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RESEARCH COMMUNICATION

The cloning and sequence of the C isoform of PtdIns4*P* 5-kinase

Nullin DIVECHA,*‡ Oanh TRUONG,† J. Justin HSUAN,† Katherine A. HINCHLIFFE* and Robin F. IRVINE*

*Inositide Laboratory, Department of Cell Signalling and Development, The Babraham Institute, Cambridge CB2 4AT, U.K., and †Ludwig Institute for Cancer Research, University College London Medical School, 91 Riding House Street, London W1P 8BT, U.K.

In this study we describe the purification and sequencing of the C isoform of platelet PtdIns4*P* 5-kinase. Subsequently a cDNA was isolated from a human circulating-leucocyte library, which when sequenced was shown to contain all of the peptides identified in the purified protein. In addition, expression of this cDNA in bacteria led to the production of a protein which was recognized by specific monoclonal antibodies raised to the bovine brain enzyme [Brooksbank, Hutchings, Butcher, Irvine and Divecha (1993) Biochem. J. **291**, 77–82] and also led to the appearance of PtdIns4*P* 5-kinase activity in the bacterial lysates. Interestingly, the cDNA showed no similarity to any of the previously cloned inositide kinases. A search of the DNA databases showed that two proteins from *Saccharomyces cerevisiae* shared close similarity to this enzyme, one of which, the *mss*4 gene product, has been implicated in the yeast inositol lipid pathway. These data suggest that the PtdIns4*P* 5-kinases are a new family of inositide kinases unrelated to the previously cloned phosphoinositide 3/4-kinases.

INTRODUCTION

PtdIns(4,5)P_2 plays a pivotal role in signal transduction. It serves as the principal substrate for the effector enzymes of two major intracellular signalling pathways, phosphoinositide (PI) 3-kinase [1] and phosphoinositidase C (PIC), and therefore is able to generate three distinct second messengers: PtdIns(3,4,5)P_3, diacylglycerol and Ins(1,4,5)P_3 [2]. Moreover, PtdIns(4,5)P_2 has recently been suggested to be an important cofactor in the activation of phospholipase D [3], to be involved in integrin-mediated cell adhesion [4] and to be implicated in cytoskeletal dynamics [5].

Given this range of activities, it is not surprising that the enzyme responsible for the formation of this key lipid, PtdIns4*P* 5-kinase, should be specifically regulated. Indeed there is evidence of its regulation by G-proteins [6–10], tyrosine phosphorylation [11–13] or serine phosphorylation [14–16]. Activation by phosphatidic acid has also been suggested [17,17a]. The major problem of defining regulation of this enzyme *in vivo* is that it is probably also sensitive to the relief of product inhibition [18], and hence can be modulated indirectly, for example, by any agonists that stimulate the hydrolysis of PtdIns(4,5)P_2 by the activation of PIC.

Although biochemical evidence is accumulating for the regulation of these enzymes, the continued lack of specific molecular tools has hampered studies. We have previously described the purification of the C isoform of PtdIns4*P* 5-kinase [19] and have raised specific monoclonal antibodies to it [20]. These monoclonal antibodies have shown that this isoform is particularly abundant in platelets.

Here we report that we have now purified and sequenced PtdIns4*P* 5-kinase C from platelets, and have isolated a cDNA clone (which encoded a protein containing all of the sequenced peptides) and expressed it in *Escherichia coli.*

MATERIALS AND METHODS

All enzymes used for molecular biology were from Promega. All radioisotopes were from Amersham. The peripheral-blood-cell library was from Stratagene and the human tissue Northern blot was from Clontech. All other reagents were of analytical grade.

