## Inositol Pyrophosphates Mediate Chemotaxis in *Dictyostelium* via Pleckstrin Homology Domain-PtdIns(3,4,5)P3 Interactions

Hongbo R. Luo,<sup>1</sup> Yi Elaine Huang,<sup>2</sup> Jianmeng C. Chen,<sup>3</sup> Adolfo Saiardi,<sup>1</sup> Miho lijima,<sup>2</sup> Keqiang Ye,<sup>5</sup> Yunfei Huang,<sup>1</sup> Eiichiro Nagata,<sup>1</sup> Peter Devreotes,<sup>2</sup> and Solomon H. Snyder<sup>1,3,4,\*</sup> <sup>1</sup>Department of Neuroscience <sup>2</sup>Department of Cell Biology <sup>3</sup>Department of Pharmacology <sup>4</sup>Department of Psychiatry and Behavioral Sciences Johns Hopkins University School of Medicine 725 N. Wolfe St. Baltimore, Maryland 21205 <sup>5</sup>Department of Pathology **Emory University** Atlanta, Georgia 30322

#### Summary

Inositol phosphates are well-known signaling molecules, whereas the inositol pyrophosphates, such as diphosphoinositol pentakisphosphate (InsP7/IP7) and bis-diphosphoinositol tetrakisphosphate (InsP8/IP8), are less well characterized. We demonstrate physiologic regulation of Dictyostelium chemotaxis by InsP7 mediated by its competition with PtdIns(3,4,5)P3 for binding pleckstrin homology (PH) domain-containing proteins. Chemoattractant stimulation triggers rapid and sustained elevations in InsP7/InsP8 levels. Depletion of InsP7 and InsP8 by deleting the gene for InsP6 kinase (InsP6K/IP6K), which converts inositol hexakisphosphate (InsP6/IP6) to InsP7, causes rapid aggregation of mutant cells and increased sensitivity to cAMP. Chemotaxis is mediated by membrane translocation of certain PH domain-containing proteins via specific binding to PtdIns(3,4,5)P3. InsP7 competes for PH domain binding with PtdIns(3,4,5)P3 both in vitro and in vivo. InsP7 depletion enhances PH domain membrane translocation and augments downstream chemotactic signaling activity.

#### Introduction

Inositol phosphates occur in many animal and plant tissues with the most extensively characterized being inositol 1,4,5-trisphosphate (IP3), which releases calcium from intracellular storage (Irvine and Schell, 2001). Higher inositol phosphates containing energetic pyrophosphate bonds have been described, especially diphosphoinositol pentakisphosphate (pp-InsP5/InsP7) and bis-diphosphoinositol tetrakisphosphate (bis-pp-InsP4/InsP8) (Glennon and Shears, 1993; Stephens et al., 1993). InsP7 is formed by a family of inositol hexakisphosphate kinases (InsP6K) including InsP6K1 (Saiardi et al., 1999), InsP6K2 (Saiardi et al., 1999; Schell et al., 1999) and InsP6K3 (Saiardi et al., 2001). A protein with InsP7 kinase activity was purified but not cloned (Huang et al., 1998), and it is unclear whether InsP8 is physiologically generated by a distinct enzyme or by one of the known InsP6Ks.

Physiologic functions of inositol pyrophosphates have not been extensively characterized. They participate in vesicular endocytosis, as deletion of InsP6K in yeast leads to selective deficits in endocytosis (Saiardi et al., 2002). Also, one of the InsP6 kinases, InsP6K1, physiologically binds to a novel protein GRAB (Guanine Exchange Factor for Rab3A). GRAB is a guanine nucleotide exchange factor for Rab3A, which mediates vesicular exocytosis (Luo et al., 2001). A role in DNA repair/recombination is suggested by findings that InsP6K deletion from yeast influences DNA hyperrecombination (Huang and Symington, 1995; Luo et al., 2002). Inositol pyrophosphates may also influence apoptosis, as deletion of one of the InsP6 kinases, InsP6K2, prevents apoptotic cell death in ovarian carcinoma cells (Morrison et al., 2001).

In most mammalian tissues levels of InsP7 are 0.5–3  $\mu$ M with InsP8 levels being substantially lower (Glennon and Shears, 1993; Stephens et al., 1993). By contrast, *Dictyostelium discoideum (D. discoideum)* displays InsP7 and InsP8 levels of about 100  $\mu$ M and 250  $\mu$ M, respectively, which are developmentally regulated (Laussmann et al., 2000). In the present study, we used *D. discoideum* as a model system to elucidate the physiological functions and molecular mechanisms of inositol pyrophosphates.

D. discoideum is a group of unicellular free-living amoebae that aggregate to form a motile multicellular organism. When D. discoideum cells overgrow their food supply and starve, they stop dividing and some cells release cAMP in pulses as a chemoattractant to elicit aggregation (Chung et al., 2001; lijima et al., 2002; Devreotes and Janetopoulos, 2003). cAMP binds a G protein-coupled cAMP receptor (cAR1) on cell membrane and induces the dissociation of a specific G protein into  $\alpha$  and  $\beta\gamma$  subunits. Released  $\beta\gamma$  subunits initiate localized activation of a phosphatidylinositol 3-kinase (PI3K) and deactivation of a phosphatidylinositol 3-phosphatase (PTEN), causing the accumulation of phosphatidylinositol 3, 4, 5 trisphosphate (PtdIns(3,4,5)P3) on the leading edge of chemotaxing cells. A subset of PH domain-containing proteins, including Crac (cytosolic regulator of adenylyl cyclase), Akt/PKB, and PhdA, are rapidly recruited to the leading edge through their specific binding with PtdIns(3,4,5)P3 and trigger downstream signaling pathways leading to chemotaxis. This PH domain translocation appears to be an essential step for directional sensing, since either decreasing the amount of PtdIns(3,4,5)P3 by disrupting PI3K genes or abnormally increasing PtdIns(3,4,5)P3 levels by disruption of the PTEN gene diminishes the ability of cells to orient and move in chemotactic gradients (Funamoto et al., 2002; lijima and Devreotes, 2002).

In the present study, we demonstrate a crucial role for InsP6K and its product InsP7 in these chemotactic events. InsP7 binds PH domain-containing proteins and

mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	  м н	- - - I	  F \	  / L	- - V	- - N	- - S			- - -	- - V	- - N	- - N	- - S		S F		 		- - D	- - R	- - - K	- - - Y	- - N	- - L	- - E		 	- - N	- - N	- - N	N		- - -	- - - N	- - N	- - N	- - N	- - N			- - N	- - N	1 1 1 50
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	  N N	- - - N	 	 	- - N	- - N	- - N		N N	-	- - N	- - N	- - N	- - N		N 1	 			- - N	- - N	M  N	C - N	V - N	C - N	Q -	T N  N N		V S M N	G P V S	Q A V N			MAN	A D E	S V A N	R E G N	A P D S	G R M D	D I A I R J N I		V V V M	LLQK	25 18 17 100
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K		P P P Q	F ] F \ F L F F		0000	V V V V V	G G G G G				M V V M	M L M L	R R K S	Y F Y V			+ 1 F 1 + 1 V -	Г V Г L Г V - ]		K K K	P P P	L L L	I V V I	S P S N	R R R R	E ( E E	QF QF L(	R F P F R F P F	Y Y Y Y	E E E E	S I T S I			E	MMM	K R K	E K R	F F F F	T T T S	P ( P ( - 1		<u>к к к к</u>	GGGN	75 68 67 140
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	V V V V T V N I	S S T H	V ( V F V F S F		E E W I	G E K S	D D D P	S I E I S F	D C D F T C L A	i Y N i H	I L F	N C S F	L L G	V I V V		Y F Y F N F		- H		S D S K	E H - N	T G D	V I - I	E V - V	Q D Q T	D I E D	D T A F P F L C		E S V S	R D S Q	E C T			R S A -	K K V	H L A	S L I	R R W	R W Q	S T T	H F N Q	RKQQ	S K T Q	125 118 113 181
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	G S H H T Q Q	G V S Q	S E L E N Q		K E D Q	E K C Q	E T T Q	K I P I Q I			S V Q	F R H Q	EQAQ	T H Q Q	S R L Q Q	E 9 - 1 A F Q 0			Q E K K S	A M E L	K K S G	S S P S	P H A P	кккк	V L S	E E L S		F	H E F Y	-   W - М	S L P S			E	V P I	P L A N	F Y F Y	QYSS	M T L N			N K T N	S G N K	171 167 162 231
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	G L N I G N N K	S S Q K	S E S C V E K N	K Q L R K	I K K	S H S	Y Y F	N I N I N I	P V P V P V		L M L	R K Q E	C C C C	H H H K	K Q Q H		2 L 2 L 4 L		S R Q R F R	M	R K C	S E S	E N E	S A Y	KKPP	D H E S	R N N N G T		Y Y H Q	K K R K	F F Y		. L . L . l / L	E	NNND	V L V L	V T V T	H S S S	H R Q S	F I Y I Y I	K Y E V T H N S	PPP	CCCC	221 217 212 263
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	V L V L V L I V	D D D D			GGGG	T T T T	R R R R		H ( H ( H ( R (			A A A	S S S	A E E	E I E			A F A F		M I M	R R R	<b>K K K</b> -	CCCC	E Q A S	Q Q Q Q S	S S T			T V C S	L I L H	G G G				GGGG	M M M M	QQQK	V V I	Y Y Y Y	QQQC		K	G G K G	271 267 262 301
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	H Y Q L Y F E M	L M L I			KKKR	YYYY	Y H Y F	GIGIGI	R I R I R	5 L ( L ( L	S S S D	I V V G	EQEN	G G G T	F I F I L I	R I R ( E I			F F	QQQH	Y F F	L F L F	H H H T	N N N S	GGGN	L R S M	D L Y L H L F F	-	- - - N	- - N	- - N	NI	N ]	- - - -	- - - N	- - S	- - N	- - D	- - N	  F I	 -	- - N	- - K	304 300 295 351
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	  N F	- - I	  S (	 - 	Ē	- - H	- - - L	- - Q	R I R I R I R I	R D R E R E		F L L I	E G E E	P P S	I I I				- F - T - F	GEAQ	L L I I	KKLE	A A S C	V V V I	L L I L	E R S	R ( R ( S ( N H		- - D	- - P	- - F	S S P	Y F Y F F F	R F R F R F	YYYY	S S S S S	S S S S	S S S S S				YYYY	D D D E	340 330 331 401
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	G K G K G K	E E E E	C   -   -	R S R P - N	E - E	L V I	R V L	L   L   N (	K H D : Q :		D A I	M E - N	G D N	L L - I	P E I P I N I			5 - 5 - 1		S S	G E P N	P E H N	S S P N	T A H N	- - N	- - N	  N M	-	- - N	- - N	- - N		N 9	 5 N	-	- - N	- - S	- - I	- - I	  S I		- - M	- - N	369 361 347 449
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	  I 0	- - - F	  S [	  - 1	S D E D	P E A S	S S P T	S A Q S	G A A	A Y A H B C	E A G T	A Y S T	G K S T	P P P P	S I G S	G I Q						- - - L	- - D	- - S	- - N	- - I		5 5	- - - T	- - D	- - S	- - Y	 		-	- - E	- - N	- - N	- - S	- - N		- - -	- - - N	388 380 360 499
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	  N N	- - D			- - I	- - S	- - V	- - S	 	  5 I	- - S	- - I	- - Q	- - R	- - N	 		<	 5 L	- - E	- - S	- - N	- - S	- - c	- - - I	- - S	  S N	 	RR	M M M M	I I I I	D I D I D I E				T T G	F C Y F	K R K H	G L G S	F Y F	G E N N G S	D D E	P T H E	404 396 382 549
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	T V V V T T E I	H H Y D			- - v	- - S	- - S	- - D	  L [	  ) S	- - D	- - S	- - D	- - E	- - M	- -	5 1	N	2 2	- - T	- - R		- - N	- - S	- - G	- - N	  F :	5 0	-	- - K	- - P	- - Q		 5 K		- - I	- - Q	- - N	- - A	- - V	  	-	- - D	410 402 388 599
mInsP6K1 mInsP6K2 mInsP6K3 dInsP6K	  F 4	- - -		  	- - -	- - T	- - - F	- - K	 		PQPE	D D D D	R A P D	GGGG	YYYYY		F					IIII	S D R	III	M V L	E T Q F	QNE		DEQV	EEEE	N S G T	Q G E F	E											433 426 410 638

#### Figure 1. Amino Acid Sequence Alignment of InsP6K Genes

Mammalian InsP6K1 (GenBank accession number AF177144), InsP6K2 (GenBank accession number AF177145), InsP6K3 (GenBank accession number AF393812), and *Dictyostelium* InsP6K (dInsP6K/I6KA, GenBank accession number AY225466) protein sequences were aligned using DNAstar software. The portions of three highly conserved regions (CR1, CR2, and CR3) are demarcated by solid lines. CR2 is the conserved inositol phosphate binding motif (Saiardi et al., 1999).

competes with their binding to PtdIns(3,4,5)P3. InsP6K deletion accelerates aggregation and increases the sensitivity of amoebae to cAMP.

## Results

#### Cloning and Deletion of D. discoideum InsP6K

Utilizing the mammalian InsP6K sequences, we searched *D. discoideum* DNA databases and identified three genes that contain the inositol phosphate binding sequence shared by both InsP6K and IPMK (Saiardi et al., 1999) (Figure 1). Phylogenetic tree analysis (data not shown) reveals that one of these resembles mammalian IPMK more than InsP6K, another resembles InsP6K

more than IPMK, while a third displays comparable similarities to InsP6K and IPMK. We cloned the cDNA for the gene most resembling InsP6K (Figure 1). The *D. discoideum* InsP6K protein (dInsP6K) possesses 637 amino acids with a molecular weight of 72 kDa and displays 26%, 30%, and 27% amino acid sequence identity with mammalian InsP6K1, InsP6K2, and InsP6K3, respectively. We observe 3 regions with substantial sequence similarity between *D. discoideum* and mammalian InsP6Ks. One of these (CR2) is the portion of the enzyme that binds inositol phosphates, while functions of the other two conserved regions are not known. The only notable difference from mammalian InsP6Ks is the enrichment of asparagine and serine containing domains, a common property of many *D. discoideum* proteins.

We deleted InsP6K gene (I6KA) by homologous recombination utilizing a BSR (blastocidin) gene as a selection marker (Figure 2A). Southern blot (Figure 2B) and Northern blot (Figure 2C) analyses confirm the deletion of InsP6K gene from the genome as well as the complete depletion of InsP6K mRNA. To establish the catalytic function of the presumed InsP6K, we demonstrated that wild-type D. discoideum extracts transform [3H]InsP6 to InsP7 (Figure 2D). Deletion of the InsP6K gene abolishes this enzymatic conversion, while the InsP6 kinase activity is restored in amoebae transformed with a vector expressing wild-type InsP6K. We also demonstrated inositol pyrophosphate formation in intact amoebae incubated with [3H] inositol revealing the formation of InsP5, InsP6, InsP7, and InsP8 in wild-type cells (Figure 2E). In cells with deletion of InsP6K, InsP5 and InsP6 are still formed, but peaks for InsP7 and InsP8 are lost. Transformation of the amoebae with wild-type InsP6K construct restores the InsP7 and InsP8 peaks. We examined the formation of other inositol phosphates including IP3, which has been implicated in development of D. discoideum (Williams et al., 1999), and found no difference between  $insp6k^{-}$  and wild-type cells (data not shown).

These experiments establish that the cloned putative InsP6K possesses InsP7 synthesizing activity. Moreover, abolition of InsP7 and InsP8 formation in amoebae lacking this enzyme establishes that it is the only inositol pyrophosphate-synthesizing enzyme in the amoebae. Thus, we have no evidence for a distinct InsP7 kinase in these cells.

To ensure that the enzymatic alterations we have observed in the cells lacking InsP6K do not derive from general metabolic disturbances, we examined various properties of the cells. Growth rate of the InsP6Kdeleted cells in HL5 medium and on bacteria lawns is the same as wild-type (data not shown). Chemotaxis and development on DB agar are readily demonstrable (data not shown). The *insp6k*<sup>-</sup> cells can form fruiting bodies with normal shape and regular size (data not shown).

# cAMP Rapidly and Potently Augments InsP7 and InsP8 Levels

Laussmann et al. (2000) reported that mass levels of InsP7 and InsP8 in D. discoideum are about 100 times higher than in mammalian tissues and are developmentally regulated, making this species a useful model system for evaluating functional roles of inositol pyrophosphates. We have labeled endogenous stores with [<sup>3</sup>H]inositol and monitored levels of InsP6, InsP7, and InsP8 at various times after initiation of starvation (Figure 3A). All three substances increase during the course of starvation, peaking at 4-8 hr and declining by 36 hr. Whereas the maximal increase of InsP6 is about 20%, InsP7 and InsP8 levels increase at least 25-fold from the vegetative state to peak levels. Accordingly, in the vegetative condition levels of inositol pyrophosphates are only about 1%-2% of InsP6, while at their peak, InsP8 levels are almost half those of InsP6.

The dynamic alterations in inositol pyrophosphate lev-

els imply regulation by signaling systems. To explore this possibility, we monitored their responses to cAMP stimulation (Figures 3B–3D). We observe pronounced rapid increases of InsP7 and InsP8 in response to cAMP. Levels of InsP7 and InsP8 increase 3- to 4-fold following cAMP application, whereas levels of InsP6 are unaltered (Figure 3C). Responses are rapid with maximal stimulation at 60 s and half maximal effects at 15–20 s. The potency of cAMP in augmenting InsP7 and InsP8 is comparable to its potency in eliciting chemotaxis with half maximal effects at about 30 nM (Figure 3D).

