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Identification of a 200 kDa polypeptide as type 3 phosphatidylinositol 4-kinase from bovine brain by partial protein and cDNA sequencing

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Abstract

Two phosphatidylinositol 4-kinase isozymes, type 3 and type 2, have been separated on hydroxylapatite after solubilizing bovine brain microsomes with Triton X-114. Employing a newly developed renaturation procedure following SDS-PAGE, we demonstrate that a 200 kDa polypeptide carries the enzymic activity of this type 3 isoform. Chromatography on hydroxylapatite, Heparin-Sepharose, Superdex 200 and finally SDS-PAGE results in an approximately 30 000-fold purification. Tryptic peptides generated from the 200 kDa polypeptide after SDS-PAGE have been sequenced and the obtained data have been used for constructing and synthesizing degenerated oligonucleotides. Polymerase chain reaction as well as screening of cDNA libraries allowed several clones to be isolated from which a 4.7 kb contiguous sequence can be built up. The open reading frame covers 4.4 kb with a 0.3 kb untranslated 3' end which yields a deduced amino acid sequence of 1,467 amino acids. The C-terminal part of ca. 300 amino acids represents the catalytic domain. Sequence alignment of this domain with the mammalian counterpart, the human type 2 phosphatidylinositol 4-kinase, the yeast kinases STT4 and PIK1, as well as with the catalytic domains of bovine, human, mouse and yeast phosphatidylinositol 3-kinases reveals a high degree of identity: 26 of these approximately 300 amino acids are invariable in all of these eight catalytic domains. Five motifs indicate nuclear localization and DNA binding properties of the enzyme. Two leucine zipper motifs (amino acids 358-386, 862-882) are detectable. Furthermore, a helix loop helix motif (amino acids 716-729) as well as two nuclear localization signals (amino acids 838-854, 345-349) indicate the presence of the type 3 isoform in the nucleus.

Keywords: Phosphatidylinositol 4-kinase; Bovine brain; Primary structure

1. Introduction

In membranes of eukaryotic cells phosphoinositide synthesis and degradation are well known signaling events by which growth factors, hormones and neurotransmitters can exert their physiological effects (for review, see Refs. [1,2]). One of these signaling pathways starts by phosphorylating PtdIns at the 4′ and at the 5′ position. The resulting product, PtdIns(4,5)P_2, is cleaved hydrolytically into two second messengers, diacylglycerol and Ins(1,4,5)P_3 by phospholipase C (for review, see Refs. [3,4]). An alternate signaling pathway involves a series of polyphosphoinositides phosphorylated at the 3′ position of the inositol ring which gives rise to the derivatives PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (for review, see Ref. [5]).

Little is known about the regulatory mechanisms of PtdIns phosphorylation. Generally, when phospholipase C is stimulated, a fall in the PtdIns(4)P and PtdIns(4,5)P_2 concentrations is followed by rapid resynthesis. There are indications that the PtdIns/PtdIns(4,5)P_2 ratio can be changed by activating protein kinase C or by increasing the cAMP level (for review, see Ref. [4]). However, the

Abbreviations: DOC, desoxycholate; PtdIns, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol; PtdIns(4)P, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4-phosphate; PtdIns(4,5)P_2, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4,5-bisphosphate; PMSF, phenylmethylsulfonyl fluoride; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; PI4K200, 1-(3-*sn*-phosphatidyl)-D-myo-inositol 4-phosphate kinase from bovine brain with an apparent molecular weight of 200 k; PI4K α, human PtdIns 4 kinase, type 2; HPLC, high performance liquid chromatography.; Enzymes: phosphatidylinositol 4-kinase (EC 2.7.1.67).

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complex metabolism of the polyphosphoinositides does not allow these alterations to be unequivocally assigned to specific enzymatic steps. Moreover, PtdIns(4)P is formed not only in plasma membranes but also in most internal membranes such as in sarcoplasmic reticular [6,7], liver endoplasmic reticular [8] and in lysosomal membranes [9] as well as in Golgi [10]. In the sarcoplasmic reticulum of skeletal [11] and smooth muscle [12], PtdIns(4)P has been characterized as an activator of the Ca^{2+} transport ATPase. Several enzymes involved in the PtdIns cycle have been identified in the nucleus [13,14].

Enzymatically but not structurally at least two different PtdIns 4-kinases have been partially characterized in mammalian tissues [5,15–17]. Type 2 is a 55-kDa enzyme present in all animal cells, whereas the type 3 isoform expresses high activity in bovine and rat brain as well as in bovine uterus. These two PtdIns 4-kinases, type 2 and 3, differ from PtdIns 3-kinase (originally termed as type 1 PtdIns kinase; for review see Refs. [5,18]) in their requirement of non-ionic detergent for enzyme activity and in their resistance to Triton X-100 inhibition up to 3%. Moreover, the type 2 enzyme is inhibited by adenosine ca. 20-fold stronger than the type 3 enzyme; furthermore the K_m values for the substrates PtdIns and ATP/Mg^{2+} are 3- to 7-fold lower for the type 2 than for the type 3 enzyme, respectively [5,15,17]. In *S. cerevisiae* multiple forms of 45 and 55 kDa have also been demonstrated and have been proven to be membrane-bound [19,20]; in addition, a soluble PtdIns 4-kinase of 125 kDa exists [21].

Two genes encoding PtdIns 4-kinases were isolated from *S. cerevisiae*. A 125-kDa form, PIK1, seems to be indispensable to cell growth [21], whereas it has been suggested that STT4, the other PtdIns 4-kinase – a 200 kDa protein – is possibly involved in the protein kinase C pathway [22].

A human type 2 PtdIns 4-kinase (PI4Kα) has recently been cloned from placental and brain libraries [23] and has been shown to be homologous to the yeast PtdIns 4-kinases, STT4 and PIK1 [21,24], as well as to PtdIns 3-kinase family genes such as bovine [25], human [26], mouse p110 [27], and yeast Vps34 [28].

