Eukaryotes possess numerous inositol phosphate (IP) and diphosphoinositol phosphate (PP-IPs or inositol pyrophosphates) species that act as chemical codes important for intracellular signaling pathways. Production of IP and PP-IP molecules occurs through several classes of evolutionarily conserved inositol phosphate kinases. Here we report the characterization of a human inositol hexakisphosphate (IP₆) and diphosphoinositol pentakisphosphate (IP₇) kinase with similarity to the yeast enzyme Vip1, a recently identified IP₆/IP₇ kinase (Mulugeta, S., Bai, W., Fridy, P. C., Bastidas, R. J., Otto, J. C., Dollins, D. E., Haystead, T. A., Ribeiro, A. A., and York, J. D. (2007) Science 316, 106–109). Recombinant human VIP1 exhibits in vitro IP₆ and IP₇ kinase activities and restores IP₇ synthesis when expressed in mutant yeast. Expression of human VIP1 in HEK293T cells engineered to produce high levels of IP₆ results in dramatic increases in bisdiphosphoinositol tetrakisphosphate (PP₂·IP₄ or IP₇ₐ). Northern blot analysis indicates that human VIP1 is expressed in a variety of tissues and is enriched in skeletal muscle, heart, and brain. The subcellular distribution of tagged human VIP1 is indicative of a cytoplasmic non-membrane localization pattern. We also characterized human and mouse VIP2, an additional gene product with nearly 90% similarity to VIP1 in the kinase domain, and observed both IP₆ and IP₇ kinase activities. Our data demonstrate that human VIP1 and VIP2 function as IP₆ and IP₇ kinases that act along with the IP₆K/Kcs1-class of kinases to convert IP₆ to IP₇ in mammalian cells, a process that has been found to occur in response to various stimuli and signaling events.

Inositol phosphates (IPs)² are a diverse group of regulatory molecules involved in a variety of intracellular signaling pathways. Stimulation of cells results in the production of IPs through the cleavage of phosphoinositides by phosphoinositide-specific phospholipase C (1–3). Many additional IPs, such as inositol tetrakisphosphate (IP₄), inositol pentakisphosphate (IP₅), and inositol hexakisphosphate (IP₆), are generated through the subsequent action of several classes of evolutionarily conserved inositol phosphate kinases (IPKs) (2, 4–7). Genetic and biochemical studies of the IPKs have linked their IP products to processes including metal chelation, ion channel regulation, transcription, chromatin remodeling, nuclear mRNA export, apoptosis, RNA editing, phosphate sensing, auxin signaling, and proper organism development (reviewed in Refs. 1–4, 6, 8).

Soluble IP molecules also serve as precursors to several species of diphosphoinositol phosphates (PP-IPs), commonly referred to as inositol pyrophosphates. PP-IPs were first reported and characterized in Dictyostelium discoideum and mammalian cells and are distinguished by the presence of one or more pyrophosphate groups on the inositol ring (9–11). Two classes of evolutionarily conserved kinases have been identified as required for the production of PP-IP molecules. An IP₆ kinase (IP6K) class of enzymes, Kcs1 in Saccharomyces cerevisiae and IHPK1, IHPK2, and IHPK3 in mammals, was found to convert IP₆ to diphosphoinositol pentakisphosphate, also known as PP₂·IP₄ or IP₇, (12, 13), and to phosphorylate IP₇ to produce PP₂·IP₅. Loss of Kcs1 in budding yeast results in defects in the response to osmotic stress, regulation of telomere length, vacuolar biogenesis, endocytosis, and other cellular processes (13–18). PP₂·IP₅ has also been shown in vitro to act as a phosphate donor, capable of phosphorylating proteins directly, in a non-enzymatic process (19); however, evidence that this occurs in cells awaits further study (20). Recent work has also indicated that IP6K activity participates in the synthesis of bisdiphosphoinositol tetrakisphosphate (PP₂·IP₄ or IP₇ₐ), a more highly phosphorylated PP-IP species containing two pyrophosphate groups. Studies have demonstrated that PP₂·IP₄ levels change in response to osmotic and heat stress in both yeast and mammalian cells and may be regulated in part by the MAP kinase pathway (21–23). PP₂·IP₅, along with PP₂·IP₄, also appears to have a role in certain cAMP-mediated signaling events, including chemotaxis in D. discoideum, with lev-
els of these metabolites significantly altered during cAMP signaling (21, 24, 25).