## InsP6K Deletion Causes Increased Chemotactic Sensitivity to cAMP

Inositol pyrophosphates have been implicated in processes like vesicular trafficking, DNA repair/recombination, and apoptosis (Huang and Symington, 1995; Luo et al., 2001, 2002; Morrison et al., 2001; Saiardi et al., 2002). InsP6K deletion in yeast disrupts several endocytotic events (Saiardi et al., 2002). Parallel events were examined in *insp6k*<sup>-</sup> null cells (data not shown). Interestingly, pinocytosis and phagocytosis function normally in the InsP6K-deleted cells. DNA repair in response to X-irradiation, ultraviolet irradiation, etoposide, MMF, cisplatin, and 4NQO also appear normal. No obvious defects are detected during the programmed cell death of prestalk cells.

Starvation elicits aggregation of D. discoideum amoebae, which is associated with cAMP-mediated chemotaxis (Chung et al., 2001; lijima et al., 2002). To explore a role for inositol pyrophosphates in chemotaxis, we examined aggregation in InsP6K deleted amoebae (Figure 4). Starvation-induced aggregation occurs more rapidly in *insp6k<sup>-</sup>* null than in wild-type amoebae especially in the early stages (Figure 4A). Upon starvation, wildtype cells take 24 hr to develop into a mature fruiting body, and streaming of chemotaxing cells toward aggregation centers appears at about 6 hr. However, in insp6k<sup>-</sup> cells, the streaming is detected as early as 5 hr after starvation. Transformation with InsP6K restores the aggregation process to the wild-type pattern. In order to ensure that these results do not simply reflect clone-to-clone variation, we utilized 3 distinct insp6k clones and observed essentially the same alteration in multiple experiments (Figure 4B).

Starvation-induced aggregation in wild-type amoebae requires proteins involved in chemotaxis and development. These proteins are sequentially expressed after the initiation of starvation, following a preset program. The rapid aggregation in *insp6k*<sup>-</sup> cells could be caused by an accelerated pace of development with earlier expression of proteins required for aggregation. We examined the expression level of two developmentally regulated proteins: cAR1 and adenylyl cyclase (ACA). Protein levels of cAR1 and ACA at different times after initiation of starvation are the same in *insp6k*<sup>-</sup> null and wild-type cells (Figure 4C), suggesting that the pace of differentiation is not altered in *insp6k*<sup>-</sup> cells and is not the cause of the accelerated aggregation.

cAMP-induced chemotaxis can only happen when the extracellular chemoattractant cAMP levels begin to oscillate spontaneously. During the early stage of development, ACA is expressed at a very low level and its prod-



#### Figure 2. Disruption of Dictyostelium InsP6K Gene

(A) Schematic representation of homologs recombination strategy for gene disruption. The solid line demarcates the portion of DNA sequence used to make probe for Southern and Northern blotting.

(B) Southern blot analysis of wild-type and *insp6k<sup>-</sup>* null (*i6kA-/Ax2*) genomic DNA. Total genomic DNA from indicated strains were extracted and digested with Clal. Digested DNA fragments (20  $\mu$ g) were separated on a 0.8% agarose gel and blotted using a <sup>32</sup>P-labeled cDNA probe against dlnsP6K. As expected, a 1 kb band for wild-type and a 2.4 kb band for *insp6k<sup>-</sup>* cells were detected.

(C) Northern blot analysis of wild-type and  $insp6k^-$  null mRNA. Indicated Ax2 cells were developed on development buffer (DB)-agar for 5 hr. Total RNA were extracted and separated on a 1% agarose-formaldehyde gel (20  $\mu$ g for each lane). dlnsP6K mRNA (2.8 kb) was detected in wild-type cells but not in  $insp6k^-$  cells.

(D) InsP6 kinase activities in wild-type and *insp6k*<sup>-</sup> cell lysates. The assay was performed as described in "Experimental Procedures". Inositol phosphates were extracted and resolved by HPLC using a Partisphere SAX column. Fractions (1 ml/min) were collected and counted. The positions of individual inositol phosphates were assigned from their elution times matching those of corresponding authentic <sup>3</sup>H-labeled standards. Data are presented as mean values from three independent experiments whose results varied less than 5%. The peaks representing InsP6 and InsP7 are indicated.

(E) HPLC analysis of inositol phosphates in *Dictyostelium* cells. Indicated cells were incubated in the presence of [ $^{\circ}$ H] inositol for 3 days to get a cell density of 6 × 10 $^{\circ}$ /ml. Inositol phosphates generated in individual strains were extracted and analyzed by HPLC as described above.

uct cAMP cannot reach a high enough amount to trigger oscillations (Parent and Devreotes, 1996). Accordingly, the rapid aggregation of  $insp6k^-$  null cells may be caused by an increased sensitivity to low amounts of

chemoattractant cAMP. To explore the sensitivity of chemotaxis, we utilized a small drop chemotaxis assay (Figure 4D). Cells and chemoattractant are placed in drops separated by about 0.5 mm with positive chemo-



Figure 3. cAMP Rapidly and Potently Augments InsP7/InsP8 Levels

(A) The amounts of InsP7 and InsP8 in *D. discoideum* cells increase dramatically during differentiation. Wild-type cells were incubated in the presence of [ $^{\circ}$ H] inositol for 3 days to reach a cell density of 1  $\times$  10 $^{\circ}$ /ml and then developed on DB-agar for indicated time. Inositol phosphates were extracted and analyzed by HPLC as described in Figure 2. All the data were normalized to the total amount of protein extracted from each sample. The results are the means ( $\pm$  SD) of three independent experiments.

(B) The amounts of InsP7 and InsP8 in *D. discoideum* cells increase immediately after chemoattractant stimulation. Wild-type cells were incubated in the presence of [<sup>3</sup>H] inositol for 3 days and then developed in DB for 5 hr. After treated with 20 mM caffeine (an inhibitor of adenylyl cyclase) for 20 min (to bring the cell to a basal state), cells were washed and then stimulated with 10  $\mu$ M cAMP. Cells were lysed at indicated time points and the inositol phosphates were extracted and analyzed by HPLC. HPLC profiles at time 0 and 60 s are presented. (C) The amounts of InsP6, InsP7, and InsP8 were plotted as a function of time after chemoattractant stimulation. The results are the means ( $\pm$  SD) of three independent experiments

(D) The dose curve of cAMP-induced elevation of InsP7 and InsP8. The experiments were performed as described above with indicated amount of cAMP. The results are the means ( $\pm$  SD) of three independent experiments.

taxis evident by a movement of cells toward the side of the drop closest to the cAMP (Konijn, 1970). Positive chemotaxis is demonstrable with 0.1 and 1.0 nM cAMP but is twice as great in *insp6k*<sup>-</sup> as in wild-type cells. Maximal responses are evident at 0.01–10  $\mu$ M concentrations of cAMP, with no differences between *insp6k*<sup>-</sup> and wild-type cells. At 0.1 and 1.0 mM cAMP levels, cAR1 saturation leads to diminished chemotaxis with no difference between wild-type and *insp6k*<sup>-</sup> cells. Our results suggest that *insp6k*<sup>-</sup> cells possess an increased sensitivity to chemoattractant and can respond more strongly than wild-type cells to low concentration cAMP gradients.

## InsP6K Deletion Causes Augmented PH Domain Membrane Translocation

The increased sensitivity of insp6k<sup>-</sup> cells suggests an alternation of chemotactic signaling pathways in these cells. Thus, we examined whether translocation of PH domains to membrane associated PtdIns(3,4,5)P3 is altered in insp6k<sup>-</sup> cells. We used the PH domain of Crac (PH<sub>crac</sub>) fused with green fluorescent protein (PH<sub>crac</sub>-GFP)



Figure 4.  $insp6k^-$  Null Cells Possess a Higher Sensitivity to Chemoattractant cAMP Stimulation

(A) *insp6k*<sup>-</sup> cells aggregate faster than wildtype cells on DB-agar plate. Exponentially growing cells of indicated strains were plated on 1.5% DB agar at  $1 \times 10^6$  cells/cm<sup>2</sup>. Pictures were taken at indicated time points. Fast aggregation phenotype of *insp6k*<sup>-</sup> null could be rescued by transforming a wild-type InsP6K construct back to null cells.

(B) Cell aggregation after 5 hr of starvation was examined with three individual  $insp6k^$ clones as well as a random insertion mutant clone. Within the same clone, each experiment was repeated at least three times and the same results were obtained each time. The figure shows the results of a representative experiment.  $insp6k^-$  cells always aggregate faster than wild-type or the random insertion mutant cells.

(C) The pace of development is not altered in *insp6k*<sup>−</sup> cells. Protein lysates from wild-type or *insp6k*<sup>−</sup> cells were prepared using cells at indicated developmental stages. The expression levels of two developmentally regulated proteins, cAR1 and ACA were examined by Western blot analysis and no big difference was detected. The equal sample loading in each lane was confirmed by Coomassie blue staining of the membrane.

