The human homologue of yeast ArgRIII protein is an inositol phosphate multikinase with predominantly nuclear localization

Marcus M. NALASKOWSKI, Christina DESCHERMEIER, Werner FANICK and Georg W. MAYR[1]

Universitaetsklinikum Hamburg-Eppendorf, Institut für Medizinische Biochemie und Molekularbiologie, Abteilung für Zellulaere Signaltransduktion, Martinistrasse 52, 20246 Hamburg, Germany

The function of the transcription regulator ArgRIII in the expression of several genes involved in the metabolism of arginine in yeast has been well studied. It was previously reported that it is also an inositol phosphate multikinase and an important factor of the mRNA export pathway [reviewed by Shears (2000) Bioessays **22**, 786–789]. In the present study we report the cloning of a full-length 1248-bp cDNA encoding a human inositol phosphate multikinase (IPMK). This protein has a calculated molecular mass of 47.219 kDa. Functionally important motifs [inositol phosphate-binding site, ATP-binding site, catalytically important SSLL (Ser-Ser-Leu-Leu) domain] are conserved between the human IPMK and yeast ArgRIII. Bacterially expressed protein demonstrated an inositol phosphate multikinase activity similar to that of yeast ArgRIII. Ins(1,4,5)P_3 is phosphorylated at positions 3 and 6 up to Ins(1,3,4,5,6)P_5. The human IPMK fused with a fluorescent protein tag is localized predominantly in the nucleus when transiently expressed in mammalian cells. A basic cluster in the protein's C-terminus is positively involved in nuclear targeting. These findings are consistent with the concept of a nuclear inositol phosphate signalling and phosphorylation pathway in mammalian cells.

Key words: Arg^{82}, inositol 1,3,4,5,6-pentakisphosphate, nuclear localization signal, nucleus.

INTRODUCTION

Four proteins (ArgRI, ArgRII, ArgRIII and Mcm1) regulate the expression of several genes involved in the metabolism of arginine in yeast. In the presence of arginine, they repress the synthesis of five anabolic enzymes and induce the synthesis of two catabolic enzymes [1,2]. Therefore ArgRIII plays an important role in the regulation of arginine metabolism, but it has additional functions. It possesses an inositol phosphate multikinase (IPMK) activity that can catalyse the ATP-dependent phosphorylation of inositol phosphates at positions 3 and 6 [3,4]. An ARGRIII deletion yeast mutant shows dramatic changes in its inositol phosphates levels, indicating that the multikinase activity is *in vivo* important for the maintenance of the balance of the different inositol phosphates [5]. Deletion yeast mutants also show an accumulation of poly(A) RNA in the nucleus caused by an impediment to nuclear mRNA export [5,6]. Therefore the deletion of ArgRIII causes changes in inositol phosphate turnover and affects mRNA dynamics.

Saiardi et al. have identified a rat IPMK (RnIPMK). Yeast ArgRIII has the highest similarity to the RnIPMK of any gene in the completely sequenced yeast genome [7]. RnIPMK is able to phosphorylate inositol phosphates at positions 1, 3 and 6. This variety of kinase activities greatly exceeds that of any previously described enzyme [7].

We report the identification of a human IPMK (HsIPMK). The enzymic activity and the intracellular distribution of this protein were examined.

EXPERIMENTAL

Materials

NRK 52E cells were purchased from Cell Lines Service, Heidelberg, Germany. The vectors pEGFP-N1 and pEYFP-C1 were from ClonTech, La Jolla, CA, U.S.A. 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI) was purchased from Sigma, Taufkirchen, Germany. All other chemicals were of highest purity available.

Construction of fusion genes

The cDNA clone IMAGE:4510867 (GenBank® accession no. BG258567; GenBank® gene info identifier (gi): 12768383) was obtained from the UK Human Genome Mapping Project Resource Centre (HGMPRC), Hinxton, Cambridge, U.K., and completely sequenced. The green-fluorescent-protein (GFP) fusion genes C-tag and N-tag were created by PCR techniques. The open reading frame was amplified using the following primer pairs (C-tag: 5′-GCTAGCATGGCAACAGAGCCACCATC-3′, 5′-AGATCTGAATTGTCTAAAATACTTCGAAGTAC-3′; N-tag: 5′-GGTACCATGGCAACAGAGCCACCATC-3′, 5′-GGATCCTCAATTGTCTAAAATACTTCGAAG-3′). A *Nhe*I/*Kpn*I restriction site was introduced at the 5′-end, a *Bgl*II/*Bam*HI restriction site at the 3′-end. The PCR products were first cloned into the pGEM T Easy vector (Promega, Mannheim, Germany). A superfluous nucleotide at position 451 was deleted using the quikchange site-directed mutagenesis kit from

Abbreviations used: DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; EYFP, enhanced yellow fluorescent protein; gi, gene info identifier; HGMPRC, Human Genome Mapping Project Resource Centre; IP, inositol phosphate; IP3K, Ins(1,4,5)P_3 3-kinase; IP6K, InsP_6 kinase; (Hs or Rn)IPMK, (human or rat) inositol phosphate multikinase; LP, long pass; MADS, **M**CM1, **a**gamous, **d**eficiens and **S**RF; MDD, metal dye detection; NLS, nuclear localization signal; SSLL, Ser-Ser-Leu-Leu; t_R, retention time.

[1] To whom correspondence should be addressed (e-mail mayr@uke.uni-hamburg.de).

The sequence of HsIPMK has been deposited with the GenBank®, DDBJ, EMBL and GSDB Nucleotide Sequence Databases under the accession no. AF432853.

Stratagene, La Jolla, CA, U.S.A. according to the manufacturer's instructions using the following primer pair (5′-GGATGTAAAGATAGGGCAAAAAAGCTATGATCCTTTTGCCTC-3′, 5′-GAGGCAAAAGGATCATAGCTTTTTTGCCCTATCTTTACATCC-3′). The open reading frame was completely resequenced and then the fragments were subcloned by the introduced restriction sites into the expression vectors pEGFP-N1 and pEYFP-C1 (ClonTech).