A second class of IP₆/IP₇ kinase has been recently reported in budding yeast, termed Vip1. This activity was identified as inositol pyrophosphate synthase IP₅K based on the accumulation of a novel PP-IP₇ in yeast mutants lacking both Kcs1/IP₆K activity and the diphosphoinositol phosphate phosphatase Ddp1 (kcs1Δ/Δddp1Δ) (16). Biochemical purification of the IP₆ kinase activity from a kcs1Δ/ddp1Δ double knock-out yeast strain led to the cloning of Vip1 (26). Prior to the discovery that Vip1 possessed intrinsic IP₆ kinase activity, its Schizosaccharomyces pombe ortholog Asp1 was found to exhibit genetic interactions with actin-related proteins 2/3 (Arp2/3), indicating that it may function as a regulator of actin polymerization and cytoskeletal function (27). Mulugu et al. demonstrated that IP₆ kinase activity was required for certain genetic interactions and proper cell morphology (26). In addition, through phosphorus NMR analysis of the products of recombinant Vip1 and Kcs1/IP₆K, it was shown that distinct PP-IP₅ species are produced (26). Given the unique activities of Vip1 and Kcs1/IP₆K, it was shown that both enzymes also act as PP-IP₇ kinases, and together, these enzymes are capable of phosphorylating IP₆ to generate PP₂-IP₄ (26).

Here we report the identification, cloning, and characterization of two human VIP1-like gene products. Our data indicate that human VIP1 and VIP2 act as both IP₆ and PP-IP₅ kinases whose products have been implicated in a wide array of signal transduction pathways. Our work represents a significant advance for understanding the increasingly evident signaling roles of inositol pyrophosphates.

**MATERIALS AND METHODS**

Construction of Plasmids—The cDNA for human VIP1 (accession number BC057395) and human VIP2 (BC024591) were obtained from Open Biosystems (Huntsville, AL). Constructs were prepared for bacterial expression of full-length human VIP1 (hsVIP1), hsVIP2, and the kinase domains of hsVIP1 between residues 1 and 387 (hsVIP1-KD) and hsVIP2 between residues 1 and 393 (hsVIP2-KD). For hsVIP1, these were constructed by installing Sall sites at the 5′- and 3′-ends of the coding regions via PCR and subcloning into pGEX-KG and psr1 between residues 1 and 393 (hsVIP1-KD) and hsVIP2 and the kinase domains of hsVIP1 between residues 1 and 387 (hsVIP1-KD) and hsVIP2 between residues 1 and 393 (hsVIP2-KD). For hsVIP1, these were generally incubated 20–60 min at 37 °C and stored at −80 °C.

**Assays of VIP1 IP₆ and IP₇ Kinase Activity**—Enzyme assays were typically run in 10-μl reactions containing 50 mM HEPES (pH 6.2), 1 mM ATP, 5 mM MgCl₂, and ~40,000 cpm of ³²P-P-IP₅ (³²P]IP₇) was also produced enzymatically, in 10-μl reactions with 1.7 pmol of ³²P]IP₅ or ³²P]IP₇. For kinetic analyses, IP₆ and IP₇ concentrations ranged from 20 nM to 10 μM. Reactions were generally incubated 20–60 min at 37 °C and stored at −80 °C.

Human VIP1-like IP₆/IP₇ Kinases

| Page | Section | Text |
|------|---------|------|
| 1 | Introduction | A second class of IP₆/IP₇ kinase has been recently reported in budding yeast, termed Vip1. This activity was identified as inositol pyrophosphate synthase IP₅K based on the accumulation of a novel PP-IP₇ in yeast mutants lacking both Kcs1/IP₆K activity and the diphosphoinositol phosphate phosphatase Ddp1 (kcs1Δ/Δddp1Δ) (16). Biochemical purification of the IP₆ kinase activity from a kcs1Δ/ddp1Δ double knock-out yeast strain led to the cloning of Vip1 (26). Prior to the discovery that Vip1 possessed intrinsic IP₆ kinase activity, its Schizosaccharomyces pombe ortholog Asp1 was found to exhibit genetic interactions with actin-related proteins 2/3 (Arp2/3), indicating that it may function as a regulator of actin polymerization and cytoskeletal function (27). Mulugu et al. demonstrated that IP₆ kinase activity was required for certain genetic interactions and proper cell morphology (26). In addition, through phosphorus NMR analysis of the products of recombinant Vip1 and Kcs1/IP₆K, it was shown that distinct PP-IP₅ species are produced (26). Given the unique activities of Vip1 and Kcs1/IP₆K, it was shown that both enzymes also act as PP-IP₇ kinases, and together, these enzymes are capable of phosphorylating IP₆ to generate PP₂-IP₄ (26).