Here we report the first characterization of the type 3 PtdIns 4-kinase from bovine brain by isolating the protein, by characterizing its enzymological properties as well as by partial protein and cDNA sequencing. The protein contains a C-terminal catalytic domain homologous to the human and yeast PtdIns 4-kinases and, surprisingly, motifs indicating nuclear localization.

2. Materials and methods

2.1. Enrichment of PtdIns 4-kinase from bovine brain

Step 1: Preparation of solubilized membrane fraction. From fresh bovine brain (100–130 g) the skin was removed and the brain was homogenized in an ultraturrax for 1 min in 350 ml buffer containing 10 mM Tris-HCl, 1 mM DTE, 0.1 mM EDTA, 0.32 M saccharose, 2 mM benzamidine, 2 mM aminocaproic acid, 0.1 mM EGTA, 0.1 mM PMSF, 2 μg/ml aprotinin, 2 μM leupeptin (pH 7.5), and was then further disrupted in a tightly fitting teflon glass homogenizer (Braun-Melsungen, FRG). This homogenate was centrifuged at $1000 \times g$ for 10 min and subsequently the supernatant (crude extract) was centrifuged at $100000 \times g$ for 70 min. The sediment was resuspended in 10 mM Tris-HCl, 1 mM DTE, 0.1 mM EDTA (pH 7.5) (buffer A) containing 2 μM leupeptin, 0.9% NaCl, and again homogenized in the ultraturrax. Membranes were collected by centrifugation at $100000 \times g$ for 60 min and resuspended in 300 ml buffer A containing 1 mM benzamidine, 1 mM aminocaproic acid, 0.05 mM EGTA, 0.05 mM PMSF, 1 μg/ml aprotinin and 1 μM leupeptin. At 2 mg protein/ml the membranes were solubilized by stirring with 0.1% (w/v) Triton X-114 at 2°C for 20 min according to Bordier [29]. Increasing the temperature to 30°C resulted in a phase separation which was accelerated by centrifugation at $15000 \times g$ for 25 min. The PtdIns 4-kinase present in the water phase (4°C) was precipitated with ammonium sulfate up to 30% saturation and collected by centrifuging it at $54000 \times g$ for 30 min. The pellet was dissolved in 5 mM Tris-HCl, 0.5 mM DTE, 0.05 mM EDTA, 0.1% Triton X-100 (pH 7.5) and dialyzed overnight against the same buffer. Protease inhibitors were added to the dialysate to reach final concentrations of 2.5 μg aprotinin/ml, 0.05 mM PMSF and 2.5 μM leupeptin. This preparation can be stored at −20°C for several months without causing a significant loss of activity.

Step 2: Hydroxylapatite chromatography 1. The dialyzed material was adjusted to 500 mM NaCl, 0.06 mM EDTA and then centrifuged for 60 min at $100000 \times g$. The supernatant was applied to hydroxylapatite (diam. 5 cm; bed volume 160 ml), pre-equilibrated with 10 volumes of buffer B (5 mM KH_2PO_4/KOH, 1 mM DTE, 500 mM NaCl (pH 7.0) flow rate 180 ml/h, during elution 240 ml/h). The column was first washed with 3500 ml of buffer B, and subsequently with 500 ml of buffer C (25 mM KH_2PO_4/KOH, 1 mM DTE, 500 mM NaCl, pH 7.0). The type 3 isoform was eluted with buffer D (65 mM KH_2PO_4/KOH, 1 mM DTE, 500 mM NaCl, pH 7.0). EGTA, aprotinin and leupeptin were added to the eluted enzyme to reach final concentrations of 0.5 mM, 2 μg/ml and 2 μM, respectively. The column was further washed with four bed volumes of buffer E (300 mM KH_2PO_4/KOH, 1 mM DTE, 500 mM KCl, pH 7.0) and the type 2 isoform was eluted with buffer F (0.2% Triton X-100 in buffer E). The pooled fractions were supplied with EGTA and protease inhibitors, as in the case of the type 3 isoform.

Step 3: Heparin-Sepharose CL-6B chromatography of the type 3 isoform. The pool eluted by buffer D was diluted with two volumes of buffer G (10 mM Tris-HCl, 1

mM DTE, 0.1 mM EDTA, 5 mM $MgCl_2$, pH 7.5) and loaded onto a 2.6 × 11 cm Heparin-Sepharose CL-6B column (Pharmacia-LKB, Freiburg) pre-equilibrated with 20 bed volumes of buffer G (flow rate 120 ml/h). The enzyme was eluted with a NaCl gradient consisting of buffer G and 1 M NaCl in buffer G (buffer H) by using the following gradient programme: 0–120 ml, 0% H; 120–170 ml, 0–30% H; 170–450 ml, 30% H; 450–750 ml, 30–60% H; 750–800 ml, 60–100% H; 800–900 ml, 100% H.

Step 4: Hydroxylapatite chromatography II of the type 3 isoform. Pooled fractions from Heparin-Sepharose chromatography were applied to a second hydroxylapatite column (1 × 6 cm, Pharmacia, HR 10/10) pre-equilibrated with 20 bed volumes of buffer I (5 mM KH_2PO_4/KOH, 1 mM DTE, pH 7.0, flow rate 45 ml/h). The enzyme was eluted using buffer I and buffer J (350 mM KH_2PO_4/KOH, 1 mM DTE, pH 7.0) according to the following programme: 0–60 ml, 20% J; 60–90 ml, 28% J; 90–140 ml, 28–86% J. The eluted fractions were immediately adjusted to 0.5 mM EGTA by preparing the test tubes with $1/1000^{th}$ volume of 0.5 M EGTA (pH 7.0). Fractions containing high activity were pooled and brought to 0.1 M NaCl, 2 μg/ml aprotinin and 2 μM leupeptin.