Construction of fusion gene derivates

The deletion mutants and the double point mutants were created by using modified quikchange site-directed mutagenesis [8]. The N-tag fusion gene was used as template. The following primer pairs were used for mutagenesis (M4: 5′-ACAGCTAATGGAAAAATAGAGCAAGACAATGGGTGGAAAAGC-3′, 5′-GCTTTTCCACCCATTGTCTTGCTCTATTTTTCCATTAGCTGT-3′; M5: 5′-TTGTCCAAGATGTATGCGAGTCAGACTTCATTGAAA-3′; 5′-TTTCAATGAAGTCTGACTCGCATACATCTTGGACAA-3′; M6: 5′-CCAAGATGTATGCGCGTCACCAGCAAATATATACAAAAAAGCATCACAG-3′, 5′-CTGTGATGCTTTTTTGTATATATTTGCTGGTGACGCGCATACATCTTGG-3′; M7: 5′-GTCACAGGAAAATATATACACAACAGCATCACAGTCAGACTTCATTG-3′, 5′-CAATGAAGTCTGACTGTGATGCTGTTGTGTATATATTTTCCTGTGAC-3′). The fusion gene derivates containing C-terminal fragments (M1 and M2) were created by the same PCR techniques as the fusion genes C-tag and N-tag using the following primer pairs (M1: 5′-GGTACCCCAACCACTACAAAATTGAAT-3′, 5′-GGATCCGGGAAGATGGTAGAAAACTTT-3′; M2: 5′-GGTACCTCTTCAGTGGGCAAAAGCTTG-3′, 5′-GGATCCCTCCAGATTTTCAACTTTCAA-3′). The fragment of the derivate M3 was created by direct annealing of two primers (5′-CCGTCACAGGAAAATATATACAAAAAAGCATCACTGAG-3′, 5′-GATCCTCAGTGATGCTTTTTTGTATATATTTTCCTGTGACGGGTAC-3′).

Cell culture, transient gene expression and fluorescence microscopy

NRK 52E cells [9] were grown in Dulbecco's modified Eagle's medium containing 10 % fetal-calf serum (Life Technologies, Paisley, Scotland, U.K.). The cell line was cultured at 37 °C in a humidified atmosphere in the presence of 5 % CO_2.

For transfection and examination, cells were seeded in chamber slides and incubated overnight. Cell transfections were performed using the LIPOFECTAMINE® method according to the manufacturer (Life Technologies, Gaithersburg, MD, U.S.A.) at 60–70 % confluency. Typically, 1 μg of DNA/ml of transfection medium was used, and transfections were performed for 5 h at 37 °C. The solution was then replaced by complete medium and cells were examined 24 h after transfection. Cells were washed twice with PBS, fixed in 3 % paraformaldehyde in PBS for 10 min at 37 °C, washed twice with PBS, stained with DAPI (10 μg/ml) in PBS for 10 min at 37 °C, and washed twice with PBS. Finally the fixed and stained cells were covered with PBS.

Fluorescence microscopy was carried out using a microscope Axiovert 25 CFL (Zeiss). For detection of the fluorescence of enhanced green fluorescent protein (EGFP) and enhanced yellow fluorescent protein (EYFP) fusion proteins a GFP filter [excitation: 450–490; beamsplitter: FT 510; emission: long pass (LP) 520] was used. For detection of the DAPI fluorescence a DAPI filter (excitation: G 365; beamsplitter: FT 395; emission: LP 420) was used. Digitized images were created by a DX30 video camera (Kappa, Gleichen, Germany). The images were processed using the program Kappa ImageBase 2.2SP2 (Kappa).

Expression and purification of recombinant protein

An expression vector was created by PCR techniques using the GFP fusion gene C-tag as template. The open reading frame of HsIPMK was amplified using the following primer pair (5′-AGCCATGGCATGGCAACAGAGCCACCATCC-3′, 5′ - AGCTCGAGTCAATTGTCTAAAATACTTCGAAG-3′). A *Nco*I restriction site was introduced at the 5′-end, and a *Xho*I restriction site at the 3′-end. The PCR product was first cloned into the pGEM T Easy vector (Promega, Mannheim, Germany). The open reading frame was completely resequenced and then the fragment was subcloned by the introduced restriction sites into a pET17b-based expression vector (Novagen, Madison, WI, U.S.A.). The recombinant fusion protein with a N-terminal Strep-tag was overexpressed in *Escherichia coli* BL21(DE3)-pLysS[pREP4] and purified at first on DEAE-Sephacel and then on phosphocellulose [10]. After 2 h of induction with 0.5 mM isopropyl β-D-thiogalactoside at 37 °C the bacterial cells were harvested by centrifugation at 4000 ***g*** for 10 min and resuspended in 1/20 of the culture volume of buffer containing 50 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 % Triton X-100, 0.5 mM benzamidine and 1 mM PMSF. After sonification for 1 min the lysate was centrifuged at 12000 ***g*** for 10 min (4 °C). The Hepes concentration in the supernatant was adjusted to 25 mM through 1:2 dilution, maintaining the concentration of the other components. The supernatant was applied on to a DEAE-Sephacel column and the IPMK was found mainly in the flow-through, whereas other proteins were largely bound. The protein was further enriched by application of the flow-through to a phosphocellulose column, washing and elution using a buffer containing 25 mM Hepes pH 7.5, 750 mM NaCl, 1 mM EDTA, 1 mM DTT and 0.1 % Triton X-100. The purified protein and BSA standard samples were separated by SDS/PAGE and the products were stained with Coomassie Blue. Digitized images were created by a JX-325 transmission scanner (Sharp, Hamburg, Germany). The amount of IPMK was quantified using the program ImageMaster1D (Pharmacia). After separation by SDS/PAGE and transfer on to a nitrocellulose membrane, HsIPMK with a N-terminal Strep-tag was detected using an avidin–alkaline phosphatase conjugate (Bio-Rad, Hercules, CA, U.S.A.) according to the manufacturer's instructions.

Assay of enzymic activity and product identification by metal-dye-detection HPLC (MDD-HPLC)

The activity of the purified enzyme was assayed in 400 μl at 30 °C in a reaction mixture containing 10 mM triethanolamine chloride, pH 7.8, 5 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 1 mM phosphoenolpyruvate, 2 units/ml pyruvate kinase, 0.5 mM ATP and 5 μM Ins(1,4,5)P_3 (Alexis, Woburn, MA, U.S.A.). The reaction was started with 180 ng of enzyme and terminated after various periods of time by the addition of 100 μl of ice-cold 50 % (w/w) trichloroacetic acid plus 20 μl of 20 % (w/v) suspension of Norit A. After addition of 1.25 μmol of EDTA and 0.25 μmol of NaF and centrifugation for 10 min at 10000 ***g*** in a refrigerated benchtop centrifuge, the supernatant was twice more treated with 20 μl of Norit A suspension, three times extracted with 1 ml of diethyl ether and the pH adjusted to $\approx$ 6.5 with Tris base. The reaction products were analysed by HPLC with metal dye detection (MDD-HPLC) [11,12]. A micro-MDD-HPLC set-up essentially as described in [13] with optimized gradient was employed. Isomers separated in the standard mixture by this gradient could be assigned by the use of appropriate commercial standard isomers precisely in the order as described in [12]. However, on the MiniQ column (3 mm internal diameter $\times$ 32 mm long) employed, a separation between Ins(1,4,5,6)P_4 [retention time (t_R) 15.18 min]