| 2 | Materials and Methods | Construction of Plasmids—The cDNA for human VIP1 (accession number BC057395) and human VIP2 (BC024591) were obtained from Open Biosystems (Huntsville, AL). Constructs were prepared for bacterial expression of full-length human VIP1 (hsVIP1), hsVIP2, and the kinase domains of hsVIP1 between residues 1 and 387 (hsVIP1-KD) and hsVIP2 between residues 1 and 393 (hsVIP2-KD). For hsVIP1, these were constructed by installing Sall sites at the 5′- and 3′-ends of the coding regions via PCR and subcloning into pGEX-KG, and the 3′-NotI sites at the ends of hsVIP1 and hsVIP1-KD and 5′-EcoRI and 3′-Xhol sites at the ends of hsVIP2 and hsVIP2-KD. All constructs were confirmed by sequencing at the Duke University DNA Analysis Facility. Mammalian expression constructs for human Gα₆,QL, human IPK1, and human IPK1 are described elsewhere (29).

| 3 | Expression and Purification of Recombinant Human VIP1 and VIP2—Constructs were transformed into BL21 DE3 Star competent Escherichia coli cells (Invitrogen), and protein was expressed by inducing at 18 °C with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for 5 h. Cells were resuspended in lysis buffer (25 mM Tris (pH 8.0), 350 mM NaCl, 1 mM dithiothreitol) with 1 mM phenylmethylsulfonyl fluoride and lysed by passing twice through a Microfluidics M110L homogenizer at ~15,000 psi. The lysate was cleared by centrifugation, and the supernatant was applied to a column of glutathione-Sepharose beads (Sigma) equilibrated in lysis buffer. After washing, protein was eluted in buffer containing 25 mM Tris (pH 8.0), 350 mM NaCl, 1 mM dithiothreitol, and 10 mM glutathione. Eluted protein was stored at −80 °C.

| 4 | Assays of VIP1 IP₆ and IP₇ Kinase Activity—Enzyme assays were typically run in 10-μl reactions containing 50 mM HEPES (pH 6.2), 1 mM ATP, 5 mM MgCl₂, and ~40,000 cpm of ³²P-P-IP₅ (³²P]IP₇) was also produced enzymatically, in 10-μl reactions with 1.7 pmol of ³²P]ATP or ³²P]IP₇. For kinetic analyses, IP₆ and IP₇ concentrations ranged from 20 nM to 10 μM. Reactions were generally incubated 20–60 min at 37 °C and stored at −80 °C.

| 5 | Human VIP1-like IP₆/IP₇ Kinases | Human VIP1-like IP₆/IP₇ Kinases

**References**

1. Exley, J. and Wilson, C. (2007) J. Biol. Chem. 282, 30755

**Author**

J. Exley and C. Wilson

**Abstract**

Human VIP1-like IP₆/IP₇ kinases play a crucial role in signal transduction pathways. The authors describe the cloning and characterization of two human VIP1-like gene products, providing insights into their enzymatic activities and potential roles in cellular processes.

**Keywords**

Human VIP1, IP₆/IP₇ kinases, signal transduction, enzyme assays, recombinant expression.
Human VIP1-like IP6/IP7 Kinases

Yeast Steady-state Inositol Labeling for HPLC Analysis—All yeast strains used were kcs1D ddp1D vip1Δ mutants in a w303 background. Strains were transformed through a standard protocol with hsVIP1 constructs in a pRS426-myc3-GFP-cup1 vector. For in vivo labeling, yeast were grown in complete synthetic medium lacking appropriate nutrients and containing 100 μM CuSO4 and 20 μCi/ml myo-[3H]inositol (American Radiolabel Corp., St. Louis, MO). After incubating until saturation, soluble inositols were harvested as described previously (30, 31). Prior to HPLC analysis, samples were diluted with 4 volumes of 10 mM NH4H2PO4 (pH 3.5). Soluble IPs were separated by HPLC on a Partisphere SAX column (4.6 × 125 mm; Whatman, Clifton, NJ) using the following buffer profile: 10 mM NH4H2PO4 (pH 3.5) for 5 min; a linear gradient of 10 mM NH4H2PO4 to 1.7 M NH4H2PO4 for 65 min; 1.7 M NH4H2PO4 for 40 min. Radiolabeled inositides eluting from the column were quantified using an inline radiation detector.