Step 5: Gel filtration on Superdex 200 HiLoad 16/60. Following hydroxylapatite chromatography II the type 3 PtdIns 4-kinase pool was concentrated 10-fold in a Centrifree™ Micropartition System (Amicon) by centrifuging at 1000 × *g* for 20 min at 4°C. After dilution of this concentrate with either 10 mM Tris-HCl, 1 mM DTE, 0.1 mM EDTA, 0.5 M NaCl (pH 7.5), or alternatively with 10 mM Tris-HCl, 1 mM DTE, 0.1 mM EDTA, 0.1 M NaCl, 0.1% CHAPS (pH 7.5), the samples were applied in two separate runs onto a Superdex 200 HiLoad (1.6 × 60 cm, flow rate 30 ml/h) pre-equilibrated with the above buffers. 1-ml fractions were collected and supplemented with CHAPS to give a final concentration of 0.1% in the case of gel filtration without CHAPS. The column was calibrated with the following standards: thyroglobulin (669 kDa), ferritin (440 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa). The exclusion volume was determined by Blue Dextran 2000.

2.2. Test of PtdIns kinase activity

PtdIns 4-kinase activity was assayed at 25°C in a reaction mixture containing 0.83 mg/ml PtdIns, 5 mM [γ^{32}P]ATP (400–800 Bq/nmol), 27 mM $MgCl_2$, 116 mM KCl, 116 mM HEPES/KOH, 1 mM EDTA, 1 mM EGTA, 1 mM DTE and 0.4% Triton X-100 (pH 7.5). In the assays for K_m (ATP/Mg^{2+}) the free [Mg^{2+}] varied between 20–25 mM; K_m values for PtdIns were calculated employing a molecular weight of 955 for the L-palmitoyl-2-linoleoyl-derivative. Product formation was proven to be linear with time and enzyme concentration. For variation of all other conditions see Varsányi et al. [7].

2.3. Renaturation of PtdIns 4-kinases following polyacrylamide gel electrophoresis in the presence of SDS

7.5% SDS-PAGE was carried out according to Laemmli [30] with a reduced SDS concentration of 0.05% in the sample as well as in the chamber buffers. Following concentration by AMICON ultrafiltration, type 3 (first activity peak from hydroxylapatite chromatography I) and type 2 (eluted by Triton X-100 as a second activity peak from hydroxylapatite chromatography I) were applied onto a polyacrylamide gel (ca. 80 μg of PtdIns 4-kinases). Following electrophoresis at +4°C, overnight traces containing samples were cut into 2-mm segments and incubated overnight at +4°C by gently shaking them in 10 volumes of renaturation buffer consisting of 10 mM Tris-HCl, 1 mM DTE, 0.1 mM EDTA, 0.1% CHAPS, 0.2% Triton X-100, 0.03% NaN_3 (pH 7.5), for type 3, and 10 mM Tris-HCl, 1 mM DTE, 0.1 mM EDTA, 0.1% sodium DOC, 0.2% Triton X-100, 0.03% sodium azide (pH 7.5), for the type 2 isoform, respectively. PtdIns 4-kinase activity was assayed in supernatants. In both cases a parallel slot was silver-stained to detect the protein, according to Merril et al. [31].

Protein was determined using the method of Bradford [32] employing bovine serum albumin as standard.

^{32}P radioactivity was determined in the LSC SAFTY cocktail (Baker Chemicals, Deventer, NL).

Free Mg^{2+} concentrations were calculated by a programme of R. Thieleczek using the stability constants of Sillen and Martell [33,34].

2.4. Protein sequencing

The protein obtained in step 4 from hydroxylapatite (see above) was subjected to a 5% SDS-PAGE (according to Laemmli) and the resulting bands were electroblotted (Semi dry Blot, Pharmacia) onto a PVDF-membrane. The membrane part containing the 200 kDa band was cut into pieces and the protein was digested with modified trypsin (Boehringer Mannheim). The resulting peptides were dissolved in 60% acetonitrile and in 100% formic acid. After the eluate had been concentrated in vacuum, the peptides were separated by reversed-phase HPLC on a mixed C_2-C_{18} column (μRPC C2/C18 PC 3.2/3, Pharmacia) applying a linear acetonitrile gradient. (84% acetonitrile, v/v, in 0.08% trifluoroacetic acid (TFA). UV absorption was monitored at 204 nm. Fractions with high absorbance were collected and re-applied onto the same column pre-equilibrated in 10 mM ammoniumacetate (pH 6.9). Chromatography was carried out in the presence of a linear acetonitrile gradient (84% acetonitrile, v/v, in 10 mM ammoniumacetate, pH 6.9) according to Aebersold et al. [35]. Sequencing of the peptides was carried out using the Edman degradation method in a gas-liquid sequenator (Applied Biosystems 476A) according to Hewick et al. [36].

2.5. PCR

The following primers, P2-P6, and antisense primers, P2a-P6a, were derived from the sequenced peptides (bold in Fig. 6B):

P2: 5′-CARCARTAYACNCARGCNATGTTYAA-3′, P2a: 5′-TTRAACATNGCYTGNGTR TAYTGYTG-3′, P3: 5′-ATGATHCARTAYTAYCARAAYGAYAT-3′, P3a: 5′-ATRTCRTTYTGRTARTAYTGDATCAT-3′, P4: 5′-CARGGNTTGATHAAYACNTAYCC-3′, P4a: 5′-GGRTANGTRTTDATCAANCCYTG-3′, P5: 5′-CARATGACNGTNGARCARAA-3′, P5a: 5′-YTTYT-GYTCTCNACNGTCATYTG-3′, P6: 5′-GTNTAYTGG-GTNGARGA-3′, P6a: 5′-YTCYTCNACCCARTANAC-3′.

PCR was carried out [37,38] using a mixture of random- and oligo dT-primed first strand cDNA (cDNA Synthesis Kit, Gibco BRL) as a template. A thermal cycler from Biometra was used. The cDNA had previously been synthesized with polyA RNA isolated from bovine cortex [39].