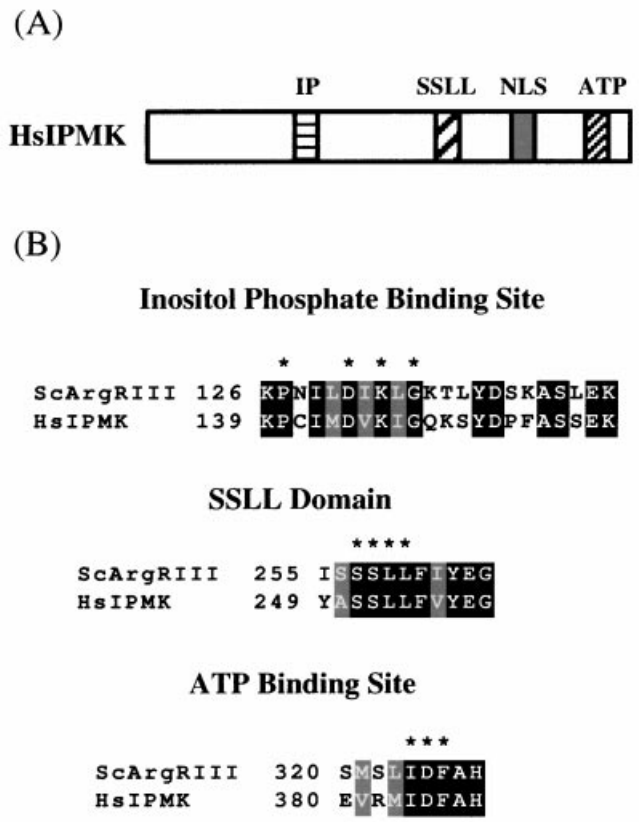

Figure 1 Domain structure of HsIPMK

The functional domains of HsIPMK and the composition of functionally important sites are shown. (**A**) The structural features of HsIPMK are schematically represented. Features depicted are: IP, inositol phosphate-binding site; SSLL, SSLL domain; NLS, nuclear localization signal; ATP, ATP-binding site. (**B**) Local sequence alignments of functionally important sites of yeast ArgRIII with the corresponding sequences in HsIPMK. Numbers refer to the position of the first amino acid in the primary sequence of the protein shown. The single-letter amino acid code is used. Conserved residues are shown by white letters on a black background and similar residues by white letters on a grey background. The invariable residues in these motifs are marked by asterisks.

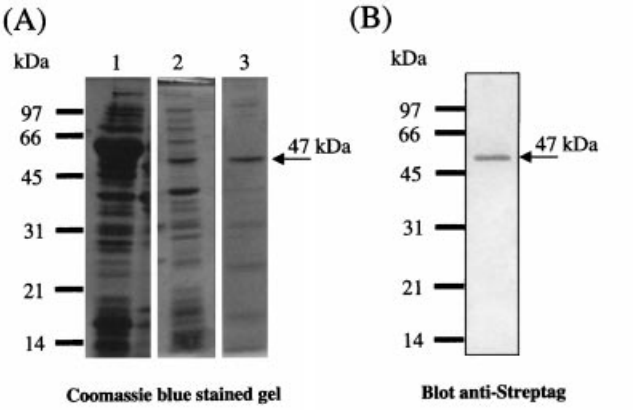

Figure 2 Purification of bacterially expressed HsIPMK

The recombinant protein was overexpressed in *E. coli* and purified as described in the Experimental section. (**A**) Sample of the supernatant (52 μg of protein, lane 1), material purified on DEAE-Sephacel (12.5 μg of protein, lane 2) and purified on phosphocellulose (3.1 μg of protein, lane 3) were separated by SDS/PAGE. The prominent band of the purified material possesses a molecular mass of approx. 47 kDa corresponding to the calculated molecular mass of HsIPMK. (**B**) Purified material was separated by SDS/PAGE and the products were transferred on to a nitrocellulose membrane. An avidin–alkaline phosphatase conjugate was used for detection of HsIPMK with a N-terminal Strep-tag. One band with a molecular mass of approx. 47 kDa was detected confirming the identity of the purified protein.

and Ins(1,2,3,4,6)P_5 (t_R 15.73 min) was observed, in contrast with the long (5 mm inner diameter × 250 mm long) MonoQ column in [12].

RESULTS

Identification and cloning of the HsIPMK

The multifunctional yeast protein ArgRIII is an IPMK, but also a transcription regulator and an important factor of the mRNA export pathway [1,5]. We used the tBLASTn program (www.ncbi.nlm.nih.gov/BLAST/) to search the human genomic sequences in the HTGS (High Throughput Genomic Sequence) database with the ArgRIII protein sequence. The chromosome 10 clone RP11-637J22 (GenBank® accession no. AL358155.10; GenBank® gi: 11322007) contains an open reading frame with considerable sequence identity. The chromosomal localization is 10q21. A hypothetical cDNA sequence was assembled by analysis of putative splice sites. Six exons contribute to the coding sequence. The open reading frame codes for a protein of 416 amino acids (calculated molecular mass 47219.40 Da). The amino acid sequence shows a 82% similarity to RnIPMK. Functionally important motifs (Figure 1A) are conserved between the mammalian IPMKs and ArgRIII. The highest similarity is found in the Ins*P*-binding site. The ATP-binding site and the catalytically important SSLL domain are also clearly conserved (Figure 1B).

This hypothetical cDNA sequence was used to search the human expressed sequence tag (EST) database with the BLASTn program. The cDNA clone IMAGE:4510867 (GenBank® accession no. BG258567; GenBank® gi: 12768383) was ordered from the UK HGMPRC and completely sequenced. The open reading frame is complete and corresponds to the hypothetical cDNA. However, a one-base insertion was found at nucleotide position 451, leading to the loss of the reading frame and to a premature stop codon. This insertion of an adenosine nucleotide in a polyadenosine stretch was probably caused by slipping of the reverse transcriptase. Quikchange mutagenesis was used to delete the superfluous nucleotide. The protein was named HsIPMK.