Multitissue Northern Blotting—DNA template corresponding to the first 625 nucleotides of the 3′-untranslated region of human VIP1 mRNA was amplified by PCR using the primers 5′-AAG TCC TGG GTG GTC CCT GAA G-3′ and 5′-CCC GGG ATG GCT ATC TGA AAT GTC TGG-3′. A random decamer primed 32P-labeled probe generated from this template was used to analyze a commercial human multitissue Northern blot (Clontech). The blot was hybridized using ExpressHyb solution (Clontech), washed under conditions described by the manufacturer, and exposed to film for 1 week at −70 °C.

Mammalian Cell Culture—HEK293T cells were obtained from the Duke University Medical Center Cell Culture Facility and were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO2. Transfections were carried out using FuGENE 6 transfection reagent (Roche Applied Science).

Subcellular Localization of Human VIP1—HEK293T cells were transfected with pCFP-hsVIP1 constructs. 48 h after transfection, cells were harvested in phosphate-buffered saline, pelleted by centrifugation, and resuspended in 50 mM Tris-HCl (pH 7.5), 100 μM phenylmethylsulfonyl fluoride. Cell extracts were prepared by sonication, and soluble and membrane fractions were separated by centrifugation at 100,000 × g. Membrane fractions were resuspended in lysis buffer by Dounce homogenization, and the levels of hsVIP1 in the soluble and membrane fractions were compared by Western blotting using an antibody to CFP (Roche Applied Science).

Fluorescence Microscopy and Western Blotting of CFP-tagged Human VIP1 and VIP2—For in vivo fluorescence microscopy, HEK293T cells were plated onto eight chamber slides at a cell density of 100,000 cells/ml and allowed to adhere overnight. The cells were transfected with CFP fusion constructs and incubated for 24 h. Cell imaging was then performed on a Nikon TE2000E inverted fluorescence microscope. Extracts were prepared by directly harvesting transfected cells with SDS-PAGE loading buffer, and CFP-hsVIP1 and CFP-hsVIP2 expression was analyzed by Western blotting extracts using an antibody to CFP.

Metabolic Labeling of Mammalian Cells—HEK293T cells were seeded onto 12-well tissue culture plates at 100,000 cells/ml. After incubation overnight, cells were washed one time with isoinositol free Dulbecco’s modified Eagle’s medium and then labeled for 72 h with 37.5 μCi/ml myo-[3H]inositol in isoinositol-free Dulbecco’s modified Eagle’s medium supplemented with 10% dialyzed fetal bovine serum. Cells were transfected after 48 h of labeling, directly into the labeling medium. Cell extracts destined for direct HPLC analysis were prepared by aspirating the labeling medium, washing the cells with phosphate-buffered saline, and adding 0.4 ml of 0.5 N HCl. HCl was left on the cells for 5 min to extract the soluble inositides and was then removed from the cells and filtered through a 0.45-μm nylon filter to remove cell debris. Samples were prepared for HPLC analysis in the same manner as yeast extracts and run with an identical column and gradient.

RESULTS

Evolutionary Conservation of VIP1 Genes—Bioinformatic analysis reported for yeast Vip1 demonstrated that this class of enzyme possesses a dual-domain structure that is evolutionarily conserved from yeast to mammals (26). The amino-terminal domain belongs to the RimK/ATP-grasp superfamily and encodes yeast Vip1’s IP6 kinase activity (Fig. 1A). A second domain within VIP1-like proteins has similarity to the histidine acid-phosphatase family of enzymes, members of which include phytase proteins capable of hydrolyzing IP6 (32, 33). According to the conserved domain structure retrieval tool (CDART), there are 69 VIP1-like sequences spanning the entire Eukaryota taxonomy that have the RimK/acid-phosphatase dual-domain structure (as of June, 2007). Notably, there is strong conservation of VIP1’s kinase domain among eukaryotic species, suggesting possible conservation of the corresponding inositol pyrophosphatase synthase activities (Fig. 1B). A catalytic Asp residue required for these kinase activities has also been identified and is extensively conserved among eukaryotes (Fig. 1, A and B). With respect to the acid-phosphatase domain, there is conservation of key histidine residues within the consensus active site motif; however, a notable mutation is present in all VIP1-like proteins at the aspartate residue adjacent to the second histidine (Fig. 1C).

In addition to VIP1’s conservation, sequence homology also revealed a closely related gene present in humans and mice, which we have termed VIP2 (Fig. 1, B and D). This gene has a similar dual-domain structure, with 86% sequence identity to mammalian VIP1 in the kinase domain.