The reaction mixture contained in 80 μl 0.2 μg cDNA, 1.25 pmol/μl sense primer, 1.25 pmol/μl anti-sense primer, 10 mM dNTP, 4 mM $MgCl_2$, 8 μl TAQ-buffer (Perkin Elmer), 2 μg T4 gene 32 protein (Boehringer Mannheim) [40,41], 5 U Ampli-TAQ (Perkin Elmer). The following programme was run: 96°C 180 s, (42°C 30 s 0.82°C/s, 72°C 105 s 0.18°C/s, 96°C 45 s 0.37°C/s)*5 cycles, (46°C 30 s 0.80°C/s, 72°C 105 s 0.87°C/s, Increment 1 s, 96°C 45 s 0.36°C/s)*40 cycles, 72°C 600 s.

Twenty reaction mixtures were composed employing all possible primer-combinations. The reaction products were analyzed by agarose-gel electrophoresis and the separated PCR fragments were blotted onto a positively charged nylon membrane (Quiabrene +, from Quiagen; Vacuum Blotter, Biometra, the conditions were specified by the manufacturer). The blots were hybridized successively with DIG-tailed oligonucleotides, P2-P6 (DIG Hybridization Manual, Boehringer Mannheim) [42]. Hybridizing PCR fragments were subcloned into *pBluescript* KS + (Stratagene) or *pUC* 19 (MBI Fermentas).

2.6. Screening of cDNA libraries

A random primed bovine brain λ gt 10 library (Clontech) and a bovine cortex oligo dT primed λ ZAP library (Stratagene) were screened with DIG-labeled PCR fragments [43] from PIK 200 subclones as probes. The tests for detection were performed according to the DIG Hybridization Manual (Boehringer Mannheim). Inserts from positive plaques were subcloned into *pBlueskript* KS + (Stratagene).

2.7. Amplification of flanking sequencing

Flanking sequences were obtained using nested-PCR with primers P17, 18 and the antisense primer P23a:

P17: 5′-TGCTGCCCAACGCCACCATC-3′, P18: 5′-ACCGCCAGCCAGCTGGTTCC-3′, P23a: 5′-AARTARTCRTACATNCCRAARTC-3′.

The PCR mixture 1 contained 0.2 μg cDNA, 0.8 pmol/μl P17 sense primer, 1.0 pmol/μl P23a anti-sense primer, 10 mM dNTP, 4 mM $MgCl_2$, 6 μl TAQ-Buffer (Perkin Elmer), 2 μg T4 Gene 32 protein (Boehringer Mannheim), 5 U Ampli-TAQ (Perkin Elmer) in a total volume of 60 μl. The following programme was employed: 96°C 180 s, (54°C 30 s 1°C/s, 72°C 120 s 0.5°C/s, 96°C 45 s 1°C/s)*5 cycles, (56°C 30 s 1°C/s, 72°C 120 s 1°C/s, increment 1 s, 96°C 45 s 1°C/s)*30 cycles, 72°C 600 s. Reaction mixture 2 contained 5 μl PCR 1, 0.8 pmol/μl P18 sense primer, 1.0 pmol/μl P23a anti-sense primer in a total volume of 60 μl. All the other components were identical to mixture 1. The same programme was used without initial cycles.

2.8. 3′-RACE PCR

For 3′-RACE PCR (Gibco-BRL) [44] the sequence of the gene-specific sense primer, 5′-GTTGCGGGGT-TAGTGAGCTTG-3′, was employed. The reaction mixture was identical to mixture 1. The following programme was

Table 1
Enrichment of type 3 PtdIns 4-kinase

Step	Protein mg	Activity nmol × min^{-1}	Specific activity nmol × min^{-1} × mg^{-1}	Yield %	Purification-fold
1000 × g supernatant	6806	17884	2.6	100	1
100000 × g pellet	5175	15927	3.1	89	1.2
NaCl washed membranes	3490	14374	4.1	80	1.6
Triton X114 solubilizate	n.d.	10365	n.d.	58	n.d.
$(NH_4)_2SO_4$	2539	9983	3.9	56	1.5
Hydroxylapatite I (1^{st} peak)	22.8	1292	22.5	7.2	8.6
Heparin-Sepharose	1.6	762	476	4.3	183
Hydroxylapatite II	0.69	478	693	2.7	266
Superdex 200	0.057	167	2900	0.9	1115

The isoform was enriched from bovine brain (starting material 120 g) as described in Section 2. The table is based on approximately 30 preparations.

employed: 96°C 180 s, (60°C 30 s 1°C/s, 72°C 360 s 1°C/s, increment 1 s, 96°C 45 s 1°C/s)*30, 72°C 600 s.

2.9. Sequencing of PI4K 200-subclones

The subcloned cDNA was sequenced with the TAQ Dye Terminator Kit (Applied Biosystems). Reaction products were detected on an A 373 DNA sequencer (Applied Biosystems). Both strands from three different and independent subclones were sequenced to verify the results, thus minimizing possible errors in the 3' half of the cDNA due to the use of Taq based PCR.

2.10. Data processing

Data processing was performed with the Lasergene Software by DNASTAR. Protein motifs were searched using PROSITE Database [45].

3. Results

A typical purification procedure for PtdIns 4-kinase from bovine brain is summarized in Table 1. About 89% of the activity present in a crude extract is sedimented at 100 000 × g. From the obtained membranes less than 10% of the activity is released by washing them with 150 mM NaCl. The enzyme can be solubilized with Triton X-114; after phase separation about 72% of the activity appears in the water phase. Already in the first step the two isoforms, type 3 and type 2, can easily be separated on hydroxylapatite in the presence of 0.5 M NaCl (Fig. 1): applying a gradient of up to 300 mM phosphate, an activity peak is eluted which represents the type 3 isoform; type 2 can only be eluted by including Triton X-100 in the elution buffer (second activity peak, Fig. 1). The two isoforms are set apart by their K_i values for adenosine which are 500 μM for the type 3 and 25 μM for the type 2 isoform. The respective K_m values of type 3 and 2 are 730 and 210 μM for PtdIns and 700 and 90 μM for ATP/Mg^{2+} (for assay conditions see Section 2.2). This characterization confirms that the first peak contains the type 3 and the second peak the type 2 isoform.