Bacterial expression of an enzymically active HsIPMK

The yeast protein ArgRIII phosphorylates inositol phosphates at positions 3 and 6 [3,4] and, on the basis of this multiple substrate specificity, has been designated IPMK. The conservation of catalytically important sites between ArgRIII and the HsIPMK (Figure 1B) is a clear indication of IPMK activity of the human protein. Therefore the HsIPMK was bacterially expressed and purified to near homogeneity (Figure 2). The enzymic activity of the recombinant protein was examined by employing Ins(1,4,5)P_3 as a substrate and analysing the isomerism of reaction products by MDD-HPLC [11–13]. The substrate was mainly phosphorylated first to Ins(1,3,4,5)P_4, and, upon longer incubation, it was completely converted into Ins(1,3,4,5,6)P_5 (Figure 3; data for longer incubation times not shown). Hence the protein is able

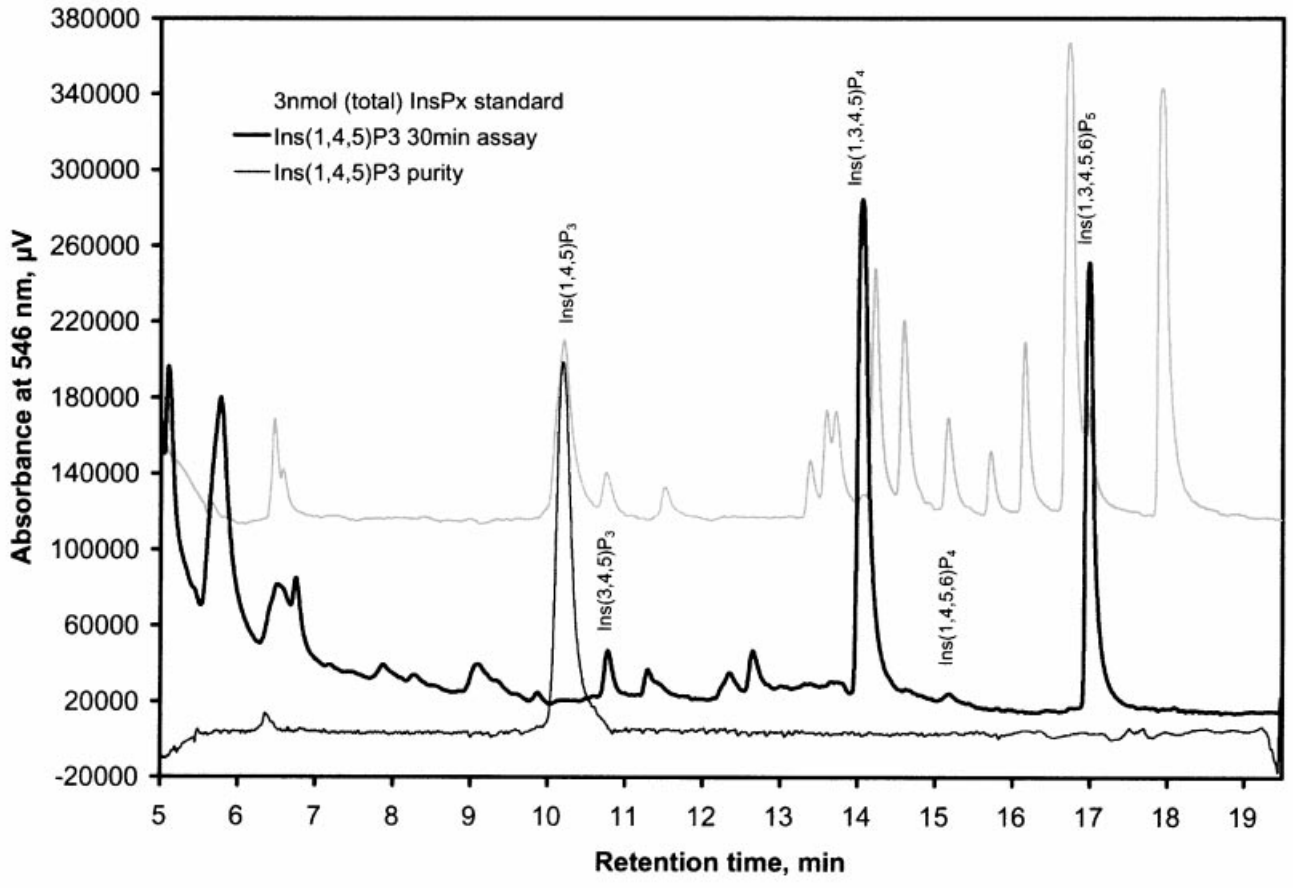

Figure 3 Products of phosphorylation of Ins(1,4,5)P_3 by HsIPMK

Recombinant enzyme was incubated, and products were analysed by MDD-HPLC, under the conditions described in the Experimental section. The products Ins(1,3,4,5)P_4 (t_R 14.06 min) and Ins(1,3,4,5,6)P_5 (t_R 16.99 min) were found in the MDD-HPLC chromatograms from all assays performed (see the middle chromatogram from a 30 min assay). A small further InsP_4 peak in the assay chromatogram (t_R 15.20 min) is eluted at precisely the position of Ins(1,4,5,6)P_4 and is evidently a second InsP_4 product formed from Ins(1,4,5)P_3 by HsIPMK. The peak succeeding Ins(1,4,5)P_3 was not present in assays without substrate and, on account of its isomeric nature, Ins(3,4,5)P_3 is likely to be a dephosphorylation product of Ins(1,3,4,5)P_4 generated by a small phosphatase contamination of IPMK or the pyruvate kinase contained in the assay mixture. Small peaks eluted before Ins(1,3,4,5)P_4 and Ins(1,4,5)P_3 respectively represent in most cases phosphorylated impurities brought in by the assay components (mainly remaining ATP and ADP not bound to charcoal), since assays incubated without substrate showed the same peaks (results not shown). The substrate employed, Ins(1,4,5)P_3 (t_R 10.22 min), is shown for its purity (lower chromatogram). A standard mixture of inositol phosphates generated by limited acid hydrolysis of InsP_6 was separated with the same gradient and is superposed as the third (upper, grey) chromatogram. Note that only a small but detectable amount of Ins(1,3,4,5)P_4 is present in this standard mixture.

to phosphorylate Ins(1,4,5)P_3 at position 3 and to further phosphorylate this product at position 6. Even upon prolonged incubations, no phosphorylation of these products to pyrophosphorylated inositol phosphates [14] or of Ins(1,3,4,5,6)P_5 to InsP_6 was observed (results not shown). However, in addition to the main detectable InsP_4 product, Ins(1,3,4,5)P_4, a second much smaller InsP_4 product was also detected (Figure 3). By precise agreement of its retention time (t_R 15.20 min) with a corresponding isomer in a standard mixture, it could be identified as Ins(1,4,5,6)P_4, which in fact has also been identified as the InsP_4 product of yeast ArgRIII when incubated with Ins(1,4,5)P_3 [4]. We could determine the apparent V_{max} for the conversion of Ins(1,4,5)P_3 into InsP_4 as 3.07 ± 0.60 units/mg and an apparent K_m for Ins(1,4,5)P_3 of 0.28 ± 0.07 μM. For the second half of the reaction transient, which is due to the conversion of InsP_4 into Ins(1,3,4,5,6)P_5, an apparent rate of 0.45 ± 0.11 unit/mg was derived. These parameter determinations were performed in triplicate or more and ranges given are $\pm$ one sample S.D. Since no pyrophosphorylated InsP_5 derivatives were formed under our assay conditions, we also performed a phosphorylation of Ins(1,4,5)P_3 by HsIPMK over 60 min under the conditions described by Saiardi et al. [7], which mainly differed from ours in having a much higher ATP concentration (10 mM versus 0.5 mM) and the presence of 20 mM phosphocreatine. Under these conditions a small amount (about 6%) of pyrophosphorylated InsP_5 (eluted at about the elution position of InsP_6) was detected by MDD-HPLC (results not shown).