IP6 and IP7 Kinase Activities of Recombinant Human VIP1 and VIP2—A full-length hsVIP1 complementary DNA (residues 1–1433) was obtained (GenBank™ accession number, AAH57395), subcloned, and expressed in bacteria as a glutathione S-transferase (GST) fusion protein. A construct spanning residues 1–387 encoding hsVIP1’s kinase domain (hsVIP1-KD) was also expressed and purified. Recombinant hsVIP1 and hsVIP1-KD fusion proteins were tested for IP6 and IP7 kinase activities using in vitro assays with the appropriate inositol phosphate substrate (Fig. 2A). These studies demonstrated that full-length hsVIP1, as well as the kinase domain alone, exhibit robust dose-dependent IP6 kinase activity capable of producing
FIGURE 1. Dual-domain structure and evolutionary conservation of the VIP1-like enzyme family. A, the dual-domain structure of yeast and human VIP1 is diagrammed. Conserved ATP-grasp and histidine acid phosphatase domains are located at residues 67–372 and 390–918, respectively, in human VIP1 and at residues 200–525 and 530–1025 in S. cerevisiae (S. cerv.). The ATP-grasp domain was found to exhibit kinase activity specific for IP6 and IP7 (PP-IP5) substrates. In yeast, this activity depended on the presence of a highly conserved catalytic aspartic acid residue, shown here.

B, evolutionary conservation of VIP1’s kinase domain across species. A multisequence alignment was performed with VIP1 homologs from Homo sapiens (hs, AAH57395), M. musculus (mm, NP_848910), Drosophila melanogaster (dm, CG14616-PE), S. cerevisiae (sc, accession NP_013514), and S. pombe (sp, SPCC1672.06c). Human and mouse VIP2 sequences (NP_056031.2 and NP_776121.2) were also aligned. A conserved catalytic aspartic acid residue required for S. cerevisiae Vip1 (scVip1’s) IP6 and IP7 kinase activity is indicated by the asterisk. Identical residues are shown in black, whereas similar residues are shown in gray.

C, the histidine acid phosphatase active site signature sequence (as obtained from the PROSITE web site) consists of two motifs with invariant catalytic residues RHXXR and HD shown in bold. Within the VIP1-like class of proteins, this signature is largely conserved in the putative acid-phosphatase domain with the notable exception of the aspartate residue in the second motif, which is substituted with isoleucine, valine, or alanine. Single letter abbreviations are used for amino acids, brackets indicate any one of the amino acids enclosed may occur, and x defines a position where any amino acid is tolerated.

D, a dendrogram illustrating the phylogeny of the VIP gene family was constructed based on sequence homology. VIP2 appears to be the product of a gene duplication occurring after divergence of D. melanogaster and mammalian VIP1 genes.
Human VIP1-like IP₆/IP₇ Kinases

IP₇ (Fig. 2A). Furthermore, both the full-length enzyme and the kinase domain are capable of phosphorylating 5-PP-IP₅, an IP₇ product of human IHPK1/IP6K, to PP₂-IP₄ (IP₈) (Fig. 2B). Of interest, the human full-length protein exhibited significantly less specific activity than the kinase domain alone (Fig. 2, A and B), a property also shared by recombinant yeast Vip1 (not shown).

To examine the relative rates of IP₆ and IP₇ kinase activities, the kinetic parameters of recombinant hsVIP1 were also determined (Fig. 2, C and D, and Table 1). With the kinase domain, a $K_m$ of 122 and 120 nM was found for IP₆ and IP₇ substrates, respectively. Corresponding $V_{max}$ values were 0.42 and 1.04 nmol/min/mg, respectively. Similar $K_m$ values were obtained for the full-length enzyme, although the turnover number ($k_{cat}$) for each substrate was 3–5-fold lower (Table 1). This difference in activity may be a result of less efficient protein folding of the much larger full-length protein in bacteria but could also be associated with a regulatory property of the putative acid phosphatase domain. Although these $K_m$ and $V_{max}$ values are ~200-fold lower than those reported for yeast Vip1, the catalytic efficiencies ($k_{cat}/K_m$) of the enzymes remain within 1 order of magnitude (Table 1) (26).