(D) The response of cells to cAMP examined using a small-drop chemotaxis assay. The small-drop assay was performed essentially as described (Konijn, 1970; lijima and Devreotes, 2002). Indicated strains were developed for 5 hr and 100–200 cells were spotted on 1.5% DB agar plates containing 3 mM caffeine. Indicated doses of cAMP solution were

spotted close to the drops of cell. After 30 min incubation, cell drops were examined for chemotaxis. Chemotactic responses were scored as positive if at least twice as many cells were localized at the edge close to the cAMP droplet compared with the opposite edge. For each cAMP concentration tested, the percentage of populations judged to have responded is plotted versus the molar cAMP concentration. At the  $10^{-9}$  and  $10^{-10}$  M cAMP concentration, *insp6k*<sup>-</sup> cells possess a higher sensitivity compared to wild-type (p < 0.01, Student's t test). At least 100 cell drops were examined for each cAMP concentration. The results are the mean of three independent experiments. Bars indicated mean  $\pm$  SEM.

as a marker for this event. Crac is a regulator of ACA whose deletion leads to a dramatic decrease of adenylyl cyclase activity in amoebae. Crac protein possesses a PH domain which enables it to bind with high-affinity to PtdIns(3,4,5)P3/PtdIns(3,4)P2 in the cell membrane (Huang et al., 2003). During cAMP-induced chemotaxis, Crac translocates to the leading edge of cells, closest to the point of chemoattractant application (Parent et al., 1998). This system has been utilized in previous studies and PH<sub>Crac</sub>-GFP has been proved to be an authentic representative of typical PtdIns(3,4,5)P3/PtdIns(3,4)P2 specific PH domains (Parent et al., 1998; lijima and Devreotes, 2002; Huang et al., 2003).

While PH<sub>Crac</sub>-GFP translocates to the leading edge of chemotaxing cells in cAMP gradient, during uniform treatment with chemoattractant it transiently translocates from cytosol to the plasma membrane (Parent et al., 1998). We explored the translocation of PH<sub>Crac</sub>-GFP to the membrane of intact amoebae in response to uniform stimulation (Figures 5A–5B). With a saturating concentration of cAMP (1  $\mu$ M), membrane translocation of PH<sub>Crac</sub>-GFP occurs instantaneously and peaks within 6–8 s. By 16 s, the original uniform cytoplasmic distribu-

tion is reestablished by an unknown adaptation mechanism. Membrane translocation is augmented in insp6k<sup>-</sup> cells, while the time course for the increase and subsequent decrease in translocation is not altered.

The PH<sub>Crac</sub>-GFP translocation was quantified by measuring membrane-associated PH<sub>Crac</sub>-GFP by Western blotting analyses (Figures 5C–5D). At every time point and every cAMP concentration tested, including the basal level, the amount of membrane-associated PH<sub>Crac</sub>-GFP in *insp6k*<sup>-</sup> cells is higher than that which is in wildtype cells. At 0.1 nM cAMP, the extent of translocation in *insp6k*<sup>-</sup> cells is more than doubled while somewhat lesser augmentations are evident at 1.0 nM and 10 nM cAMP. Concentration-response relationships for cAMP regulation of PH<sub>Crac</sub>-GFP translocation and of chemotaxis are quite similar (see Figure 4). Augmentation of PH<sub>Crac</sub>-GFP translocation in *insp6k*<sup>-</sup> cells parallels and may account for the augmentation of cAMP-elicited chemotaxis in these cells.

Increased PH<sub>Crac</sub>-GFP translocation could be caused by elevated levels of PtdIns(3,4,5)P3 in plasma membrane. Accordingly, we monitored the formation of phosphatidylinositol in cells exposed to <sup>32</sup>P (Figure 5E). Basal



Figure 5. PH<sub>Crac</sub>-GFP Translocation Is Enhanced, but the Levels of PtdIns(3,4,5)P3 are Not Altered in *insp6k*<sup>-</sup> Cells Compared with Wild-Type Cells

(A) Fluorescent images of cAMP stimulated cells. Wild-type or  $insp6k^-$  cells expressing PH<sub>crac</sub>-GFP were developed for 5 hr and examined for PH<sub>crac</sub>-GFP membrane translocation by stimulation with 1  $\mu$ M cAMP (Parent et al., 1998). The images were captured at each of the indicated time points.

(B) Quantitative analysis of the chemoattractant-mediated translocation of  $PH_{Crac}$ -GFP. The average membrane fluorescence intensities in (A) were measured with IPLab software as previously described (lijima and Devreotes, 2002). Intensities from "0 sec" frame was subtracted from each of the other frames and plotted as a function of time after chemoattractant stimulation. The results are the means of seven wild-type or *insp6k*<sup>-</sup> cells in independent experiments.

(C) Western blot analysis of membrane associated  $PH_{Crac}$ -GFP. Suspensions of developed wild-type or *insp6k*<sup>-</sup> cells were stimulated with indicated dosage of cAMP as described above. At each of the indicated time points, aliquots of cells were filter-lysed into ice-cold PM buffer and the membrane fractions were collected by microcentrifugation. The membrane associated  $PH_{Crac}$ -GFP was analyzed by Western blotting using anti-GFP antibody. The amount of  $PH_{Crac}$ -GFP in total cell lysate was also examined to evaluate the expression level of recombinant  $PH_{Crac}$ -GFP. Essentially, the same amount of  $PH_{Crac}$ -GFP fusion protein was expressed in wild-type and *insp6k*<sup>-</sup> cells.

(D) Quantification of Western blot signals detected in (C). The relative amounts of the membrane associated  $PH_{Crac}$ -GFP were quantified using NIH Image software. All samples were normalized to the signal detected for unstimulated (time point 0) wild-type cells. Data presented are the means ( $\pm$  SD) of three independent experiments.

(E) Changes in the level of PtdIns(3,4,5)P3 in response to chemoattractant. After metabolically labeled with <sup>32</sup>P for 1 hr, the indicated developed cells were stimulated with 1  $\mu$ M cAMP. At each indicated time point, the reaction was stopped and the lipids were extracted and analyzed by TLC as described previously (Huang et al., 2003). The figure shows the result of a representative experiment that was repeated three times.

and stimulated levels of PI, PIP2 and PtdIns(3,4,5)P3 are the same in *insp6k*<sup>-</sup> cell and wild-type cells. As previously reported (Huang et al., 2003), PtdIns(3,4,5)P3 level is elevated in cells with deletion of PTEN and is almost undetectable in *pi3k1-/pi3k2*- double null cells.

## Competition by InsP7 for Crac Binding to PtdIns(3,4,5)P3 Diminishes Crac Membrane Translocation

The augmented PH<sub>Crac</sub>-GFP translocation in insp6k<sup>-</sup> cells suggests that InsP7 might physiologically inhibit PH domain translocation. Inositol phosphates are hydrophilic molecules and cannot pass the plasma membrane. To examine their influence on  $\mathsf{PH}_{\mathsf{Crac}}\text{-}\mathsf{GFP}$  translocation, we utilized an in vitro cell free system, employing GTP<sub>y</sub>S to directly stimulate G proteins thereby activating PI3 kinase to synthesize PtdIns(3,4,5)P3 and trigger PH domain translocation (Huang et al., 2003). This system enabled us to include inositol phosphates in the reaction samples and monitor their ability to inhibit stimulation by GTP<sub>Y</sub>S of membrane-associated PH<sub>Crac</sub>-GFP by Western blotting analyses (Figure 6A). Ins(1,3,4,5)P4 and InsP7 inhibit translocation with an IC50 of about 1 µM, while InsP6 is only 1%-2% as potent and inositol hexasulfate (IS6) is essentially inactive.

To monitor directly the binding of Crac to PtdIns(3,4,5)P3, we incorporated PtdIns(3,4,5)P3 in liposomes which were incubated with purified recombinant  $PH_{Crac}$ -GFP fusion protein, and the amount of  $PH_{Crac}$ -GFP monitored by Western blotting analyses in centrifuged liposomes (Figure 6B). In this system, InsP7 inhibits  $PH_{Crac}$ -GFP binding to PtdIns(3,4,5)P3 with an IC50 of about 50 nM, while InsP6 is about 2% as potent and IS6 inactive. Consistent with previously reported (Venkateswarlu et al., 1998), Ins(1,3,4,5)P4 also possesses a stronger inhibition effect than InsP6.