Purification of the platelet PtdIns4*P* 5-kinase

Platelet cytosol from pig blood was isolated as described in Cullen et al. [21,22] and was loaded on a Q-Sepharose column. The column was developed with a linear salt gradient of buffer A [20 mM Tris/HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, 0.01 % Triton X-100, 10 % glycerol, phenylmethanesulphonyl fluoride (17 μg/ml)] to buffer A + 0.5 M NaCl. The active fractions were pooled and applied to a phosphocellulose column. The column was washed with buffer A + 0.3 M NaCl and developed with a linear gradient of A–A + 1.5 M NaCl. The active fractions, which were eluted at approx. 1 M NaCl, were pooled, desalted on a gel-filtration column (Sephadex G25) and applied to a heparin–Sepharose column. This was developed with a linear gradient of A–A + 1 M NaCl. The active fractions were pooled, desalted and applied to a Resource-Q column. Elution was achieved using a gradient of A–A + 0.5 M NaCl. The active fractions were analysed by SDS/PAGE, pooled, made 50 % with respect to glycerol and stored at −20 °C.

Microsequencing and cloning of the cDNA coding for PtdIns4*P* 5-kinase

The purified protein was separated by SDS/PAGE and the 53 kDa protein was excised and subsequently digested with lysyl-endoproteinase and endoproteinase Asp-N. The peptides were purified by HPLC using serial anion-exchange and octadecyl reverse-phase columns developed with an acetonitrile gradient. Fractions were applied to an automated Applied Biosystems 477A sequencer modified as described in [23]. A number of overlapping peptides were found, one of which was identical with a protein sequence encoded by a partial uncharacterized cDNA from a human adult bone-marrow library. Oligonucleotide primers (5′-GACATCCTTACTCATTATGAT-3′ and 5′-CGT-CAAGATGTGGCCAAT-3′) were designed to this region, and

Abbreviations used: PI, phosphoinositide; PIC, phosphoinositidase C.

‡ To whom all correspondence should be sent.

PCR was used to generate a 150 bp fragment that was subsequently used to screen a peripheral-blood-cell cDNA library; 1×10^6 bacteriophage plaques were screened as detailed in the manufacturer's instructions. Three clones were identified, which after excision of the phagemid were recognized by the PCR probe. The largest of these contained an insert of 4.1 kb (ND1) in the expression plasmid pBK-CMV phagemid, which was sequenced after the generation of deletions and by the use of specific oligonucleotide primers.

Expression of the PtdIns4*P* 5-kinase in bacteria

Either *E. coli* containing the ND1 cDNA, or the identical vector containing an unrelated insert, were grown overnight. After a 10-fold dilution, the bacteria were grown for 1 h before addition of isopropyl β-D-thiogalactoside (0.1 mM). Growth was continued for a further 4 h, after which the bacteria were washed twice with Tris-buffered saline (TBS), resuspended in 0.04 of the original volume of TBS/1 mM EGTA/1 mM EDTA, and disrupted by sonication. Cellular debris was removed by centrifugation and the supernatant assayed for PtdIns4*P* 5-kinase activity. In some cases the PtdIns4*P* 5-kinase was purified in a single step by batchwise chromatography using phosphocellulose. Briefly, 10 ml of cleared bacterial lysate was added to 2 ml of pre-activated phosphocellulose and allowed to bind for 1 h while rotating. The resin was washed with 50 ml of buffer A and the activity was eluted by addition of 1 ml of A + 1.5 M NaCl. This purified activity was used for determination of the specificity of the enzyme.

Assay for PtdIns4*P* 5-kinase activity

The assay was carried out in a final volume of 200 ml, containing 5 μM PtdIns4*P*, 50 μM PtdOH, 100 μM ATP together with 5 μCi of [γ-^{32}P]ATP. Assays were carried out for 10 min and quenched by addition of 1 ml of chloroform/methanol (1:1, v/v). The phases were split by addition of 250 μl of 2.4 M HCl. The bottom phase was dried and chromatographed on TLC plates preactivated by dipping in potassium oxalate (1 %) and heating at 110 °C for 1 h. The plates were developed in chloroform/methanol/water/ammonia (45:35:8:2, by vol.) for 2.5 h, dried and then exposed to film. Identification of lipids was carried out by inclusion of standards. In some cases, after TLC, the PtdIns(4,5)P_2 spot was deacylated [24] and the head group was analysed by chromatography on a Partisphere SAX column as described by Stephens et al. [1].