HsIPMK tagged with GFP is localized within the nucleus of NRK 52E cells

The yeast protein ArgRIII is localized in the nucleus [1]. This localization is appropriate to its tasks in transcription regulation and mRNA export. A knowledge of the intracellular distribution of HsIPMK might give an insight into its probably similar functions. NRK 52E cells were transiently transfected

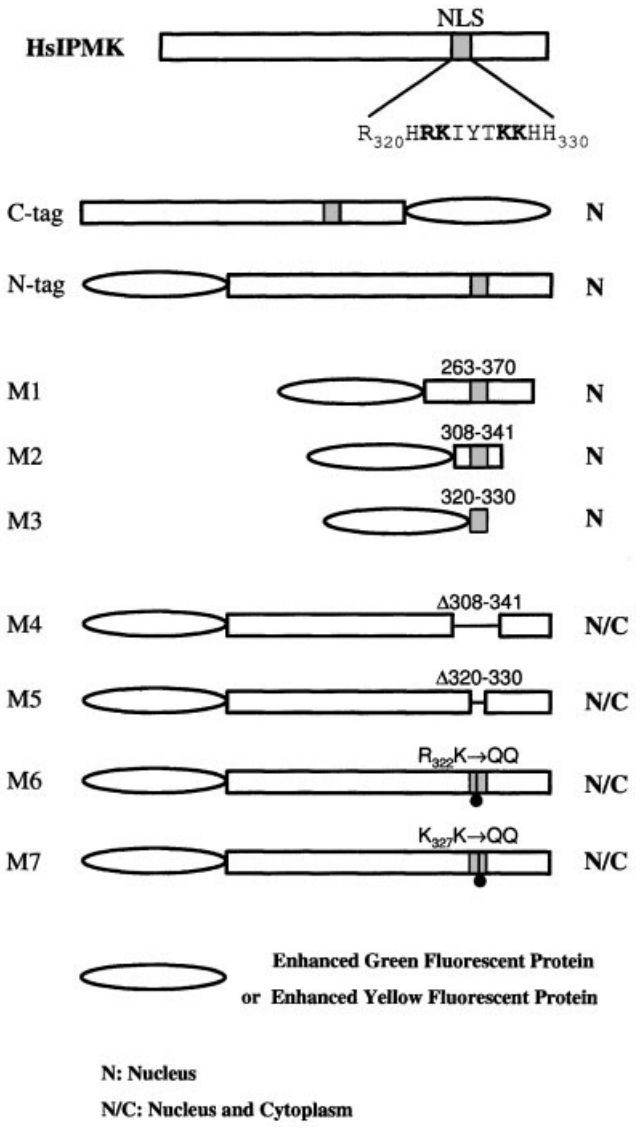

Figure 4 Structure of GFP–IPMK fusion proteins

The structure of GFP–IPMK fusion proteins and their derivatives is as follows: C-tag, IPMK with C-terminal EGFP; N-tag, IPMK with N-terminal EYFP; M1–M3, fusion proteins consisting of EYFP and various portions of the C-terminal region of IPMK (the positions of the first and the last amino acid of each portion are indicated.); M4 and M5, fusion proteins consisting of EYFP and IPMK missing different portions of the C-terminal region (deletions are represented by thin lines separating the boxes. The positions of the first and the last deleted amino acid are indicated.); M6 and M7, fusion proteins consisting of EYFP and IPMK with amino acid substitutions ($R^{322}K \rightarrow QQ$; $K^{327}K \rightarrow QQ$) in the C-terminal region (substitutions are marked by ●).

with the HsIPMK cDNA fused with a C-terminal (pEGFP-N1) and a N-terminal fluorescent protein tag (pEYFP-C1) respectively (Figure 4; C-tag, N-tag). Both arrangements of protein sequences in the fusion protein were used to rule out the possibility of steric effects of the fused fluorescent protein leading to protein misfold. The linker sequence consists mainly of small amino acids to ensure high flexibility. Both fusion proteins were localized predominantly within the nuclei of the transfected cells. The EYFP alone was distributed homogeneously between nucleus and cytoplasm. The nuclear localization was verified by co-staining of the DNA in the nucleus with DAPI. As clearly shown in the overlays the fluorescence of the fusion protein is

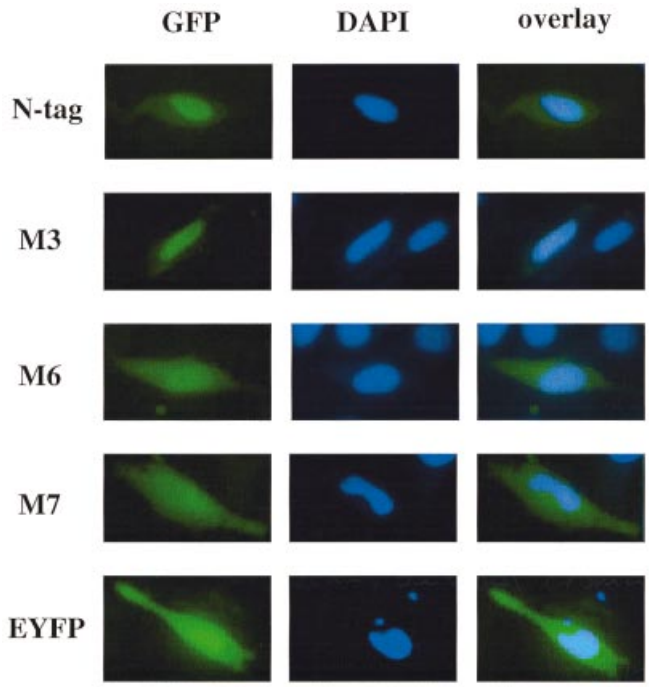

Figure 5 Intracellular localization of GFP–IPMK fusion proteins

NRK 52E cells were transiently transfected, fixed and the nuclei stained with DAPI. Fusion proteins (green) and DAPI (blue) were visualized by fluorescence microscopy as described in the Experimental section. An overlay of the digitized images was created showing the areas of co-localization (pale blue). The structure of the different fusion proteins and derivates is further characterized in Figure 4. The cellular localization of the EYFP when expressed alone was examined as control.

confined to the sole organelle stained by DAPI, the nucleus (Figure 5).