In another approach, we monitored the binding of purified recombinant  $PH_{Crac}$ -GFP to [<sup>3</sup>H]Ins(1,3,4,5)P4 as the disposition of its phosphate groups mimics PtdIns(3,4,5)P3 (Figure 6C). We monitored binding by coprecipitation of [<sup>3</sup>H]Ins(1,3,4,5)P4 with  $PH_{Crac}$ -GFP fusion protein. Like the liposome findings, InsP7 has a similar effect as Ins(1,3,4,5)P4 and inhibit binding with an IC50 of about 60 nM, while InsP6 is about 3% as potent and IS6 inactive. The IC50 of InsP7 and InsP6 obtained using crude cell lysate (Figure 6A) is somewhat higher than what is observed using purified  $PH_{Crac}$ -GFP (Figures 6B and 6C). This might reflect binding by inositol phosphates in cell lysates to a variety of targets or their degradation, lowering the concentration of free inositol phosphates.

## InsP7 Competes for Binding of the PH Domains of Several Mammalian Proteins to PtdIns(3,4,5)P3

We wondered whether the competition by InsP7 for CRAC binding to PtdIns(3,4,5)P3 involves selectively the PH domain of CRAC or might be a relatively universal phenomenon evident with other PH domain containing proteins. Accordingly, we explored competition for PH domains of 3 mammalian proteins, Akt, PIKE and TIAM. As in the experiments with Crac, we monitored the binding of purified recombinant  $PH_{Akt}$ ,  $PH_{PIKE}$ , and  $PH_{TIAM}$  to [<sup>3</sup>H]Ins(1,3,4,5)P4 whose phosphate group disposition

mimics PtdIns(3,4,5)P3 (Figure 7A). For Akt and TIAM, the relative potencies of InsP7, InsP8, and IS6 in competing for [<sup>3</sup>H]Ins(1,3,4,5)P4 are similar to the Crac PH domain. InsP7 is most potent, with similar concentration-response relationships as Ins(1,3,4,5)P4, InsP6 is about 3% as potent and IS6 is virtually inactive. The behavior of PIKE is somewhat similar, though InsP7 is less potent than InsP4 and only a few fold more potent than InsP6.

We also utilized liposomes to evaluate the binding of PH<sub>Akt</sub>, PH<sub>PIKE</sub> and PH<sub>TIAM</sub> to PtdIns(3,4,5)P3. As in the experiments with Crac, we incorporated PtdIns(3,4,5)P3 into liposomes incubated with purified, recombinant PH<sub>Akt</sub>, PH<sub>PIKE</sub> and PH<sub>TIAM</sub> and monitored the amount of the PtdIns(3,4,5)P3 associated proteins by Western blots of centrifuged liposomes (Figure 7B). The absolute and relevant potencies of the various inositol derivatives are essentially the same as in the experiments utilizing [<sup>3</sup>H]Ins(1,3,4,5)P4.

In summary, InsP7 potently and selectively competes for interactions with PtdIns(3,4,5)P3 of PH domains of several mammalian proteins, raising the possibility that InsP7 competition is a universal regulatory mechanism.

## PH<sub>Crac</sub> Associates with Inositol Phosphates In Vivo

To explore interactions of inositol phosphates with Crac in intact cells, we transformed cells with a construct expressing PH<sub>Crac</sub>-GFP, incubated them with [<sup>3</sup>H]inositol and examined levels of radiolabeled inositol phosphates in immunoprecipitated PH<sub>Crac</sub>-GFP (Figures 8A–8B). [<sup>3</sup>H]InsP6, [<sup>3</sup>H]InsP7, and [<sup>3</sup>H]InsP8 associate with precipitated PH<sub>Crac</sub>-GFP, while these substances do not precipitate in the presence of preimmune serum or in cells expressing GFP not associated with PH<sub>Crac</sub>. The supernatant of immunoprecipitated Crac contains substantial levels of [<sup>3</sup>H]InsP6 but relatively low levels of InsP7 and InsP8 in contrast to the much greater levels of InsP7 and InsP8 compared to InsP6 in the immunoprecipitate. This finding further substantiates the selective association of InsP7 and InsP8 with PH<sub>Crac</sub>.

## InsP6K Deletion Rescues the Aggregation Defect Caused by a PI3 Kinase Inhibitor

The ability of InsP7 to compete with PtdIns(3,4,5)P3 for binding to PH<sub>Crac</sub> in multiple models strongly suggests that the augmented PH domain translocation, rapid aggregation, and the enhanced sensitivity of chemotaxis in *insp6k*<sup>-</sup> cells reflect loss of InsP7 which would physiologically inhibit these processes. Inhibition of PtdIns(3,4,5)P3 formation by PI3 kinase inhibitors leads to defects in chemotaxis and aggregation (Funamoto et al., 2001). Accordingly, we evaluated the influence of InsP6K deletion on the aggregation defect caused by LY294002, a PI3 kinase inhibitor (Figure 8C). In order to ensure that LY294002 specifically inhibit PI3 kinase and does not affect InsP7/InsP8 level, we measured InsP7/ InsP8 in both LY294002-treated and untreated cells and no difference was detected (data not shown).

At 9 hr after starvation in the absence of drug, streaming toward aggregation centers is complete in wild-type cells. LY294002 substantially delays starvation-induced aggregation. We employed 150  $\mu$ M of the drug, because in agar plates many drugs penetrate poorly into *D. dis*-





(A) GTP- $\gamma$ -S induced PH<sub>Crac</sub>-GFP membrane translocation is inhibited by InsP7. Wild-type cells were filter-lysed and PH<sub>Crac</sub>-GFP membrane translocation was induced by incubating the cell lysates with a supernatant containing PH<sub>Crac</sub>-GFP fusion protein in the presence or absence of 10  $\mu$ M GTP- $\gamma$ -S (lijima and Devreotes, 2002). Indicated dosages of IS6, InsP6, InsP7, or Ins(1,3,4,5)P4 were included in the above incubation mixtures to assess their ability to block the PH<sub>Crac</sub>-GFP translocation. After 5 min incubation, the membrane fractions were isolated and membrane associated PH<sub>Crac</sub>-GFP protein was analyzed by Western blotting using anti-GFP antibody (left image). The GTP- $\gamma$ -S treated samples were normalized to those of without treatment and this fold increase was plotted versus the molar concentration of indicated drug used in each sample (right image). Data presented are the means (±SD) of three independent experiments.

(B) Cosedimentation of  $PH_{Crac}$ -GFP fusion protein and PtdIns(3,4,5)P3 containing lipid vesicles is blocked by InsP7. The assay was performed in the presence of indicated amount of IS6, InsP6, InsP7, or Ins(1,3,4,5)P4. Vesicle-associated  $PH_{Crac}$ -GFP fusion protein was pulled-down by centrifugation and visualized (left image) and quantified as described above. Percentage of the initial binding in the absence of drug was plotted versus the molar concentration of indicated drug used in each sample (right image). Data presented are the means (±SD) of three independent experiments.

(C) InsP7 competes with Ins(1,3,4,5)P4 for  $PH_{Crac}$ -GFP binding. The binding was performed in a mixture consisting of 20 nM purified  $PH_{Crac}$ -GFP fusion protein, 10 nM [H3] Ins(1,3,4,5)P4, and indicated amount of IS6, InsP6, Ins(1,3,4,5)P4, or InsP7 as a competitor. InsP4-PH<sub>Crac</sub>-complex was precipitated by polyethylene glycol. The number of counts in each pellet was determined by scintillation counting and plotted versus the concentration of indicated drug used in the corresponding binding reaction. Data presented are the means (±SD) of three independent experiments.

*coideum* amoebae (Williams et al., 1999). In the presence of this drug, streaming of wild-type cells is not evident until 11 hr after starvation. This delay is largely reversed in *insp6k*<sup>-</sup> cells. Transformation of the cells with wildtype InsP6K gene reinstitutes the LY294002-induced delay in aggregation. These findings indicate that loss of InsP7 in the *insp6k*<sup>-</sup> null cells diminishes physiologic competition by InsP7 for PtdIns(3,4,5)P3 influences on aggregation. We have replicated these results in liquid culture in which a lower concentration of LY294002 (40  $\mu$ M) can be employed (Figure 8D).

### Discussion

In the present study we demonstrate potent, rapid, and selective augmentation of InsP7/InsP8 through the G



Figure 7. InsP7 Is a Competitor for PtdIns(3,4,5)P3 Binding to Several Mammalian PH Domains

(A) InsP7 competes with Ins(1,3,4,5)P4 for PH domain binding, The binding assay was performed as described in Figure 6C. Akt PH domain (PH<sub>Akt</sub>), PIKE PH domain (PH<sub>FIKE</sub>), and TIAM PH domain (PH<sub>TIAM</sub>) were expressed as GST fusion proteins. Data presented are the means ( $\pm$ SD) of three independent experiments.

(B) Cosedimentation of PH domain fusion proteins and PtdIns(3,4,5)P3 containing lipid vesicles is blocked by InsP7. The experiments were performed as described in Figure 6B. PH domain fusion proteins were visualized by Western blotting using anti-GST antibody. Data presented are the means ( $\pm$ SD) of three independent experiments.

protein-coupled cAMP receptor. Also, InsP7 physiologically competes with PtdIns(3,4,5)P3 for binding to PH domains and regulates chemotactic responsiveness (Figure 8E). In  $insp6k^-$  cells, the production of InsP7 and InsP8 is abolished, leading to augmented PH domain membrane translocation, and enhanced sensitivity of null cells to chemoattractant cAMP stimulation.