Recombinant full-length human and mouse VIP2 complementary DNAs (GenBank accession numbers: NP_056031.2 and NP_776121.2) were also subcloned, expressed as GST fusion proteins in bacteria, and purified. These enzymes both exhibited robust IP₆ and IP₇ activities, with catalytic efficiencies for the two substrates 5–30-fold higher than those of hsVIP1. Although VIP2's increased in vitro activity may have physiological relevance, it should be noted that differences in the recombinant proteins' expression and stability in bacteria could

![FIGURE 2. In vitro IP₆ and IP₇ kinase activity of human VIP1. Varying amounts of full-length hsVIP1 (FL) and KD were incubated with 0.5 μM of either IP₆ (A) or IP₇ (B) produced by human IP6K, as well as trace amounts of ³²P-radiolabeled substrate. Reactions were resolved on polyethyleneimine-cellulose TLC plates and visualized on a Phosphorimager. Dose-dependent kinase activity is seen with all constructs and substrates, but the full-length protein appears about five times less active than the kinase domain alone. Kinetic parameters were also determined for each construct, and a velocity curve is shown for hsVIP1-KD’s IP₆ kinase activity (C) and IP₇ kinase activity (D). Corresponding Lineweaver-Burk plots are in the inset. Kinetic parameters are reported in Table 1.](image)

**TABLE 1**

| Substrate | Construct | $K_m$ (μM) | $V_{max}$ (nmol/min/mg) | $k_{cat}$ (10⁻¹ s⁻¹) | $k_{cat}/K_m$ (10⁻³ s⁻¹) |
|-----------|-----------|------------|-------------------------|----------------------|--------------------------|
| IP₆       | hsVIP1    | 0.12       | 0.03                    | 1.1                  | 0.88                     |
|           | hsVIP1-KD | 0.12       | 0.42                    | 5.0                  | 4.1                      |
|           | mmVIP2    | 0.23       | 1.70                    | 10.6                 | 8.0                      |
|           | scVIP1*   | 17.63      | 22.63                   | 593                  | 3.37                     |
|           | scVIP1-KD*| 20.66      | 98.79                   | 1441                 | 6.97                     |
| IP₇       | hsVIP1    | 0.10       | 0.13                    | 4.0                  | 3.9                      |
|           | hsVIP1-KD | 0.12       | 1.04                    | 12.3                 | 10.3                     |
|           | hsVIP2    | 0.19       | 1.38                    | 38.0                 | 20.4                     |
|           | mmVIP2    | 0.54       | 5.23                    | 163                  | 30.1                     |

*a Values taken or derived from those reported in Mulugu et al. (26).
Human VIP1-like IP₆/IP₇ Kinases

In Vivo IP₆ Kinase Activity of hsVIP1 Expressed in Yeast—To determine whether the IP₆ and IP₇ kinase activities associated with human VIP1 are retained in a cellular environment, hsVIP1’s kinase activities were also observed through in vivo studies. Yeast mutants overexpressing hsVIP1 were radiola
ebeled with myo-[³H]inositol, and extracts were analyzed through HPLC. These studies were done in a kcs1Δ ddp1Δ vip1Δ triple mutant, which lacks IP₆ kinase and diphosphoinositol phosphate phosphate activities but possesses a high intracellular concentration of IP₆ (26). Although no IP₇ was observed in this mutant transfected with vector alone (Fig. 3A, top trace), when either full-length or kinase domain hsVIP1 constructs were overexpressed, a significant peak of IP₇ was detected (Fig. 3A, middle and lower traces). The VIP1 kinase domain appeared to be more active than full-length protein, as judged by the amount of IP₇ produced, recapitulating catalytic differences observed in in vitro activities. Although the acid phosphatase domain was also overexpressed in yeast, no change in soluble inositol levels was observed (not shown). These data demonstrate that hsVIP1 possesses cellular IP₆ kinase activity capable of producing IP₇. It is important to note that exposing this activity may require loss of both Kcs1 and Ddp1 activities, and the lack of Kcs1 kinase activity is a likely explanation for the failure to observe the formation of IP₇ in these cells.

In Vivo IP₇ Kinase Activity of hsVIP1 Expressed in Mammalian Cells—To further examine hsVIP1 and hsVIP2’s in vivo inositol pyrophosphate synthase activity, a myo-[³H]inositol radiolabeled HEK293T cell line was transfected with full-length and kinase domain CFP-hsVIP1 fusion constructs. However, hsVIP1-transfected cells showed minimal increases in the levels of either IP₆ or IP₇ under normal conditions, with their levels less than 3% of IP₆ under these conditions. This lack of observable in vivo kinase activity was not without precedent, however, as other inositol polyphosphate kinases do not produce detectable levels of their products under normal cellular conditions, even when overexpressed (29, 34, 35). Additionally, the failure to observe an effect when monitoring rapidly fluxing metabolites may be due to an inability to trap turnover of substrate or products; for example, measuring agonist-induced stimulation of IP₃ is difficult in the absence of treating cells with the phosphatase inhibitor lithium.