Immunoblotting

Proteins were separated by SDS/PAGE [25], transferred to nitrocellulose as described by Towbin et al. [26] and blocked by incubation in TBS/5 % dried milk/0.1 % Tween 20. The blot was then incubated with primary antibody for 2 h, washed six times with the above solution and incubated with a secondary antibody conjugated to horseradish peroxidase (Sigma). After washing three times in the above solution without milk, the blot was rinsed with TBS, and then revealed with the ECL reagent (Amersham).

RESULTS

The 53 kDa protein isolated from pig platelets is a PtdIns4*P* 5-kinase

The purification procedure was based on our previous purification of a PtdIns4*P* 5-kinase from bovine brain [19]. When the fractions from the Resource-Q column containing the PtdIns4*P* 5-kinase activity were analysed by SDS/PAGE, a protein with a molecular mass of 53 kDa was found (Figure 1a). Immunoprobing of this protein using the previously characterized monoclonal antibodies to the bovine brain enzyme showed that two different monoclonal antibodies recognized this band (Figure 1b). Immunoprecipitation using these antibodies showed a correlation between the presence of this band in the supernatant and the presence of PtdIns4*P* 5-kinase activity. Immunoprecipitation followed by assaying of the supernatant for radioactivity incorporated into PtdIns(4,5)P_2 gave the following results: no antibody 3795 c.p.m., irrelevant antibody 3684 c.p.m., +monoclonal antibodies to PtdIns4*P* 5-kinase 200 c.p.m. Immunoblotting of the supernatant after immunoprecipitation demonstrated that all of the 53 kDa epitope was removed by the monoclonal antibodies, but not by the other treatments. Head-group analysis of the product of this enzymic reaction showed that the enzyme was indeed a 5-kinase (results not shown). These data unequivocally demonstrate that the 53 kDa protein is a PtdIns4*P* 5-kinase. This protein was then used to generate peptide sequence (see the Materials and methods section) after excision of the 53 kDa protein from an SDS/PAGE gel.

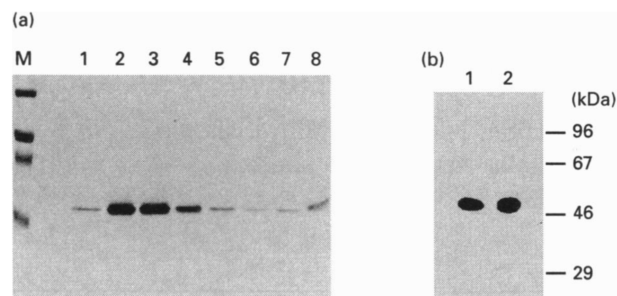

Figure 1 Pig platelets contain a 53 kDa protein which is a PtdIns4*P* 5-kinase

(**a**) After chromatography on a Resource-Q column, the fractions containing PtdIns4*P* 5-kinase activity were electrophoresed on a 10%-acrylamide gel and stained with Coomassie Brilliant Blue R250. The elution of the 53 kDa protein mirrored the elution of the activity, with lanes 2 and 3 containing the highest activity. Lane M contains the molecular-mass markers, of 200 kDa, 96 kDa, 67 kDa and 46 kDa. (**b**) The pooled fractions from the Resource-Q column (lanes 1 and 2 from **a**) were separated by SDS/PAGE, transferred to nitrocellulose and immunoprobed with two different monoclonal antibodies raised against the bovine brain PtdIns4*P* 5-kinase. The 53 kDa protein was recognized by both of these antibodies. In other studies, the platelet enzyme was immunoprobed with three other monoclonal antibodies, all of which recognized this protein. These data suggest that the 53 kDa protein is the pig homologue of the bovine brain PtdIns4*P* 5-kinase.