Levels of InsP7 and InsP8 in *D. discoideum* are about 100 times those of mammalian tissues but the combined levels of InsP7 and InsP8 are still only about half those of InsP6. While InsP6 can also compete with PtdIns(3,4,5)P3 for binding to  $PH_{Crac}$ , it is only about 1%–2% as potent as InsP7 so that the physiologic ligand for these processes is most likely InsP7. We do not know the molar potency of InsP8 in influencing PtdIns(3,4,5)P3-PH<sub>Crac</sub> interactions, as we lacked adequate supplies of unlabeled InsP8 to examine its actions.

Chemotaxis is a process by which cells move up a chemoattractant gradient. A frequent problem in explaining chemotactic events is accounting for the ability of small cells to sharply localize the response to the leading edge of motile cells in minute gradients of a

substance, often less than 10%, between the front and the back of the cells. In D. discoideum and leukocytes, chemotaxis is mediated by G protein-coupled receptors. Receptors are uniformly localized along the cell membrane during chemotaxis and are activated at both the leading and the trailing edge of moving cells with only a slightly higher receptor occupancy at the front (reviewed in Devreotes and Janetopoulos, 2003). Accordingly, the sharply localized response at the leading edge of chemotactic cells cannot be solely explained by "positive signals" elicited by receptor activation. "Negative regulators" presumably suppress the positive responses outside the leading edge, whose strength should match the "positive signals" elicited by receptors and be coregulated by receptor activation. As levels of InsP7, a physiological suppressor of chemotactic signaling, are regulated by receptor occupancy (Figure 3), InsP7 may serve as a negative regulator of chemotaxis.

The pleckstrin homolog domain is a divergent protein module of approximately 120 amino acids found in many proteins involved in signal transduction (Lemmon and Ferguson, 2000). PH domains often mediate protein-pro-



Figure 8. Inositol Polyphosphates Associate with  $PH_{Crac}$  In Vivo

(A) Western blot analysis of PH<sub>Crac</sub>-GFP and GFP. Wild-type cells expressing PH<sub>Crac</sub>-GFP or GFP was cultured in HL5 media containing 100 $\mu$ Ci/ml [<sup>3</sup>H] inositol for 3–4 days to get a cell density of 6 × 10<sup>6</sup>/ml. Cells were filter-lysed and the lysate was incubated with Protein A-Sepharose bound rabbit anti-GFP or preimmune serum for 2 hr. Bound proteins (20% of the pellet) were visualized by immunoblotting with mouse monoclonal anti-GFP antibody.

(B) HPLC analysis of inositol phosphates associate with PH<sub>Crac</sub>. Inositol phosphates were extracted from the immunoprecipitated pellets (80% of the pellet, see above) as well as the supernatant and resolved by HPLC as described in Figure 2. Peaks detected before 30 min are mixtures of various species of free inositol, InsP, InsP2, InsP3, and InsP4.

(C) Disruption of InsP6K rescues the aggregation defect caused by LY294002 on DB agar. Exponentially growing cells of indicated strains were plated on 1.5% DB agar at 1  $\times$  10<sup>6</sup> cells/cm<sup>2</sup>. To examine cell development in the presence of a PI3K inhibitor LY294002, 150  $\mu$ M drug was included in the DB agar.

(D) Cell aggregation in DB buffer. Cells of indicated strains were plated on 6-well plate in DB buffer containing indicated drug.  $6 \times 10^6$  cells were plated in each well. In liquid buffer, the aggregation of wild-type cells was blocked by 40  $\mu$ M LY294002. *insp6k*<sup>-</sup> cells possess a resistance to the same amount of drug.

(E) Proposed model of the role of InsP7 in negatively regulating PH domain-containing protein functions during chemotaxis. Please refer to "Discussion" for a detailed discussion of the proposed model. Briefly, upon receptor activation by a chemoattractant cAMP, G protein is dissociated into  $\alpha$  and  $\beta\gamma$  subunits. The  $\beta\gamma$  subunit will regulate PI3K and PTEN activity and trigger an instant increase of membrane PtdIns(3,4,5)P3 production. A set of PH domain-containing proteins are then translocated onto the membrane through their specific binding with PtdIns(3,4,5)P3 and subsequently activate various signal transduction pathways mediating chemotaxis. Since InsP7 can compete with PtdIns(3,4,5)P3 for PH domain binding, high concentration of InsP7 will occupy more PH domain-containing proteins and prevent their recruiting onto the membrane and consequently inhibit their activation. On the contrary, low concentration of InsP7 will leave more proteins available for binding to PtdIns(3,4,5)P3 and therefore enhance their activation. Thus, the sensitivity of cells to cAMP stimulation can be modulated by regulating the intracellular concentration of InsP7.

tein or protein-phosphoinositide interactions (Lemmon and Ferguson, 2000). A subset of PH domains, including those in Btk, Akt/PKB, PLC- $\gamma$ , Gab1, and Grp1, drive membrane translocation of their host proteins through specific, high-affinity recognition of Ptdlns(3,4,5)P3 and Ptdlns(3,4)P2, the products of PI3K (Cantley, 2002). This membrane translocation is crucial for these proteins to fulfill their functions in PI3K-mediated physiological processes such as cell survival, growth, differentiation, polarization, chemotaxis, and membrane trafficking (Hemmings, 1997). Translocation of PH domains was previously thought to be dependent solely upon concentrations of PtdIns(3,4,5)P3 in the membrane (Maehama and Dixon, 1999; Cantley, 2002). Our study provides another level of regulation for this membrane translocation process, namely relative levels of InsP7 and PtdIns(3,4,5)P3.

*D. discoideum* Crac protein binds to PtdIns(3,4,5)P3 and PtdIns(3,4)P2 through its PH domain and in this way behaves very much like many other PH-domain proteins in mammalian as well as other species (Hemmings, 1997; Downward, 1998). Our observation that InsP7 is a physiologic regulator of interactions of  $PH_{Crac}$  with PtdIns(3,4,5)P3 may be indicative of a general phenomenon. We have demonstrated similar competition by InsP7 with PtdIns(3,4,5)P3 for binding to several mammalian PH-domain containing proteins including PIKE, Akt, and Tiam.

Numerous instances of inositol phosphate binding to regulatory proteins have been reported. Several Ins(1,3,4,5)P4 binding proteins, including GAP1<sup>IP4BP</sup>, α-centaurin, GAP1<sup>m</sup>, and p42(IP4), interact physiologically with PtdIns(3,4,5)P3) (Cullen et al., 1995; Hammonds-Odie et al., 1996). Most inositol polyphosphate binding proteins have been characterized in terms of their binding to InsP6 with numerous reports of clathrinassociated proteins such as AP2 and AP180 binding to InsP6 (Voglmaier et al., 1992; Norris et al., 1995; Ye et al., 1995). Binding of InsP6 to AP180 can negatively regulate clathrin cage assembly activity (Norris et al., 1995; Ye et al., 1995). InsP6 also binds to several other proteins associated with the turnover of synaptic and other vesicles populations including synaptotagmin, protein 4.1, ß arrestin, Golgi coatomer, and yeast coatomer (Ali et al., 1995; Mehrotra et al., 1997) Furthermore, InsP6 can bind a cell-matrix protein Syndecan-4 and regulate its function by competing for its binding with PtdIns(4,5)P2 (Couchman et al., 2002). InsP6 also binds the Ku70 subunit of the DNA-PK holoenzyme and is required for DNA-PK mediated nonhomologous DNA end joining (NHEJ) (Hanakahi and West, 2002; Ma and Lieber, 2002). In the case of AP180, InsP7 binds with substantially higher affinity than InsP6 (Ye et al., 1995; Saiardi et al., 2002). In most other instances, InsP7 and InsP8 have not been evaluated for binding potencies, simply because samples of pure InsP7 and InsP8 have not been readily available. We suggest that many presumed InsP6 binding proteins are physiologically associated with InsP7 and/or InsP8. Moreover, in a number of cases PtdIns(3,4,5)P3 or PtdIns(4,5)P2 might be physiologic ligands for proteins characterized as InsP6 binding proteins with InsP7 and InsP8 acting as physiologic modulators, analogous to PtdIns(3,4,5)P3-InsP7 competition for Crac. Alternatively, InsP7 might be the physiologic ligand whose interactions are modulated by PtdIns(3.4.5)P3.