A basic cluster in the C-terminus of the HsIPMK is positively involved in nuclear targeting

The maximum size of proteins allowed to passively translocate through the nuclear pore complex is ≈ 40 kDa. Larger proteins require a nuclear localization signal (NLS) [15]. Because the HsIPMK tagged with a fluorescent protein has a molecular mass of ≈ 75 kDa, active NLS-directed transport across the nuclear membrane must occur. Consensus sequences for the classical monopartite and bipartite NLS have been defined (Figure 6A) [16]. No sequence was found within the HsIPMK that precisely fits to one or the other consensus. However, many proteins have been described up to now being efficiently transported into the nucleus without possessing a classical NLS [17]. Since most known non-classical NLSs have a high content of basic residues, we searched for such clusters in the HsIPMK. Indeed, there is a basic cluster in the C-terminus with five basic amino acids (lysine or arginine) in a segment of nine. We found that the position of the basic residues is conserved between this sequence and a fragment from the human c-Myc protein (Figure 6B), for which a weak NLS activity was shown [18].Therefore the putative effect of this tentative NLS on cellular topology of proteins was further examined.

Three fragments with decreasing length (M1, M2, M3) always containing the basic cluster in central location were tagged with a fluorescent protein. Fragments with variably sized flanking

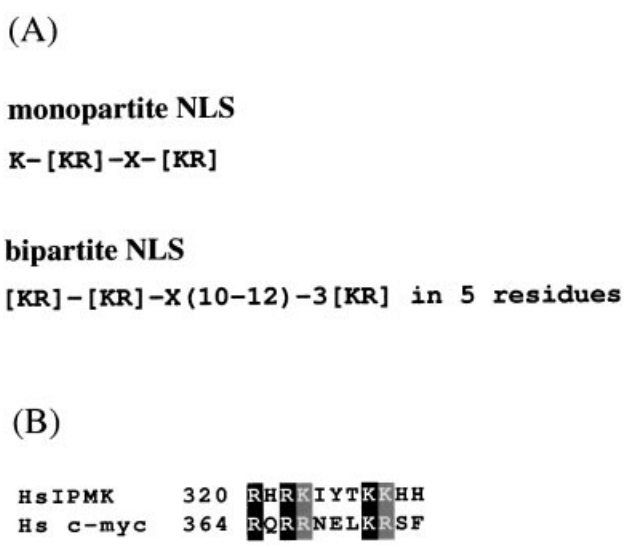

Figure 6 NLS of HsIPMK

HsIPMK possesses a functional NLS in its C-terminus. (**A**) The consensus sequences of the two classical NLSs are shown in the single-letter amino acid code. (**B**) Local sequence alignment of the NLS in the HsIPMK with a sequence in the human c-Myc protein, that possesses weak nuclear-localization activity. Numbers refer to the position of the first amino acid in the primary sequence of the protein shown. The single-letter amino acid code is used. Conserved residues are shown as white letters on a black background; similar residues are shown by white letters on a grey background.

sequences were used in order to rule out the possibility of protein misfold effects caused by disrupted secondary structures. All fragments fused with fluorescent protein were located predominantly in the nucleus like the full-length fusion protein (Figure 4). Therefore, the basic cluster (amino acids 320–330) in fact is a minimal sequence sufficient for nuclear localization.

Next we constructed deletion mutants (M4, M5) lacking the NLS. These mutants were equally distributed between cytoplasm and nucleus. The deletion of an internal sequence is a drastic operation, which can lead to misfolding and thus changes in the three-dimensional structure of a protein. These changes can mask a further NLS without its direct deletion. Therefore we carried out a less drastic strategy to functionally eliminate the putative NLS using double mutations of basic amino acids to polar non-basic amino acids. In both mutants (M6, M7) lysine and arginine residues in dipeptide segments were mutated to glutamine residues. Steric effects of these exchanges are unlikely, because the residues are similar in their steric demands and their hydrophilicity. Both double mutants were equally distributed between cytoplasm and nucleus (Figure 4).

DISCUSSION

HsIPMK can phosphorylate Ins(1,4,5)P_3 first either at its 3- or its 6-hydroxy group respectively and, in a subsequent InsP_4 phosphorylation reaction, at the remaining 6- or 3-hydroxy group respectively. The reason for the strongly differing intermediate InsP_4 concentrations is not necessarily that the pathway via Ins(1,3,4,5)P_4 is the strongly preferred one. In contrast, a lower K_m and/or higher V_{max} for the conversion of the minor (detectable) intermediate Ins(1,4,5,6)P_4 into InsP_5 as compared with the conversion of Ins(1,3,4,5)P_4 into InsP_5 may be the reason for its low concentration under near-steady-state conditions. Therefore HsIPMK possesses a substrate specificity that is similar to, although not identical with, that of yeast ArgRIII and RnIPMK. The isomer Ins(1,3,4,5)P_4 is the strongly preferred intermediate of human (the present results) and RnIPMK [7], in contrast with the preference of Ins(1,4,5,6)P_4 shown by yeast ArgRIII [4]. The intermediate Ins(1,4,5,6)P_4 is also produced by HsIPMK (the present results), but only in a small amount probably preventing its detection in the analysis of RnIPMK products. The rat and yeast enzyme lead also to pyrophosphorylated inositol phosphates [7,14], albeit with a much lower rate. Such products were not detected in our assay with HsIPMK, even after prolonged incubation time. Our assay employed a relatively low concentration of ATP (0.5 mM) as compared with that of Saiardi et al. [7], as well as a low concentration of phosphoenolpyruvate (1 mM) and pyruvate kinase instead of 20 mM phosphocreatine and phosphocreatine kinase. This, together with more salt in our assay, may explain the absence of pyrophosphorylation under our conditions. To test this assumption, the HsIPMK was assayed under conditions essentially as described by Saiardi et al. [7]. Now in addition to the described inositol phosphate products, a small amount of pyrophosphorylated InsP_5 (apparently two or three PP-InsP_4 isomers) were detected (results not shown). Therefore the observed divergence is probably caused by the different assay conditions. In summing up, the human enzyme represents an effective Ins(1,4,5)P_3 3-kinase with low K_m and high specific activity, as well as an Ins(1,4,5,6)P_4 3-kinase, an Ins(1,4,5)P_3 6-kinase, and an Ins(1,3,4,5)P_4 6-kinase. Of all the latter activities the Ins(1,3,4,5)P_4 6-kinase may be the less effective one, since only this intermediate strongly accumulates during the *in vitro* reaction phase near steady state. An evolutionary reason for this behaviour may be that the latter isomer is not only a reaction intermediate on the pathway to Ins(1,3,4,5,6)P_5, but plays some genuine physiological roles itself [19].