The cDNA ND1 codes for the 53 kDa protein

Screening of the translated DNA database yielded a sequence within which was a peptide which matched exactly with one of our overlapping sequenced peptides (DILTHY-FIGHILT; see Figure 2). On the basis of this cDNA, oligonucleotides (see the Materials and methods section) were prepared and a 150 bp fragment corresponding to the sequence of this peptide was generated by PCR from the circulating lymphocyte library. This PCR product was then used to screen the above library. Three clones were identified, one of which contained a 4.1 kb insert. After sequencing approx. 2 kb of this insert, an open reading frame was found which encoded a protein of 406 amino acids, with a deduced molecular mass of 46 kDa (Figure 2). All of the peptides that we had sequenced were found in this open reading

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TTACACTTTATACTTCCGGCTCGAATATTGTGTGGAATTGTGANCGGATAACAATTTCAC  60
ACAGGAAACANCTATGACCTTGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAA 120
CAAAAGCTGGAGCTCGCGCGCCTGCAGGTCGACACTAGTGGATCCAAAGAATTCGGCACG 180
AGGCGACGGGCGGAGCGGAGCGCGGCGCGCCGGGGCCGCCGCCGGGGGGATCGGCTGCCT 240
CCCCGGGCCGGGTGTAGAGAGGGCGGGTCCCCGGCCTCGGGAGCACGGCGGTGGAGGGGA. 300
               M  A  T  P  G  N  L  G  S  S  V  L  A  S  K
CATAGGAGGCGGCC ATGGCGACCCCCGGCAACCTAGGGTCCTCCGTCCTGGCGAGCAAG 360
T  K  T  K  K  K  H  F  V  A  Q  K  V  K  L  F  R  A  S  D
ACCAAGACCAAGAAGAAGCACTTCGTAGCGCAGAAAGTGAAGCTGTTTCGGGCCAGCGAC 420
P  L  L  S  V  L  M  W  G  V  N  H  S  I  N  E  L  S  H  V
CCGCTGCTCAGCGTCCTCATGTGGGGGGTAAACCACTCGATCAATGAACTGAGCCATGTT 480
Q  I  P  V  M  L  M  P  D  D  F  K  A  Y  S  K  I  K  V  D
CAAATCCCTGTTATGTTGATGCCAGATGACTTCAAAGCCTATTCAAAAATAAAGGTGGAC 540
N  H  L  F  N  K  E  N  M  P  S  H  F  K  F  K  E  Y  C  P
AATCACCTTTTTAACAAAGAAAACATGCCGAGCCATTTCAAGTTTAAGGAATACTGCCCG 600
M  V  F  R  N  C  G  K  R  F  G  I  D  V  Q  D  F  Q  N  S
ATGGTCTTCCGTAACTGCGGGAAGAGGTTTGGAATTGATGTTCAAGATTTCCAGAATTCC 660
L  T  R  S  A  P  L  P  N  D  S  Q  A  R  S  G  A  R  F  H
CTGACCAGGAGCGCACCCCTCCCCAACGACTCCCAGGCCCGCAGTGGAGCTCGTTTTCAC 720
T  S  Y  D  K  R  Y  M  I  K  T  I  T  S  E  D  V  A  E  M
ACTTCCTACGACAAAAGATACATGATCAAGACTATTACCAGTGAAGACGTGGCCGAAATG 780
H  N  I  L  K  K  Y  H  Q  Y  I  V  E  C  H  G  I  T  L  L
CACAACATCCTGAAGAAATACCACCAGTACATAGTGGAATGTCATGGGATCACCCTTCTT 840
P  H  L  L  G  M  Y  R  L  N  V  D  G  V  E  I  Y  V  I  V
CCCCACTTGTTGGGCATGTACCGGCTTAATGTTGATGGAGTTGAAATATATGTGATAGTT 900
T  R  N  V  F  S  H  R  L  S  V  Y  R  K  Y  D  L  K  G  S
ACAAGAAATGTATTCAGCCACCGTTTGTCTGTGTATAGGAAATACGACTTAAAGGGCTCT 960
T  V  A  R  E  A  S  D  K  E  K  A  K  E  L  P  T  L  K  D
ACAGTGGCTAGAGAAGCTAGTGACAAAGAAAAGGCCAAAGAACTGCCAACTCTGAAAGAT 1020
N  D  F  I  N  E  G  Q  K  I  Y  I  D  D  N  S  K  K  V  F
AATGATTTCATTAATGAGGGCCAAAAGATTTATATTGATGACAACAGCAAGAAGGTCTTC 1080
L  E  K  L  K  K  D  V  E  F  L  A  Q  L  K  L  M  D  Y  S
CTGGAAAAACTAAAAAAGGATGTTGAGTTTCTGGCCCAGCTGAAGCTCATGGACTACAGT 1140
L  L  V  G  I  H  D  V  E  R  A  E  Q  E  E  V  E  C  E  E
CTGCTGGTGGGAATTCATGATGTGGAGAGAGCCGAACAGGAGGAAGTGGAGTGTGAGGAG 1200
N  D  G  E  E  E  G  E  S  D  G  T  H  P  V  G  T [P  P  D
AACGATGGGGAGGAGGAGGGCGAGAGCGATGGCACCCACCCGGTGGGAACCCCCCCAGAT 1260
S  P] G  N  T  L  N  S  S [P  P  L  A  P] G  E  F  E  P  N
AGCCCCGGGAATACACTGAACAGCTCACCACCCCTGGCTCCCGGGGAGTTCGAGCCGAAC 1320
I  D  V  Y  G  I  K  C  H  E  N  S  P  R  K  E  V  Y  F  M
ATCGACGTCTATGGAATTAAGTGCCATGAAAACTCGCCTAGGAAGGAGGTGTACTTCATG 1380
A  I  I  D  I  L  T  H  Y  D  A  K  K  K  A  A  H  A  A  K
GCAATTATTGACATCCTTACTCATTATGATGCAAAAAAGAAAGCTGCCCATGCTGCAAAA 1440

