation (50 krad) does not change the intracellular distribution of InsP$_6$K, suggesting that the distribution of InsP$_6$K is not regulated by DNA damage (data not shown).

DISCUSSION

Our findings establish that inositol pyrophosphates, InsP$_7$ and InsP$_8$, mediate stimulated homologous recombination in protein kinase C mutant yeast. The inositol pyrophosphates are required whether the recombination utilizes pop-out or gene conversion mechanisms. Since the pop-out and gene conversion mechanisms differ markedly, these findings imply a relatively general role for inositol pyrophosphates in recombination. In this study, we employed a model of recombination that occurs in yeast with a mutant protein kinase C. As we did not evaluate other forms of recombination, we do not know the extent to which our findings can be generalized to all types of stimulated recombination. We have not found evidence for inositol pyrophosphates regulating basal levels of recombination which are not altered in yeast with deletion of *yInsP$_6$K*.

Why does mutation of *pkc1-4* lead to recombination in yeast? Recombination in yeast is typically stimulated by damage to DNA, by enhanced transcription, or by alterations in proteins of the cyclin family, which can cause a prolonged S phase. Substrates of PKC in yeast have not been characterized, so that it is difficult to know whether PKC phosphorylates yeast cyclins or transcription factors and alters them in a fashion that would influence recombination. In mammals, PKC phosphorylates so many different proteins that one cannot formulate discrete testable models to explain the yeast recombination.

All of our experiments modulating InsP$_6$K activity involved the formation of both inositol pyrophosphates, InsP$_7$ and InsP$_8$. We are unable to ascertain the relative roles of InsP$_7$ or InsP$_8$, since they are both formed in our experiments. A InsP$_7$K activity has been purified but not cloned from mammalian tissues (*10*). We do not know whether such an

enzyme generates $InsP_8$ in yeast or whether $yInsP_6K$ forms both $InsP_7$ and $InsP_8$. Utilizing a different strain of yeast, we observed some residual $InsP_7$ and $InsP_8$ formation following deletion of *yInsP$_6$K* (*11*). In the present study, we employed a different strain in which $InsP_7$ and $InsP_8$ formation is virtually abolished in mutants lacking $yInsP_6K$.

How might inositol pyrophosphates influence recombination? DNA recombination involves cleavage and re-ligation of DNA fragments, which is catalyzed by many regulated enzymes. The new 3′,5′-phosphodiester bond is formed between the 5′-phosphate group and the 3′-hydroxyl group in the nick. Accordingly, it is unlikely that $InsP_7$ or $InsP_8$ regulates the recombination by donating their phosphate groups directly onto DNA. However, $InsP_7$ or $InsP_8$ might regulate DNA recombination by modifying the functions of involved proteins. We have previously provided evidence that the pyrophosphate groups can be donated to proteins, providing a novel means of protein phosphorylation that might by relevant to discrete groups of proteins, such as those involved in nuclear or synaptic events (S. Voglmaier and S. H. Snyder, unpublished data). In these studies, incubation of [^{32}P]InsP7 (^{32}PP-InsP5) with rat brain lysate for 1 h at 37 °C revealed phosphorylation of several proteins. IP_6Ks in brain lysate also have ATP synthase activity so that [^{32}P]-ATP can be formed from [^{32}P]InsP7 and ADP (*7*). We cannot determine whether the ^{32}P-labeled proteins were directly phosphorylated by [^{32}P]$InsP_7$ or indirectly labeled by [^{32}P]-ATP (data not shown).

Alternatively, the inositol pyrophosphates might regulate protein activation, analogous to the way in which GTP regulates activity of G proteins. It is also possible that the energy derived from hydrolysis of inositol pyrophosphates alters energy dynamics in selected microenvironments of the cell.

While our experiments employed yeast, homologous recombination also participates in DNA repair in mammalian cells. The other predominant mode of DNA repair in mammalian cells involves nonhomologous end-joining (NHEJ) processes (*27*). Interestingly, West and associates (*15*) showed that $InsP_6$ stimulates NHEJ through DNA-dependent protein kinase and suggested that the physiologically active inositol phosphates might be $InsP_7$ and $InsP_8$. Homologous recombination and end-joining differ in many ways, but share certain elements such as the MRE11−RAD50 complex (*28*) which might provide a site whereby inositol pyrophosphates could influence both homologous recombination and NHEJ.

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