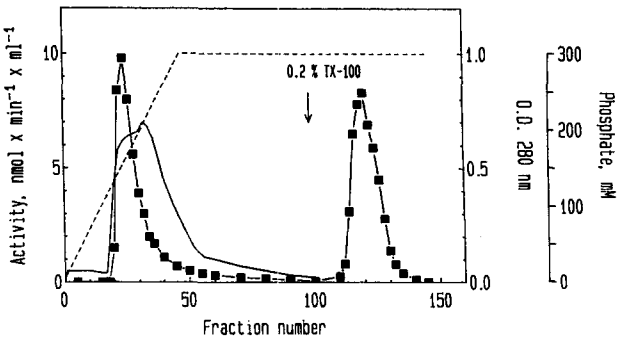


Fig. 1. Separation of PtdIns 4-kinase isoforms by hydroxylapatite chromatography I. Triton X-114 solubilized and ammonium sulfate precipitated PtdIns 4-kinase sample (see Section 2) was employed. Fractions of 15 ml were collected and PtdIns 4-kinase activity (■) was assayed. Protein (unbroken line) was recorded at 280 nm. The linear phosphate gradient is indicated by a dashed line. The arrow indicates the inclusion of 0.2% Triton X-100 into the elution buffer.

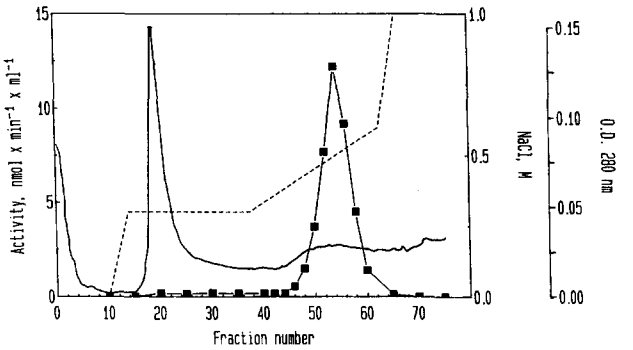


Fig. 2. Heparin-Sepharose chromatography. The type 3 PtdIns 4-kinase eluted from hydroxylapatite I (first peak from Fig. 1) was pooled and chromatographed on Heparin-Sepharose. PtdIns 4-kinase activity (■) and protein (unbroken line) were assayed in 12-ml fractions as described in Section 2. The NaCl gradient is indicated by the dashed line.

Several consecutive chromatographic steps on hydroxylapatite, on Heparin-Sepharose (Fig. 2), and on a second hydroxylapatite (Fig. 3) result in a ca. 300-fold purification. Finally gel filtration over Superdex 200 (Fig. 4) yields a more than 1000-fold overall enrichment (Table 1). SDS inhibits the type 3 isoform at 0.005% (w/v) to an extent of ca. 98%. However, a combination of 0.1% CHAPS with 0.2% Triton X-100 almost completely overcomes this SDS inhibition; 90% of the residual activity can be assayed in the presence of these two detergents. Therefore, SDS-PAGE has been carried out at a reduced SDS concentration resulting in a final SDS concentration of 0.005% in the renaturation assay. Using this method one main activity peak of the type 3 isoform can be seen, coinciding with a 200 kDa polypeptide (Fig. 5A). About 20% of the activity loaded onto the gel is recovered.

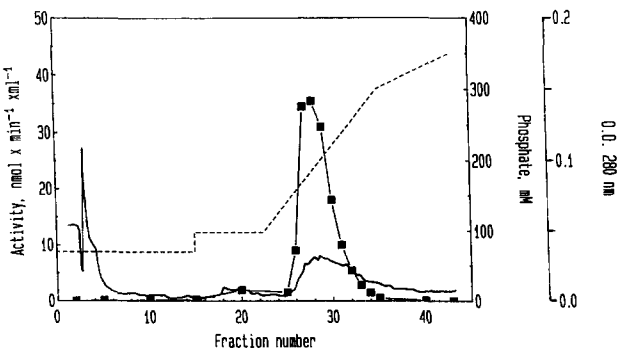


Fig. 3. Hydroxalapatite chromatography II. A pool of active samples from Heparin-Sepharose was chromatographed on a hydroxylapatite column. In fractions (1 ml) PtdIns 4-kinase activity (■) and protein distribution (unbroken line) were assayed as described in Section 2. The phosphate gradient profile is indicated by the dashed line.

Employing the same reduced SDS concentration in SDS-PAGE the type 2 enzyme is inhibited only to 7%. In presence of 0.1% DOC and 0.2% Triton X-100 the activity is enhanced to 120%. Following treatment of gel slices with this DOC-Triton X-100 solution, a main activity peak appears coinciding with an apparent molecular weight of 55 kDa (Fig. 5B). About 90% of the loaded activity can be recovered. The 200 kDa type 3 and the 55 kDa type 2 PtdIns 4-kinases are the only activities detectable in the Triton X-114 solubilizate of brain microsomes.