T  V  K  H  G  A  G  A  E  I  S  T  V  N  P  E  Q  Y  S  K
ACTGTTAAACATGGCGCTGGCGCGGAGATCTCCACCGTGAACCCAGAACAGTATTCAAAG 1500
R  F  L  D  F  I  G  H  I  L  T  •
CGCTTTTTGGACTTTATTGGCCACATCTTGACGTAACCTCCTGCGCAYCTCGGACAGCAT 1560
GAACATTGGATGGACAGAGGTGGCTTCGGTGTAGGAAAAATGAAAACCAAACTCAGTGAA 1620
GTACTCATCTTGCAGGAAGCAAACCTCCTTGTTTACATCTTCAGGCCAAGATGACTGATT 1680
TGGGGGCTACTCGCTTTACAGCTACCTGATTTTCCCAGCATCGTTCTAGCTATTTCTGAC 1740
TTTGTGTATATGTGTGTGTGTGTGTGTTGGGGGGGGGTGAGTGTGTGCCCGCGTGTGCAT 1800
TTAAAGCATAAATTAATTAAACAGCCACTTCGGTCA                         1836
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Figure 2 The clone ND1 codes for a 46 kDa protein containing all of the sequenced peptides

Analysis of the ND1 clone showed the presence of one open reading frame starting with an ATG within a putative Kozak consensus sequence: 315 base pairs were sequenced 5′ of this start sequence, and at least five stop codons were present in all reading frames within that region. The Figure shows the DNA sequence and the translation of the whole reading frame, with amino acids in single-letter code above the corresponding DNA sequence. Peptides that were found by sequencing of the protein are underlined. The underlined DNA sequence delineates the region that differs from the published sequence of Boronenkov and Anderson [37]. The two putative SH3-domain binding motifs are boxed. The • denotes the presence of a stop codon in the sequence. No other sequence similarities were found within this reading frame.

frame (Figure 2). Although the peptides were derived from an enzyme isolated from pig platelets and the clone was from a human peripheral-blood-leucocyte library, conservation of the amino acids was extremely high. In fact, from 234 amino acids that were sequenced only three did not match. This suggests that ND1 codes for the 53 kDa protein that we isolated from pig platelets. No common domain structures such as PH, CAL B, SH2 or SH3 domains were found when the sequence was analysed by using a motifs package. There was also no conserved ATP-binding site, which one might have expected in a lipid kinase. There were, however, two small proline-rich sequences which fit a consensus defining ligands able to associate with SH3 domains (Figure 2; [27,28]). Surprisingly, there was no similarity between this clone and any of the previously cloned inositide kinases [29–31].