Therefore, to trap rapidly equilibrating or masked IP and PP-IP molecules, a strategy was devised to genetically perturb inositol metabolism through manipulation of both G-proteins and a variety of mammalian IPKs, the results of which are presented elsewhere (29). Using this system, we have genetically engineered cells to produce high levels of IP₆ and IP₇, as a means to potentially stimulate VIP1-dependent pathways. Briefly, cells were transfected with an activated G-protein (Goαq) as a means to stimulate phospholipase C (36), along with Ipkl, an I(1,3,4,5,6)P₅ 2-kinase that synthesizes IP₆ (30, 34, 35). Under these conditions, the levels of IP₆ increased as compared with control (not shown); however, the levels of IP₇ remained below 3% of the IP₆ and IP₇ was not detectable above background (Fig. 3B, left column, upper trace). Expression of hsVIP1 in these cells did not result in detectable changes in IP₇ or IP₈ (Fig. 3B, left column, middle trace), and expression of hsVIP1-KD resulted in the appearance of a small peak of IP₈ (Fig. 3B, left column, lower trace). In contrast, when cells were engineered to overexpress activated G-protein, Ipkl, and the IP₆ kinase IHPK1, levels of IP₇ were dramatically elevated (Fig. 3B, right column, top trace). When hsVIP1 or hsVIP1-KD was overexpressed in these cells, a significant elevation in the level of IP₇ was observed (Fig. 3B, right column, middle and lower traces). The IP₈ levels we observed appear higher than those previously reported in mammalian cells (22–24). These data indicate that both IP6K and hsVIP1 collaborate to convert IP₆ to IP₇ in mammalian cells.

In addition, we examined the effects of overexpression of the hsVIP2 kinase domain in similarly activated cells (Fig. 3C). Although no detectable changes in IP₇ or IP₈ levels were detected in the absence of IHPK1, coexpression of this IP₆ kinase with hsVIP2-KD resulted in an IP₇ peak of similar size to that observed with hsVIP1. Human VIP2 therefore appears to possess an in vivo inositol pyrophosphate kinase activity similar to VIP1’s.

Tissue Distribution of hsVIP1 mRNA—A multitissue Northern blot analysis was performed to determine the distribution of hsVIP1 mRNA transcription in human tissues (Fig. 4A). A single transcript having a molecular size expected for full-length human VIP1 (6.2 kb) was observed in most tissues tested, and it appeared most abundant in skeletal muscle, brain, and heart (normalized to total polyadenylated RNA loaded).
Subcellular Localization of hsVIP1 and hsVIP2—To investigate hsVIP1’s subcellular localization, human HEK293T cell lines were transfected with a CFP-hsVIP1 fusion construct. Extracts were then prepared from transfected cells and fractionated by ultracentrifugation into soluble and membrane components. After Western blotting against CFP, bands corresponding to full-length human VIP1 were observed in over 80% of the antigen in the soluble fraction, whereas the membrane fraction contained less than 20% of the total (Fig. 4B).

To determine hsVIP1 and hsVIP2’s subcellular localizations, HEK293T cells expressing full-length CFP-hsVIP1 and CFP-hsVIP2 fusion proteins were evaluated by fluorescence microscopy (Fig. 4C). In transfected cells, localization appeared primarily cytosolic for both enzymes, with apparent nuclear exclusion relative to CFP controls. Based on a Western blot analysis of extracts made from these cells immediately following microscopy, CFP-hsVIP1 and CFP-hsVIP2 proteins were expressed at similar levels, almost exclusively as full-length fusion proteins (Fig. 4D). This indicates that the fluorescent signal reflects localization of full-length protein and not products of degradation or incomplete translation. Although the Western blot of fractionated hsVIP1 extracts shows a majority of CFP antigen migrating as free CFP, and not a complete fusion protein, it appears that this is a result of proteolysis that occurred during the fractionation process.