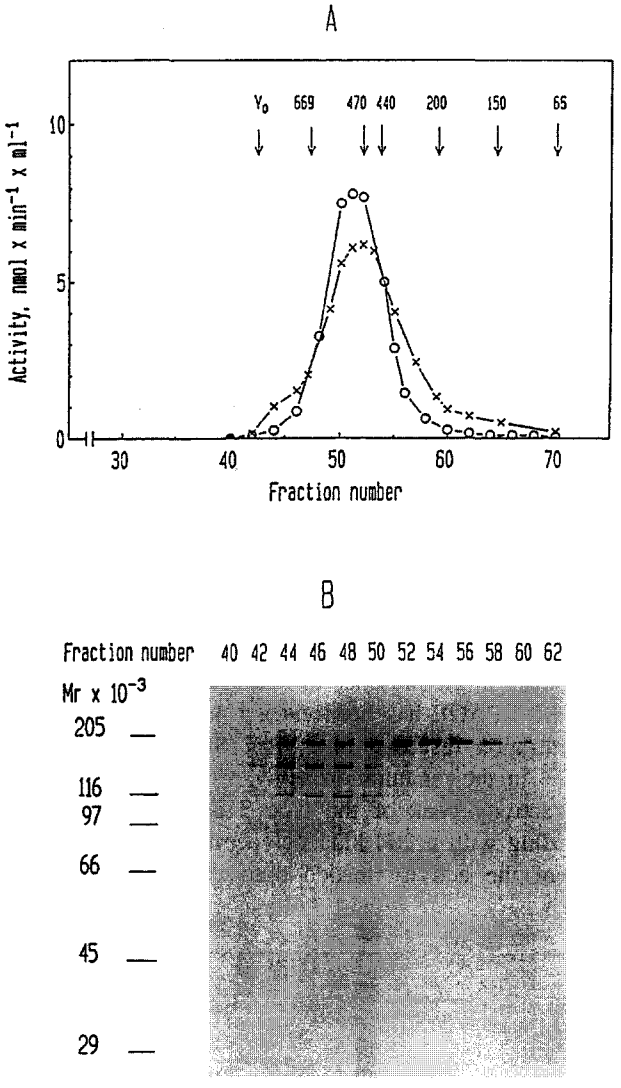


Fig. 4. Gel filtration of the type 3 PtdIns 4-kinase over Superdex 200 HiLoad. Following concentration and appropriate dilution of the type 3 containing pool after hydroxylapatite II chromatography (see Section 2) the samples were applied onto a Superdex 200 HiLoad column (16/60) in absence (○) and presence (×) of CHAPS in the buffer, respectively. A: PtdIns 4-kinase activity was assayed in fractions of 1 ml as described in Section 2. B: Silver staining of polypeptides following SDS-gelelectrophoresis was carried out according to Merril et al. [31]: 200 μl of fractions following gel filtration in presence of CHAPS were precipitated by TCA, centrifuged, the pellet was dissolved in 3% SDS, 10% glycerol, 5% 2-mercaptoethanol and electrophoresed. High molecular weight standards from Sigma were run in parallel and the corresponding apparent molecular weights are indicated.

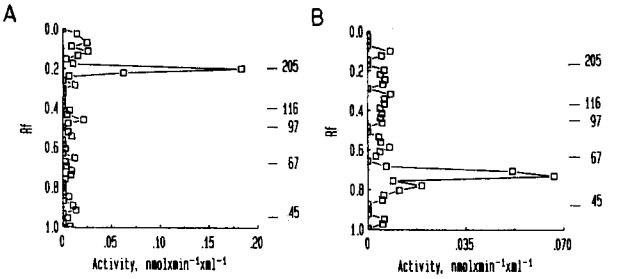


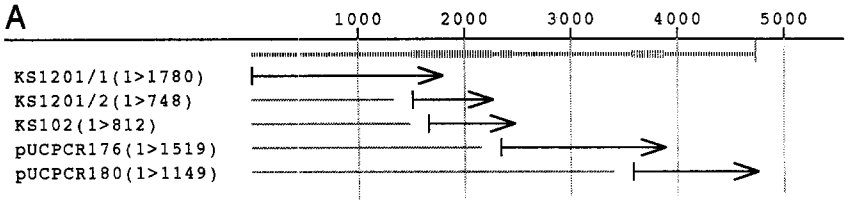
Fig. 5. Renaturation of PtdIns 4-kinase activity after SDS-gelelectrophoresis. Samples of the type 3 (A) and type 2 (B) PtdIns 4-kinases have been prepared for a modified electrophoresis as described in Section 2. Molecular weight standards were run in parallel as described in the legend of Fig. 3. Activities were determined following renaturation, as described in Section 2.

Gel filtration of the native type 3 enzyme in the presence or absence of CHAPS, independent of ionic strength yields an activity maximum which indicates an apparent molecular weight of 470–490 kDa. The activity increase and decrease parallels the elution of a 200 kDa polypeptide, as determined by SDS-gel electrophoresis (Fig. 4). We therefore conclude that this 200-kDa polypeptide represents a monomeric form of the type 3 PtdIns 4-kinase which dimerizes in the native state.

In order to identify the primary structure of this PtdIns 4-kinase, the 200-kDa protein was blotted onto PVDF membranes and the protein was digested with trypsin. The resulting peptides were separated and purified by HPLC (for experimental details see Section 2). The isolated peptides which were identified by microsequence analysis are shown in bold letters in Fig. 6B.

These peptide sequences were used to construct and synthesize the degenerated oligonucleotides P2 to P6. PCR products could be obtained with these primers and first strand cDNA template, synthesized with polyA-RNA isolated from bovine brain cortex. The products generated by primers P2/P4 show one clear hybridization signal with P5 being used as a probe. The 812b fragment (KS 102) was subcloned and sequenced (Fig. 6A). The deduced amino acid sequence contains additional peptide sequences which were identified by peptide sequencing (see Fig. 6B). This subclone was used to synthesize hybridization probes which were employed to screen cDNA libraries from cortex and brain. In the random primed library two different positive clones were found (KS 1201 and KS 1201/2) which overlap with the clone KS 102. One 3′-flanking clone, pUC PCR 176, was amplified using nested PCR with the first strand cDNA as template; the 3′-end was amplified by 3′-RACE PCR, thus obtaining the clone pUC PCR 180. From all the clones a 4.7 kb contiguous sequence was built up (Fig. 6A). The open reading frame covers 4.4 kb with a 0.3 kb untranslated 3′-end which yields a deduced amino acid sequence of 1467 amino acids for which a molecular weight of 166,178 is calculated.