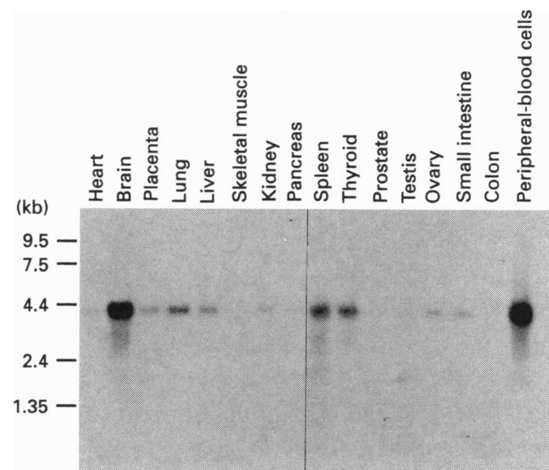

Figure 3 The mRNA coding for the PtdIns4*P* 5-kinase C isoform is highly expressed in brain and in peripheral-blood leucocytes

A human tissue Northern blot was probed with a 150 bp ^{32}P-labelled probe, followed by washing under high stringency. The exposure shown was for 30 h. On a longer exposure a mRNA of 2.3 kb was also shown to be present in most tissues, being most prominent in the testis.

Distribution of the mRNA, studied by Northern analysis, showed that a 4.1 kb mRNA was widely distributed, being present in most tissues, except notably skeletal muscle and small intestine. Interestingly, the mRNA was present in high levels both in brain and in peripheral-blood leucocytes (Figure 3). This pattern of distribution is similar to that seen for the PtdIns4*P* 5-kinase C enzyme by using the antibodies to immunoprobe various murine tissues (results not shown). On a long exposure a smaller mRNA of 2.3 kb was also shown to be present in testis.

Clone ND1 codes for a PtdIns4*P* 5-kinase

Lysates from bacteria containing either the ND1 clone or the identical vector containing an unrelated insert were separated by SDS/PAGE and immunoprobed with monoclonal antibodies against the bovine brain PtdIns4*P* 5-kinase. A protein with a molecular mass of 46 kDa was recognized by the antibody which was only present in lysates from bacteria containing the ND1 clone (Figure 4a, lane 1). It is noticeable that the protein recognized in the bacterial lysates had a molecular mass of 46 kDa, whereas the purified protein electrophoresed with a molecular mass of 53 kDa. This may be due to post-translational modification of the protein in eukaryotes. When these lysates were assayed for PtdIns4*P* kinase activity, only those from bacteria containing the ND1 clone (Figure 4b, lane 2) were able to produce PtdInsP_2. Chromatography of these lysates on phosphocellulose yielded a fraction from the ND1 lysates which was able to phosphorylate PtdIns4*P* to produce PtdInsP_2 (Figure 4b, lanes 6 and 7). No PtdIns4*P* kinase activity was found in bacterial lysates either from wild-type (Figure 4b, lane 1) or from those expressing an unrelated insert (Figure 4b, lane 3), even