As shown in the present study, HsIPMK is localized predominantly in the nucleus. It can be concluded that a basic cluster in the protein's C-terminus is positively involved in the nuclear targeting, because its functional elimination changes the predominantly nuclear localization to an equal distribution between nucleus and cytoplasm (the present results). This still nuclear localization cannot be explained by passive diffusion, because of the fusion protein's size [15]. The nuclear targeting of proteins by two or more NLSs with equal [31] or different strength [18] is well known. Therefore we searched the protein for a further NLS marked by a high content of basic residues. A candidate sequence, namely a cluster (amino acids 107–112) containing three basic amino acids (lysine or arginine) in a segment of six, was further investigated. The examination of a double mutant with lysine residues in a dipeptide segment mutated to glutamine residues ($K^{107}K \rightarrow QQ$) revealed an intracellular distribution similar to that of the wild-type (results not shown). Therefore this cluster seems not necessary for nuclear localization, but probably its activity is only additional and its functional elimination is masked by the strong activity of the C-terminal cluster. To further investigate this hypothesis we created a mutant with an additional mutation of the C-terminal cluster ($K^{107}K \rightarrow QQ$ / $K^{327}K \rightarrow QQ$) known to eliminate its activity. The intracellular distribution of this mutant was comparable with that of the double mutant M7 ($K^{327}K \rightarrow QQ$) (results not shown). In consideration of these results, a functional NLS activity of the examined cluster seems unlikely. With no further candidate sequence remaining, it should be considered that the mutants with functionally eliminated NLS (M6, M7) are translocated into the nucleus together with an interaction partner by a co-transport mechanism [32]. Nuclear localization is not common among the inositol phosphate-metabolizing kinases. Non-nuclear localizations are reported for

the isoform A and B of the Ins(1,4,5)P_3 3-kinase (IP3K). The IP3K isoform A associates with F-actin and dendritic spines via its N-terminus [20]. The isoform B has been shown to be localized to internal membranes of the endoplasmic reticulum and Golgi apparatus [21]. The intracellular localization of the three isoforms of the mammalian InsP_6 kinase (IP6K) have been determined. The IP6K isoform 2 is predominantly nuclear, and the isoform 1 occurs at similar densities in nucleus and cytoplasm, whereas the isoform 3 is predominantly cytoplasmic [22]. Apoptosis appears to be influenced by the isoform 2. Deletion of this isoform reduces interferon-β-induced apoptosis in tumour cells in culture, whereas cells overexpressing this isoform are more affected by interferon-β-induced cell death [23].

It is reasonable to assume that mammalian inositol phosphate kinases in the nucleus possibly act together with other enzymes (e.g. nuclear phospholipase C [24]) forming a nuclear inositol phosphate signalling and phosphorylation pathway, ending up with highly phosphorylated inositol phosphates part of which are pyrophosphorylated [25,26] by nuclear IP6K isoform 2 [27]. In yeast a phospholipase C-dependent inositol phosphate kinase pathway leading up to InsP_6 is required for efficient mRNA export and normal cell growth [6]. The IP3K necessary for this pathway has been shown to be identical with ArgRIII [4]. The yeast protein ArgRIII, on the one hand, possesses IPMK activity, and, on the other hand, is a transcription regulator. Intriguingly, the latter function has been shown by enzymically inactive yeast mutants not to require IPMK activity [2]. ArgRIII stabilizes the transcription factors ArgRI and Mcm1 through direct interaction with their MADS (**M**CM1, **a**gamous, **d**eficiens and **S**RF)-box domains [1]. These two proteins, together with the arginine sensor ArgRII, form the ArgR–Mcm1 transcription complex [29]. The binding of freely available arginine by ArgRII leads to a change in the steric orientation, enabling the complex to bind specific DNA motifs that are located in the promoters of genes coding for important enzymes of arginine metabolism. The DNA-bound complex induces the expression of catabolic genes and represses the expression of anabolic genes [1]. According to first investigations with an ArgRIII–β-galactosidase fusion protein using isolated nuclei from yeast cells, 60 % of ArgRIII was localized in the nucleus [30]. These results were uncertain, because this technique is prone to an outflow of β-galactosidase from the nuclei during the experimental procedure. Recently the intracellular localization was further investigated in a more reliable way using an ArgRIII–GFP fusion protein. Detection of the GFP fluorescence in living yeast cells revealed a predominantly nuclear localization [1]. This localization of ArgRIII in the nucleus is a requirement to allow the interaction with the ArgR–Mcm1 transcription complex, its regulatory target. Future experiments have to clarify whether HsIPMK localized also predominantly in the nucleus has a similar transcription-regulatory function in addition to its enzymic activity. A possible approach is the identification of nuclear interaction partners, probably MADS-box proteins like ArgRI and Mcm1 [1].

On the basis of the nuclear localization of IPMK it is now certain that mammalian nuclei contain the complete set of enzymic activities for the biosynthesis of Ins(1,3,4,5,6)P_5 from nuclear Ins(1,4,5)P_3. Since InsP_6 is a better substrate of nuclear IP6K isoform 2 than Ins(1,3,4,5,6)P_5 [27], it is possible that this isomer is also synthesized in mammalian nuclei. The synthesis of InsP_6 by an Ins(1,3,4,5,6)P_5-2-kinase in yeast [6] and *Candida albicans* [28] has been well studied. The yeast protein is localized in the nucleus and in a punctate pattern at the nuclear periphery, which is typical of the yeast nuclear pore complex [6]. A recently identified homologue [33] still awaits investigation of its probably similar localization.

We thank Bettina Serreck for excellent technical assistance. This paper is based on the Doctoral Thesis of M.M.N. in the Faculty of Chemistry at the University of Hamburg. Financial support by the Deutsche Forschungsgemeinschaft (grant MA 989/3-1) given to G.W.M., the Graduiertenkolleg 336 and the Fonds der Chemischen Industrie is gratefully acknowledged.