A



B

```
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GAC GTG CTC ATC ATC GAC CAG CTG GGC TGC CTG GTC ATC ACC GGA AAC CA  100
D  V  L  I  I  D  Q  L  G  C  L  V  I  T  G  N  Q                  33
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  Y  I  Y  Q  E  V  W  N  L  F  Q  Q  I  S  V  K  A               50
CC AGC TCG GTC GTG TAC TCG GCC ACC AAG GAC TAC AAG GAC CAT GGC TAC  200
   S  S  V  V  Y  S  A  T  K  D  Y  K  D  H  G  Y                  66
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R  H  C  S  L  A  V  I  N  A  L  A  N  I  A  G  N                  83
C ATC CAG GAT GAG CAC CTC GTG GAC GAG CTG CTC ATG AAC CTG CTG GAG C  300
  I  Q  D  E  H  L  V  D  E  L  L  M  N  L  L  E  L               100
TG TTT GTG CAG CTG GGG CTG GAG GGC AAG CGG GCC AGT GAG CGG GCC AGC  350
   F  V  Q  L  G  L  E  G  K  R  A  S  E  R  A  S                  116
GAG AAG GGG CCC GCC CTC AAG GCC TCC AGC AGT GCG GGG AAC CTG GGC GT  400
E  K  G  P  A  L  K  A  S  S  S  A  G  N  L  G  V                  133
G CTC ATC CCT GTG ATC GCA GTG CTC ACC CGG CGG CTG CCG CCC ATC AAG G  450
  L  I  P  V  I  A  V  L  T  R  R  L  P  P  I  K  E               150
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   P  K  P  R  L  Q  K  L  L  R  D  F  G  L  Y  S                  166
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V  L  M  G  F  A  V  E  G  S  G  L  W  P  E  E  W                  183
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  Y  E  G  V  C  D  I  A  T  K  S  P  L  L  T  F  P               200
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N  D  T  V  T  P  A  E  L  S  E  L  R  S  T  I  V                  233
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  N  L  L  D  P  A  P  E  V  S  A  L  I  G  K  L  D               250
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   F  A  M  S  T  Y  L  L  S  V  Y  R  L  E  Y  M                  266
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R  V  L  R  S  S  D  P  A  R  F  Q  V  M  F  C  Y                  283
C TTC GAG GAC AAG GCC ATC CAG AAG GAC AAG TCC GGG ATG ATG CAG TGT G  900
  F  E  D  K  A  I  Q  K  D  K  S  G  M  M  Q  C  V               300
TG ATC GCC GTC GCC GAC AAG GTG TTC GAC GCC TTC CTG AAC ATG ATG GCG  950
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  F  L  L  V  S  F  N  H  V  H  K  R  I  R  R  V  A               350
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G TCG CTG AGT GCT GAC ATC CAC AAG GAC CAG CCA TAC TAC GAC ATT CCC G 1200
  S  L  S  A  D  I  H  K  D  Q  P  Y  Y  D  I  P  D               400
AC GTG CCC TAC CGC ATC ACC GTC CCG GAC ACC TAC GAG GCC CGA GAG AGC 1250
   V  P  Y  R  I  T  V  P  D  T  Y  E  A  R  E  S                  416
ATC GTC AAA GAC TTC GCC GCG CGC TGC GGG ATG ATC CTT CAG GAG GCC AT 1300
I  V  K  D  F  A  A  R  C  G  M  I  L  Q  E  A  M                  433
G AAG TGG GCG CCC ACG GTC ACC AAG TCC CAC CTG CAG GAG TAC CTG AGC A 1350
  K  W  A  P  T  V  T  K  S  H  L  Q  E  Y  L  S  K               450
AG CAC CAG AAC TGG GTA TCG GGG CTG TCG CAG CAC ACG GGG CTG GCC ATG 1400
   H  Q  N  W  V  S  G  L  S  Q  H  T  G  L  A  M                  466
GCC ACC GAG AGC GTC CTG CAC TAC GCC GGC TAC AAC AAG CAG AGC ACG AG 1450
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   L  L  A  G  K  N  G  V  E  V  P  F  M  R  E  M                  616
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   P  C  A  P  E  V  T  P  H  Y  I  W  I  D  F  L                  666
GTA CAG CGG TTT GAG ATT GCC AAG TAC TGC AGT TCC GAC CAG GTG GAG AT 2050
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  F  C  S  L  L  Q  R  S  L  S  L  S  I  G  G  T  A               700
CG GGC AGC ATG AAC CGG CAT GTG GCG GCC ATC GGG CCC CGC TTC AAG CTG 2150
   G  S  M  N  R  H  V  A  A  I  G  P  R  F  K  L                  716
CTG ACC CTC GGC CTG TCC CTG CTG CAC GCG GAC GTG CTG CCC AAC GCC AC 2200
L  T  L  G  L  S  L  L  H  A  D  V  L  P  N  A  T                  733
C ATC CGC AAC GTG CTC CGC GAG AAG ATC TAC TCC ACC GCC TTT GAC TAC T 2250
  I  R  N  V  L  R  E  K  I  Y  S  T  A  F  D  Y  F               750
TC AGC TGC CCG CCG AGG TTC CCG ACC CAG GGG GAG AAG AGG CTG CGT GAG 2300
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  Y  L  T  A  S  Q  L  V  P  P  D  N  Q  D  T  R  S               800
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GCC CCC GAG CTC AGC CAC GTG CTG TGC TGG GCG CCC GCG GAC CCA CCC AC 2950