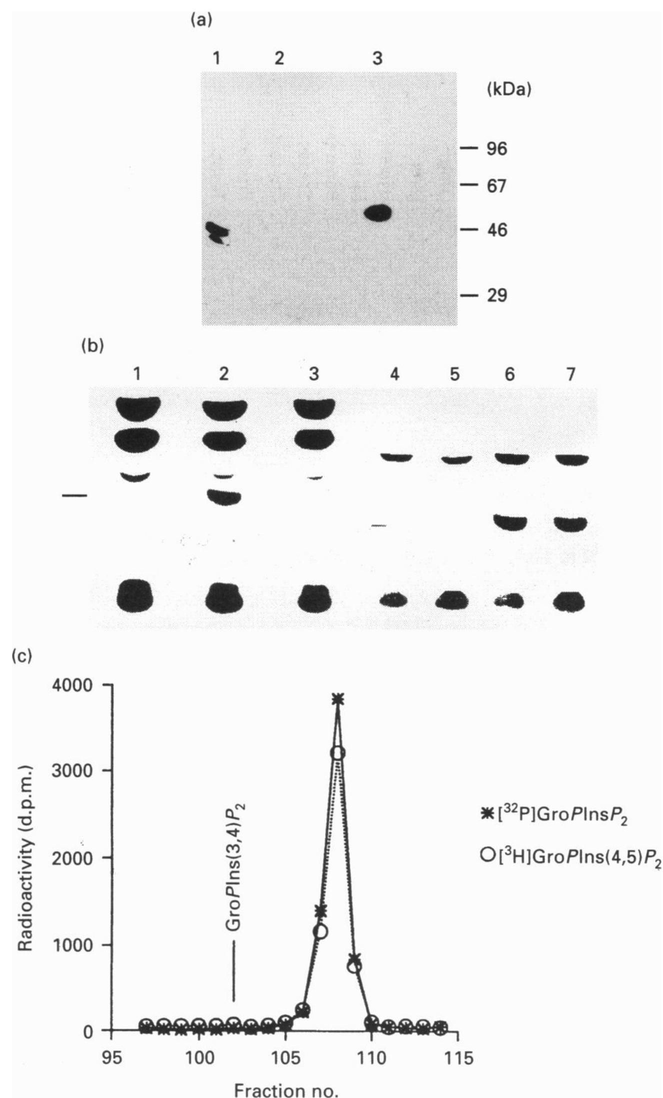

Figure 4 ND1 codes for a PtdIns4P 5-kinase

(**a**) Lysates from bacteria carrying ND1 (lane 1) or an unrelated insert (lane 2) were separated by SDS/PAGE, trasferred to nitrocellulose and immunoprobed with a mixture of monoclonal antibodies raised against the bovine brain PtdIns4P 5-kinase. A protein of molecular mass 46 kDa was recognized only in bacteria carrying ND1. Lane 3 contains purified platelet PtdIns4P 5-kinase as a positive control. The difference in electrophoretic mobility may represent post-translational modification of the purified protein. (**b**) Lysates from wild-type bacteria (lane 1) or from those containing ND1 (lane 2) or an irrelevent insert (lane 3) were assayed for PtdIns4P 5-kinase activity. In some cases, to remove other contaminants, the lysates from ND1 bacteria or from those containing an unrelated insert were chromatographed on phosphocellulose. The fraction eluted with 1.5 M NaCl from ND1 (lanes 6 and 7) or from the non-related insert (lanes 4 and 5) was assayed for PtdIns4P 5-kinase activity. The horizontal lines denote the migration of authentic PtdIns(4,5)P_2. (**c**) The [^{32}P]PtdIns(4,5)P_2 generated from the ND1 bacteria after chromatography on phosphocellulose was deacylated and chromatographed, after addition of authentic Gro$P$Ins(4,5)P_2 labelled with ^{3}H, on a Partisphere SAX column. The co-elution of these compounds unequivocally demonstrates that the clone ND1 codes for a PtdIns4P 5-kinase.

after chromatography on phosphocellulose columns (Figure 4b, lanes 4 and 5). To determine unequivocally that this activity was a 5-kinase, the ^{32}P-labelled PtdInsP_2 was deacylated and chromatographed with a ^{3}H-labelled glycerophosphoinositol 4,5-bisphosphate [Gro$P$Ins(4,5)P_2] internal standard. Both the ^{32}P-labelled product and the ^{3}H standard co-chromatographed identically in a gradient that would have separated Gro$P$Ins(4,5)P_2 from Gro$P$Ins(3,4)P_2 (Figure 4c).