In the present study, we established InsP7 and InsP8 as intracellular signaling molecules and demonstrated that their production in the cells is highly regulated. During D. discoideum development, a 25-fold accumulation of InsP7 and InsP8 was observed by both massmeasurement (Laussmann et al., 2000) and a [3H]-inositol labeling. Moreover, InsP7 and InsP8 level in developed D. discoideum cells can be rapidly and potently augmented by cAMP through a G protein-coupled cAMP receptor. In mammalian systems, InsP7 and InsP8 are also dynamic molecules with very rapid turnover rates (Menniti et al., 1993). Depleting Ca<sup>2+</sup> stores with thapsigargin elicits a rapid decrease in InsP7 and InsP8 levels in primary cultured hepatocytes by an unknown mechanism (Glennon and Shears, 1993). Turnover of InsP8 can also be regulated by  $\beta$ -adrenergic receptors through a cAMP-mediated mechanism in a smooth muscle cell line (Safrany and Shears, 1998). Recently, an increase of InsP6K kinase activity (Morrison et al., 2001), as well as a dramatic elevation of intracellular InsP7 and InsP8 level (H.R.L. and S.H.S., unpublished data), was detected during apoptotic cell death in ovarian carcinoma cells. All these findings are consistent with roles of InsP7 and InsP8 as highly regulated cellular messenger molecules.

The regulation of the production of InsP7 and InsP8 is still not fully understood and conceivably involves the modulation of the enzymatic activities of InsP6K or/and inositol phosphate phosphatases by a variety of intracellular or extracellular stimuli. A family of diphosphoinositol polyphosphate phosphatases (DIPPs) has been identified and cloned recently, may be responsible for removal of the  $\beta$ -phosphate from the pyrophosphates in InsP7 and InsP8 (Caffrey et al., 2000). Cellular and molecular mechanisms controlling the relevant kinases and phosphatases remain to be investigated.

#### **Experimental Procedures**

#### Gene Disruption and Rescue

The D. discoideum strain Ax-2 cells were cultured axenically in HL5 medium at 22°C. Cell maintenance and transformation was carried out as previously described (lijima and Devreotes, 2002). To construct the InsP6K (I6KA gene) disruption vector, the Dictyostelium InsP6K genomic DNA was amplified by PCR and inserted into a pBluescript vector between Sall and Notl sites. An EcoR1 site, as well as an adjacent Hind III site, was engineered in the middle of the genomic DNA and a blasticidin S resistance (BSR) cassette was then inserted between these two sites. Gene disruption by homoloas recombination and PCR-based positive colony screening was performed as described previously by lijima et al. (2002). To rescue the insp6k- null (i6kA-/Ax2) phenotypes, the full-length InsP6K cDNA was amplified by PCR from a Dictyostelium 3' RACE cDNA library, which was kindly provided by Dr. Tian Jin. The InsP6K ORF was subcloned into the BgIII and Notl site of a Dictyostelium expression vector pJK1 and the resulting plasmid was then transformed into insp6k<sup>-</sup> null cells by electroporation. Southern Blot, Northern Blot, and Western Blot analyses were performed essentially as described (Luo et al., 2001).

#### InsP6 Kinase Assay

Ax2 cells were developed on DB-agar for 5 hr. 5  $\times$  10<sup>6</sup> cells were homogenized in 500  $\mu$ l lysis buffer (20 mM HEPES [pH 6.8], 100 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.1% CHAPS, 5 mM DTT, 15% Glycerol, 5  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 6  $\mu$ g/ml chymostatin, 0.7  $\mu$ g/ml pepstatin, and 1 mM PMSF) and centrifuged at 10,000  $\times$  g for 20 min to remove insoluble materials. The resulting lysate was used for InsP6 kinase assay as described previously (Saiardi et al., 2001).

#### Radiolabeling and Detection of Inositol Phosphates in Intact Cells

Ax2 cells were cultured in HL5 media containing 100 $\mu$ Ci/ml [<sup>3</sup>H]inositol (NEN, Boston, MA). Cell culture (2 ml) were seeded at 10<sup>5</sup> cells/ml and grown at 22°C for 3–4 days to get a cell density of 6  $\times$  10<sup>6</sup>/ml. Cells were harvested and washed twice with ice-cold DB buffer. Inositol phosphates were extracted and resolved by HPLC as described previously (Saiardi et al., 2002).

#### Ins(1,3,4,5)P4 Binding Assay

The ability of InsP6 or InsP7 to compete with Ins(1,3,4,5)P4 for binding to PH<sub>Crac</sub> was assessed using a polyethylene glycol precipitation procedure. The PH<sub>Crac</sub>-GFP fusion protein used in this study was prepared as previously described (Huang et al., 2003). Akt PH domain (PH<sub>Akt</sub>), PIKE PH domain (PH<sub>PIKE</sub>), and TIAM PH domain (PH<sub>TIAM</sub>) were expressed as GST fusion proteins and were purified as previously described (Wang et al., 1997; Ye et al., 2000). The binding mixtures consist of 20 nM purified PH-GFP fusion protein,

10 nM [<sup>3</sup>H] Ins(1,3,4,5)P4 (NEN, Boston, MA), 20 mM Tris [pH 7.5], 100 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, 5 mg/ml  $\gamma$ -globulin, and indicated amount of InsP6 or InsP7 as a competitor. A total volume of 50  $\mu$ l binding mixture was incubated for 10 min at 4°C. InsP4-PH domain complex was precipitated by the addition of 35  $\mu$ l ice-cold polyethylene glycol (Mr 3,350). After 10 min incubation at 4°C, samples were centrifuged for 10 min at 10,000 g and the supernatants were aspirated. The pellets were solubilized in 1 ml 1% SDS and 8 ml LCS-cocktail. The number of counts was determined for each sample by scintillation counting.

## Cosedimentation Assays for PH Domain Binding to Lipid Vesicles

Ptdlns(3,4,5)P3 containing lipid vesicles were prepared by mixing phosphatidylcholine, phosphatidylethanolamine, and Ptdlns(3,4,5)P3 at the proportions of 40:10:1, drying the mixture under nitrogen gas, and resuspending to a final concentration of 0.2 mg total phospholipid/ml in liposome buffer (50 mM HEPES [pH 7.25], 100 mM NaCl, and 0.5 mM EDTA). Resuspended lipids were sonicated in a bath sonicator until a homogeneous suspension was formed (about 5 min). Lipid vesicles were collected by centrifugation at 50,000 g for 10 min and were resuspended to a final concentration of 3 mg/ ml in liposome buffer. Binding assay was performed in a total volume of 500  $\mu$ l PM buffer containing 40  $\mu$ l lipid vesicle suspension, 5  $\mu$ g purified recombinant PH domain fusion protein, and indicated amount of InsP6, InsS6, or InsP7. Vesicle/protein mixtures were incubated on ice for 2 hr, collected, washed, and the vesicle associated PH domain fusion protein was analyzed by Western blot.

#### Acknowledgments

The authors thank Tian Jin for providing the RACE cDNA library, Linnan Tang for providing anti-GFP antibody, Linfeng Chen and Chris Janetopoulos for stimulating discussions. This work was funded by USPHS grants MH-18501 to S.H.S., GM-28007 to P.N.D., and Research Scientist grant DA-00074 to S.H.S.

Received: March 18, 2003 Revised: July 14, 2003 Accepted: July 17, 2003 Published: September 4, 2003

#### References

Ali, N., Duden, R., Bembenek, M.E., and Shears, S.B. (1995). The interaction of coatomer with inositol polyphosphates is conserved in Saccharomyces cerevisiae. Biochem. J. *310*, 279–284.

Caffrey, J.J., Safrany, S.T., Yang, X., and Shears, S.B. (2000). Discovery of molecular and catalytic diversity among human diphosphoinositol-polyphosphate phosphohydrolases. An expanding Nudt family. J. Biol. Chem. 275, 12730–12736.

Cantley, L.C. (2002). The phosphoinositide 3-kinase pathway. Science 296, 1655–1657.

Chung, C.Y., Funamoto, S., and Firtel, R.A. (2001). Signaling pathways controlling cell polarity and chemotaxis. Trends Biochem. Sci. *26*, 557–566.

Couchman, J.R., Vogt, S., Lim, S.T., Lim, Y., Oh, E.S., Prestwich, G.D., Theibert, A., Lee, W., and Woods, A. (2002). Regulation of inositol phospholipid binding and signaling through syndecan-4. J. Biol. Chem. 277, 49296–49303.

Cullen, P.J., Hsuan, J.J., Truong, O., Letcher, A.J., Jackson, T.R., Dawson, A.P., and Irvine, R.F. (1995). Identification of a specific Ins(1,3,4,5)P4-binding protein as a member of the GAP1 family. Nature *376*, 527–530.

Devreotes, P., and Janetopoulos, C. (2003). Eukaryotic chemotaxis: distinctions between directional sensing and polarization. J. Biol. Chem. *278*, 20445–20448.

Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. Curr. Opin. Cell Biol. *10*, 262–267.

Funamoto, S., Milan, K., Meili, R., and Firtel, R.A. (2001). Role of phosphatidylinositol 3' kinase and a downstream pleckstrin homol-

ogy domain-containing protein in controlling chemotaxis in Dictyostelium. J. Cell Biol. 153, 795–810.

Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R.A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. Cell *109*, 611–623.

Glennon, M.C., and Shears, S.B. (1993). Turnover of inositol pentakisphosphates, inositol hexakisphosphate and diphosphoinositol polyphosphates in primary cultured hepatocytes. Biochem. J. 293, 583–590.

Hammonds-Odie, L.P., Jackson, T.R., Profit, A.A., Blader, I.J., Turck, C.W., Prestwich, G.D., and Theibert, A.B. (1996). Identification and cloning of centaurin-alpha. A novel phosphatidylinositol 3,4,5-trisphosphate-binding protein from rat brain. J. Biol. Chem. 271, 18859– 18868.

Hanakahi, L.A., and West, S.C. (2002). Specific interaction of IP6 with human Ku70/80, the DNA-binding subunit of DNA-PK. EMBO J. *21*, 2038–2044.

Hemmings, B.A. (1997). PtdIns(3,4,5)P3 gets its message across. Science 277, 534.

Huang, K.N., and Symington, L.S. (1995). Suppressors of a Saccharomyces cerevisiae pkc1 mutation identify alleles of the phosphatase gene PTC1 and of a novel gene encoding a putative basic leucine zipper protein. Genetics *141*, 1275–1285.

Huang, C.F., Voglmaier, S.M., Bembenek, M.E., Saiardi, A., and Snyder, S.H. (1998). Identification and purification of diphosphoinositol pentakisphosphate kinase, which synthesizes the inositol pyrophosphate bis(diphospho)inositol tetrakisphosphate. Biochemistry *37*, 14998–15004.

Huang, Y.E., lijima, M., Parent, C.A., Funamoto, S., Firtel, R., and Devreotes, P. (2003). Receptor-mediated regulation of PI3K3 confines PI(3,4,5)P3 to the leading edge of chemotaxing cells. Mol. Biol. Cell *14*, 1913–1922.

lijima, M., and Devreotes, P. (2002). Tumor suppressor PTEN mediates sensing of chemoattractant gradients. Cell *109*, 599–610.

lijima, M., Huang, Y.E., and Devreotes, P. (2002). Temporal and spatial regulation of chemotaxis. Dev. Cell 3, 469–478.

Irvine, R.F., and Schell, M.J. (2001). Back in the water: the return of the inositol phosphates. Nat. Rev. Mol. Cell Biol. 2, 327–338.

Konijn, T.M. (1970). Microbiological assay of cyclic 3',5'-AMP. Experientia 26, 367–369.

Laussmann, T., Pikzack, C., Thiel, U., Mayr, G.W., and Vogel, G. (2000). Diphospho-myo-inositol phosphates during the life cycle of Dictyostelium and Polysphondylium. Eur. J. Biochem. *267*, 2447–2451.

Lemmon, M.A., and Ferguson, K.M. (2000). Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem. J. 350, 1–18.

Luo, H.R., Saiardi, A., Nagata, E., Ye, K., Yu, H., Jung, T.S., Luo, X., Jain, S., Sawa, A., and Snyder, S.H. (2001). GRAB: a physiologic guanine nucleotide exchange factor for Rab3A, which interacts with inositol hexakisphosphate kinase. Neuron *31*, 439–451.

Luo, H.R., Saiardi, A., Yu, H., Nagata, E., Ye, K., and Snyder, S.H. (2002). Inositol pyrophosphates are required for DNA hyperrecombination in protein kinase c1 mutant yeast. Biochemistry *41*, 2509–2515.

Ma, Y., and Lieber, M.R. (2002). Binding of inositol hexakisphosphate (IP6) to Ku but not to DNA-PKcs. J. Biol. Chem. 277, 10756–10759.

Maehama, T., and Dixon, J.E. (1999). PTEN: a tumour suppressor that functions as a phospholipid phosphatase. Trends Cell Biol. 9, 125–128.

Mehrotra, B., Elliott, J.T., Chen, J., Olszewski, J.D., Profit, A.A., Chaudhary, A., Fukuda, M., Mikoshiba, K., and Prestwich, G.D. (1997). Selective photoaffinity labeling of the inositol polyphosphate binding C2B domains of synaptotagmins. J. Biol. Chem. 272, 4237–4244.

Menniti, F.S., Miller, R.N., Putney, J.W., Jr., and Shears, S.B. (1993). Turnover of inositol polyphosphate pyrophosphates in pancreatoma cells. J. Biol. Chem. *268*, 3850–3856.

Morrison, B.H., Bauer, J.A., Kalvakolanu, D.V., and Lindner, D.J.

Norris, F.A., Ungewickell, E., and Majerus, P.W. (1995). Inositol hexakisphosphate binds to clathrin assembly protein 3 (AP-3/AP180) and inhibits clathrin cage assembly in vitro. J. Biol. Chem. 270, 214–217.

Parent, C.A., and Devreotes, P.N. (1996). Molecular genetics of signal transduction in Dictyostelium. Annu. Rev. Biochem. *65*, 411–440. Parent, C.A., Blacklock, B.J., Froehlich, W.M., Murphy, D.B., and Devreotes, P.N. (1998). G protein signaling events are activated at the leading edge of chemotactic cells. Cell *95*, 81–91.

Safrany, S.T., and Shears, S.B. (1998). Turnover of bis-diphosphoinositol tetrakisphosphate in a smooth muscle cell line is regulated by beta2-adrenergic receptors through a cAMP-mediated, A-kinase-independent mechanism. EMBO J. *17*, 1710–1716.

Saiardi, A., Erdjument-Bromage, H., Snowman, A.M., Tempst, P., and Snyder, S.H. (1999). Synthesis of diphosphoinositol pentakisphosphate by a newly identified family of higher inositol polyphosphate kinases. Curr. Biol. 9, 1323–1326.

Saiardi, A., Nagata, E., Luo, H.R., Snowman, A.M., and Snyder, S.H. (2001). Identification and characterization of a novel inositol hexakisphosphate kinase. J. Biol. Chem. *276*, 39179–39185.

Saiardi, A., Sciambi, C., McCaffery, J.M., Wendland, B., and Snyder, S.H. (2002). Inositol pyrophosphates regulate endocytic trafficking. Proc. Natl. Acad. Sci. USA 99, 14206–14211.

Schell, M.J., Letcher, A.J., Brearley, C.A., Biber, J., Murer, H., and Irvine, R.F. (1999). PiUS (Pi uptake stimulator) is an inositol hexakisphosphate kinase. FEBS Lett. *461*, 169–172.

Stephens, L., Radenberg, T., Thiel, U., Vogel, G., Khoo, K.H., Dell, A., Jackson, T.R., Hawkins, P.T., and Mayr, G.W. (1993). The detection, purification, structural characterization, and metabolism of diphosphoinositol pentakisphosphate(s) and bisdiphosphoinositol tetrakisphosphate(s). J. Biol. Chem. *268*, 4009–4015.

Venkateswarlu, K., Gunn-Moore, F., Oatey, P.B., Tavare, J.M., and Cullen, P.J. (1998). Nerve growth factor- and epidermal growth factorstimulated translocation of the ADP-ribosylation factor-exchange factor GRP1 to the plasma membrane of PC12 cells requires activation of phosphatidylinositol 3-kinase and the GRP1 pleckstrin homology domain. Biochem. J. 335, 139–146.

Voglmaier, S.M., Keen, J.H., Murphy, J.E., Ferris, C.D., Prestwich, G.D., Snyder, S.H., and Theibert, A.B. (1992). Inositol hexakisphosphate receptor identified as the clathrin assembly protein AP-2. Biochem. Biophys. Res. Commun. *187*, 158–163.

Wang, D.S., Deng, T., and Shaw, G. (1997). Membrane binding and enzymatic activation of a Dbl homology domain require the neighboring pleckstrin homology domain. Biochem. Biophys. Res. Commun. 234, 183–189.

Williams, R.S., Eames, M., Ryves, W.J., Viggars, J., and Harwood, A.J. (1999). Loss of a prolyl oligopeptidase confers resistance to lithium by elevation of inositol (1,4,5) trisphosphate. EMBO J. *18*, 2734–2745.

Ye, W., Ali, N., Bembenek, M.E., Shears, S.B., and Lafer, E.M. (1995). Inhibition of clathrin assembly by high affinity binding of specific inositol polyphosphates to the synapse-specific clathrin assembly protein AP-3. J. Biol. Chem. *270*, 1564–1568.

Ye, K., Hurt, K.J., Wu, F.Y., Fang, M., Luo, H.R., Hong, J.J., Blackshaw, S., Ferris, C.D., and Snyder, S.H. (2000). Pike. A nuclear GTPase that enhances PI3-kinase activity and is regulated by protein 4.1N. Cell *103*, 919–930.

#### Accession Numbers

The GenBank accession number for *Dictyostelium* InsP6K gene (I6KA) reported in this paper is AY225466.