REFERENCES

1 El Bakkoury, M., Dubois, E. and Messenguy, F. (2000) Recruitment of the yeast MADS-box proteins, ArgRI and Mcm1 by the pleiotropic factor ArgRIII is required for their stability. Mol. Microbiol. **35**, 15–31
2 Dubois, E., Dewaste, V., Erneux, C. and Messenguy, F. (2000) Inositol polyphosphate kinase activity of Arg82/ArgRIII is not required for the regulation of the arginine metabolism in yeast. FEBS Lett. **486**, 300–304
3 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
4 Odom, A. R., Stahlberg, A., Wente, S. R. and York, J. D. (2000) A role for nuclear inositol 1,4,5-trisphosphate kinase in transcriptional control. Science **287**, 2026–2029
5 Saiardi, A., Caffrey, J. J., Snyder, S. H. and Shears, S. B. (2000) Inositol polyphosphate multikinase (ArgRIII) determines nuclear mRNA export in *Saccharomyces cerevisiae*. FEBS Lett. **468**, 28–32
6 York, J. D., Odom, A. R., Murphy, R., Ives, E. B. and Wente, S. R. (1999) A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science **285**, 96–100
7 Saiardi, A., Nagata, E., Luo, H. R., Sawa, A., Luo, X., Snowman, A. M. and Snyder, S. H. (2001) Mammalian inositol polyphosphate multikinase synthesizes inositol 1,4,5-trisphosphate and an inositol pyrophosphate. Proc. Natl. Acad. Sci. U.S.A. **98**, 2306–2311
8 Wang, W. and Malcolm, B. A. (1999) Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange Site-Directed Mutagenesis. Biotechniques **26**, 680–682
9 De Larco, J. E. and Todaro, G. J. (1978) Epithelioid and fibroblastic rat kidney cell clones: epidermal growth factor (EGF) receptors and the effect of mouse sarcoma virus transformation. J. Cell. Physiol. **94**, 335–342
10 Bertsch, U., Haefs, M., Moller, M., Deschermeier, C., Fanick, W., Kitzerow, A., Ozaki, S., Meyer, H. E. and Mayr, G. W. (1999) A novel A-isoform-like inositol 1,4,5-trisphosphate 3-kinase from chicken erythrocytes exhibits alternative splicing and conservation of intron positions between vertebrates and invertebrates. Gene **228**, 61–71
11 Mayr, G. W. (1988) A novel metal-dye detection system permits picomolar-range h.p.l.c. analysis of inositol polyphosphates from non-radioactively labelled cell or tissue specimens. Biochem. J. **254**, 585–591
12 Mayr, G. W. (1990) Mass determination of inositol phosphates by high-performance-liquid-chromatography with postcolumn complexometry (metal-dye-detection). In Methods in Inositide Research (Irvine, R. F., ed.), pp. 83–108, Raven Press, New York
13 Guse, A. H., Goldwich, A., Weber, K. and Mayr, G. W. (1995) Non-radioactive, isomer-specific inositol phosphate mass determinations: high-performance liquid chromatography-micro-metal-dye detection strongly improves speed and sensitivity of analyses from cells and micro-enzyme assays. J. Chromatogr. B Biomed. Appl. **672**, 189–198
14 Zhang, T., Caffrey, J. J. and Shears, S. B. (2001) The transcriptional regulator, Arg82, is a hybrid kinase with both monophosphoinositol and diphosphoinositol polyphosphate synthase activity. FEBS Lett. **494**, 208–212
15 Cole, C. N. and Hammell, C. M. (1998) Nucleocytoplasmic transport: driving and directing transport. Curr. Biol. **8**, 368–372
16 Fontes, M. R., Teh, T. and Kobe, B. (2000) Structural basis of recognition of monopartite and bipartite nuclear localization sequences by mammalian importin-alpha. J. Mol. Biol. **297**, 1183–1194
17 Christophe, D., Christophe-Hobertus, C. and Pichon, B. (2000) Nuclear targeting of proteins: how many different signals? Cell. Signaling **12**, 337–341
18 Dang, C. V. and Lee, W. M. (1988) Identification of the human c-myc protein nuclear translocation signal. Mol. Cell. Biol. **8**, 4048–4054
19 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
20 Schell, M. J., Erneux, C. and Irvine, R. F. (2001) Inositol 1,4,5-trisphosphate 3-kinase A associates with F-actin and dendritic spines via its N terminus. J. Biol. Chem. **276**, 37537–37546
21 Soriano, S., Thomas, S., High, S., Griffiths, G., Dsantos, C., Cullen, P. and Banting, G. (1997) Membrane association, localization and topology of rat inositol 1,4,5-trisphosphate 3-kinase B: implications for membrane traffic and Ca^{2+} homoeostasis. Biochem. J. **324**, 579–589

22 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
23 Morrison, B. H., Bauer, J. A., Kalvakolanu, D. V. and Lindner, D. J. (2001) Inositol hexakisphosphate kinase 2 mediates growth suppressive and apoptotic effects of interferon-beta in ovarian carcinoma cells. J. Biol. Chem. **276**, 24965–24970
24 Cocco, L., Martelli, A. M., Gilmour, R. S., Rhee, S. G. and Manzoli, F. A. (2001) Nuclear phospholipase C and signaling. Biochim. Biophys. Acta **1530**, 1–14
25 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
26 Mayr, G. W., Radenberg, T., Thiel, U., Vogel, G. and Stephens, L. R. (1992) Phosphoinositol diphosphates: non-enzymatic formation *in vitro* and occurrence *in vivo* in the cellular slime mold *Dictyostelium*. Carbohydr. Res. **234**, 247–262
27 Saiardi, A., Caffrey, J. J., Snyder, S. H. and Shears, S. B. (2000) The inositol hexakisphosphate kinase family. Catalytic flexibility and function in yeast vacuole biogenesis. J. Biol. Chem. **11**, 24686–24692
28 Ives, E. B., Nichols, J., Wente, S. R. and York, J. D. (2000) Biochemical and functional characterization of inositol 1,3,4,5,6-pentakisphosphate 2-kinases. J. Biol. Chem. **275**, 36575–36583
29 Amar, N., Messenguy, F., El Bakkoury, M. and Dubois, E. (2000) ArgRII, a component of the ArgR–Mcm1 complex involved in the control of arginine metabolism in *Saccharomyces cerevisiae*, is the sensor of arginine. Mol. Cell. Biol. **20**, 2087–2097
30 Bercy, J., Dubois, E. and Messenguy, F. (1987) Regulation of arginine metabolism in *Saccharomyces cerevisiae*: expression of the three ARGR regulatory genes and cellular localization of their products. Gene **55**, 277–285
31 Imagawa, M., Sakaue, R., Tanabe, A., Osada, S. and Nishihara, T. (2000) Two nuclear localization signals are required for nuclear translocation of nuclear factor 1-A. FEBS Lett. **484**, 118–124
32 Melen, K. and Julkunen, I. (1997) Nuclear cotransport mechanism of cytoplasmic human MxB protein. J. Biol. Chem. **272**, 32353–32359
33 Verbsky, J. W., Wilson, M. P., Kisseleva, M. V., Majerus, P. W. and Wente, S. R. (2002) The synthesis of inositol hexakisphosphate: characterization of human inositol 1,3,4,5,6-pentakisphosphate 2-kinase. J. Biol. Chem. DOI 10.1074/jbc.M205682200

Received 25 February 2002/23 May 2002; accepted 23 May 2002
Published as BJ Immediate Publication 31 May 2002, DOI 10.1042/BJ20020327