DISCUSSION

The cloning of this PtdIns4P 5-kinase represents a significant step forward: not only does it provide the tools required for a molecular analysis of the enzyme and its functions *in vivo*, but it means that the structure of PI 3/4-kinases is no longer the only paradigm for inositide kinases. In this study we purified a PtdIns4P 5-kinase from platelets, sequenced this protein and isolated a cDNA clone which when expressed in bacteria gave rise to a 46 kDa protein. This protein was recognized by specific antibodies raised against the bovine brain enzyme and also had PtdIns4P 5-kinase activity. The cloning has suggested the presence of two SH3-domain binding motifs, which may throw some light on the mechanisms of regulation. One of the common steps that occurs on activation of a signal-transduction pathway appears to be the formation of a multi-protein complex which leads to the compartmentation of the key components of that pathway (e.g. recruitment of PICγ and of PI 3-kinase to receptors). In the case of the activation of the PIC or the PI 3-kinase pathway, this microcompartmentation would perhaps require the continued resynthesis of the substrate, PtdIns(4,5)P_2, and thus one might expect that the enzymes required for this would also become compartmentalized. PtdIns4P 5-kinase C, through the presence of the SH3 binding motif, may be able to associate with the SH3 domains present in PICγ and PI 3-kinase. Moreover, in recent studies the phosphatidylinositol-transfer protein has been shown to be required for receptor activation of the PIC pathway [32]. It has been suggested that this protein acts to shuttle substrates from one enzyme of that pathway to the next. It would obviously be more efficient if the components were first recruited to a common point. Indeed, there is evidence showing that a PtdIns4P 5-kinase activity is constitutively associated with the EGF (epidermal growth factor) receptor, although the mechanism for this association is not known [11]. Recruitment of both the EGF receptor and inositide kinases to the cytoskeleton has also been demonstrated [33]. Another example of recruitment of this enzyme during receptor activation has come from work on platelets. Here, activation by thrombin leads to aggregation and to the association of a PtdIns4P 5-kinase activity with the cytoskeletal fraction in an integrin-dependent fashion [34,35]; we have demonstrated that it is the C isoform of the enzyme that is recruited in this case (N. Divecha and K. A. Hinchliffe, unpublished work).

A search of the DNA database showed that, although there were no similarities to previously cloned inositide kinases, there were two proteins from *Saccharomyces cerevisiae* which did share similarity. One of these proteins, mss4 [35a], shows 32% identity in a 360-amino-acid overlap. However, there are regions of 58% identity, which increases to 78% when conserved residues are taken into account. The *mss4* gene was initially identified as a gene able to suppress a mutation in the *sst4* gene. The *sst4* gene product has been shown to be a PtdIns 4-kinase, the enzyme which provides the substrate for PtdIns4P 5-kinase [36]. Interestingly, mss4 is unable to surpress a null mutation of the *sst4* gene. These data, together with the similarity to our cloned PtdIns4P 5-kinase, are consistent with *mss4* coding for a PtdIns4P 5-kinase. The other protein, FAB1, also shows a high degree of similarity over similar regions of the kinase. While we were preparing this manuscript, there was a report from Boronenkov and Anderson [37], describing the cloning of an erythrocyte PtdIns4P 5-kinase. Their sequence is nearly identical with ours and demonstrates that the type II enzyme [38] is very similar to

the PtdIns4*P* 5-kinase C isoform, as previously suggested by immunological data [19]. The sequences differ in a region between amino acids 297 and 311. However, the presence of the peptide DGTHPV in our sequence strongly suggests that this is merely due to a frame shift due to an extra T at position 1206 and the omission of a G at position 1245 (see Figure 2).

The cloning of PtdIns4*P* 5-kinase C thus defines a new family of inositide kinases. It is likely that different isoforms of the enzyme are required for different functions, suggesting that the PtdIns4*P* 5-kinase family will resemble the other families of enzymes involved in signal transduction. The molecular elucidation of the other members of this family will lead to an understanding of the regulation of this key enzyme.

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