D I S C U S S I O N

Our study identifies two human gene products belonging to the VIP1-like class of enzymes that are capable of both in vitro and in vivo production of IP₆ and IP₇. A mammalian diphosphoinositol pentakisphosphate kinase (PPIP5K or IP7K) activity was described over a decade ago (11, 24, 37–39). Our demonstration of hsVIP1 and hsVIP2’s innate IP₇ kinase activity suggests that one or both of these enzymes is the previously recognized human IP₇ kinase. Together with the IP6K/Kcs1 class of kinases, VIP1 and VIP2 appear capable of producing significant amounts of cellular IP₆. The role of human VIP1 for IP₆ production also indicates possible involvement with several previously reported examples of inositol pyrophosphate signaling. Production of IP₆ has been associated with environmental stress responses, including hyperosmotic and heat stress, as well as with certain cAMP-mediated signaling events (21–25). Some of these responses may be mediated by MAP kinase pathways, suggesting one possible regulatory mechanism for IP7 kinase activity (22, 23). Further studies examining the importance of hsVIP1 and hsVIP2 to the regulation of IP₆ levels are needed to explore the enzyme family’s precise involvement in this metabolic pathway.

In addition to its IP₇ kinase activity, we found that hsVIP1, like its previously characterized yeast ortholog, exhibits IP₆ kinase activity detectable both in vitro and when overexpressed in mutant yeast (26). Through biochemical analysis, the enzyme was found to possess similar, specific affinities for IP₆ and IP₇, with Kₘ values within reported ranges of intracellular IP₆ and IP₇ levels in yeast and mammalian cells (39, 40). The catalytic efficiencies of the enzyme’s two kinase activities, while relatively weak, are also similar and in the same range as other IPKs (41, 42). Considering the IP₆ kinase activity detectable in yeast and with recombinant protein, the failure to detect significant IP₆ kinase activity in mammalian cells under our conditions is notable. Although it is possible that IP₆ kinase activity is not a relevant activity in mammalian cells, the distinct products observed in different systems could also be a result of some mechanism in mammalian cells regulating or inhibiting IP₆ kinase activity under normal conditions. In this respect, it is conceivable that changes in cellular conditions due to external stress or signaling events could lead to a change in hsVIP1’s specificity or enzymatic activity. A shift in subcellular localization is another possible mechanism for the regulation of hsVIP1’s activities, although we have not yet observed any such change. Additionally, although the specificity and relevance of the response is unclear, the reported activation of IP₇ production under osmotic stress suggests a possible connection to regulation of kinase activity (22, 23).

The extensive evolutionary conservation of the VIP1-like family of kinases in eukaryotic cells is consistent with an ancient fundamental biological role for these enzymes. As both human and yeast enzymes have now been shown to possess IP₆ and IP₇ kinase activities, and with biological functions reported for both IP₇ and IP₆ products, a conserved signaling role for this class of enzymes appears increasingly likely (22–24, 26, 43). The two mammalian enzymes identified in this family, VIP1 and VIP2, have so far
been shown to possess similar kinase activities and subcellular localizations. Although mouse and human VIP2 may exhibit more robust IP₁ and IP₃ kinase activities in vitro, no significant difference has been observed in vivo activity. It therefore remains unclear whether or not these paralogs have distinct functions in the cell.

With respect to the acid-phosphatase domain of VIP1-like family members, it remains unclear whether this domain possesses enzymatic activity. This domain appears to have some function based on: 1) reports that expression of a mutant protein in which a conserved active site histidine residue has been substituted with alanine results in conditional cellular toxicity (26) and 2) the strict evolutionary conservation of the domain within the VIP1-like class of proteins across the entire eukaryotic taxonomy has been demonstrated. Inspection of the VIP1-like acid-phosphatase domain sequences indicates a strong overall sequence similarity to the hundreds of bona fide acid-phosphatases including most of the residues within the active site motif (Fig. 1C).

However, the mechanism of action of acid-phosphatases requires two histidine residues and an aspartate proton donor, which is adjacent to the second histidine (44–47). Substitution of this aspartate residue in E. coli phytase decreases catalytic activity over 100-fold (47). Of interest, all members of the VIP1-like class have an aspartate substitution, which is replaced with isoleucine, valine, or alanine (Fig. 1C). Remarkably, another member of the acid-phosphatase family is the enzyme multiple inositol phosphophosphate phosphatase (48). Inspection of multiple inositol polyphosphate phosphatase sequence from a variety of organisms indicates that it also has a substitution at this aspartate residue, typically an alanine. Thus, it appears that in order for VIP1-like members to be active phosphatases, a unique mechanism may be utilized or a proton donor residue may be present from outside of this motif.

During the course of preparing this study, we have become aware of another study that has independently purified and cloned a mammalian diphosphoinositol pentakisphosphate (IP₅) kinase activity and found it to be identical to hsVIP1 (49). This additional report is consistent with our findings concerning hsVIP1’s PP-IP₅ kinase activity and provides additional evidence for the significance of hsVIP1’s activities.

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