A  P  E  L  S  H  V  L  C  W  A  P  A  D  P  P  T                  983
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  G  L  S  Y  F  S  S  M  Y  P  P  H  P  L  T  A  Q              1000
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   Y  G  V  K  V  L  R  S  F  P  P  D  A  I  L  F                 1016
TAC ATT CCC CAG ATT GTG CAG GCG CTC AGG TAT GAC AAG ATG GGC TAC GT 3100
Y  I  P  Q  I  V  Q  A  L  R  Y  D  K  M  G  Y  V                 1033
G CGG GAG TAC ATC CTG TGG GCC GCC TCC CAG TCC CAG CTA CTG GCA CAC C 3150
  R  E  Y  I  L  W  A  A  S  Q  S  Q  L  L  A  H  Q              1050
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   F  I  W  N  M  K  T  N  I  Y  V  D  E  E  G  H                 1066
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Q  K  D  P  D  I  G  D  L  L  E  Q  L  V  E  E  I                 1083
C ACA GGC TCC CTC TCC GGC CCG GCC AAG GAC TTC TAC CAG CGG GAG TTC G 3300
  T  G  S  L  S  G  P  A  K  D  F  Y  Q  R  E  F  D              1100
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  V  Q  P  G  C  Y  L  P  S  N  P  E  A  I  V  L  D              1150
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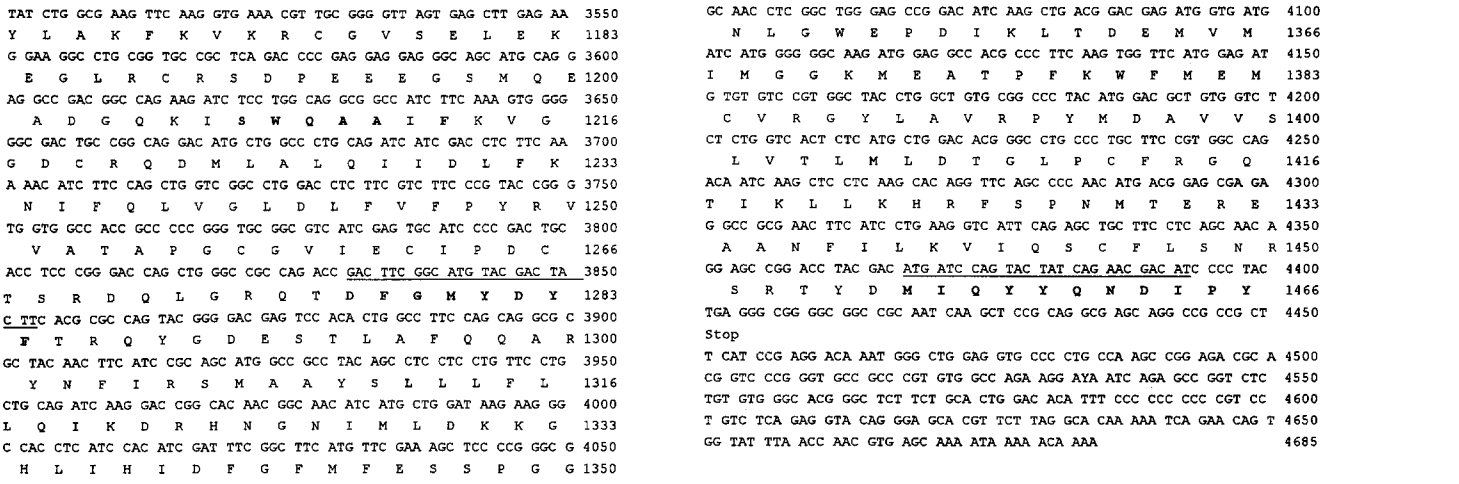

Fig. 6. A: Clonemap of PI4K200. The first dashed line represents the contiguous sequence, enlarged dashed lines indicate overlapping clones. B: cDNA and deduced amino acid sequence of PI4K200. The amino acid sequence in single-lettered code is indicated below the nucleotide sequence. Sequenced peptides are bold. Oligonucleotides derived from peptides for PCR are underlined. The cDNA sequence is reported including the non-coding region up to base 4685; downstream the sequence has not yet been refined.

Based on homology, the C-terminal part of this sequence obviously represents the catalytic domain of this type 3 PtdIns kinase. Sequence alignment of about the last 300 amino acids with the corresponding domains of the mammalian counterpart PI4Kα, the yeast enzymes STT4 and PIK1 as well as with catalytic domains of the bovine, human, mouse and yeast PtdIns 3-kinases reveals a high degree of sequence identity (Fig. 7). 26 of these approximately 300 amino acids are invariable in all of these eight catalytic domains. The histogram shown in Fig. 7 demonstrates that a consensus sequence can be built from all eight sequences. The greatest similarity of approximately 98% is found between the bovine PI4K200 and the human PI4Kα. The yeast kinase STT4 is approximately 20% more similar to the bovine enzyme than the yeast enzyme PIK1. The degree of similarity of the catalytic domains of all the PtdIns 3-kinases is in the same range as that of PIK1 (not shown).

Two leucine zipper motifs (amino acids 358-386 and 862-882) are detectable, one located near one of the two nuclear localization signals (amino acids 345-349 and 838-854) [46]. Furthermore, a helix loop helix motif (amino acids 716-729) as found in DNA binding proteins is observed. Many potential phosphorylation sites for cAMP/cGMP-dependent protein kinases, casein kinase 2, protein kinase C as well as protein tyrosine kinases can be localized.

4. Discussion

PtdIns 4-kinase activity has been found to be enriched with brain microsomes consistent with the observations made by Endemann et al. [15]. This lipid kinase activity is still associated with the membranes even in the presence of salt, but it can be solubilized and transferred into the water phase following Triton X-114 treatment. Indeed, the enzyme contains α-helical hydrophobic regions which can potentially cause membrane association (not shown). However, once the enzyme has been transferred into the water phase, it no longer requires the presence of detergents. The dimeric form (see below) is apparently stable in aqueous solution.

It has been reported that type 3 PtdIns 4-kinase sediments faster than β-amylase and slower than apoferritin, indicating a M_r between 200 and 443 kDa [15]. The results reported here yield a slightly higher apparent molecular weight of 470-490 kDa both in the absence and presence of CHAPS (see Fig. 4). So far, the polypeptide harboring the type 3 activity has not yet been identified. When employing our newly developed renaturation procedure in presence of CHAPS and Triton X-100, a 200 kDa protein exhibits type 3 enzymic activity. Thus, the native enzyme might be a dimer and this association is apparently not reversed by CHAPS.

Enzyme purification, as employed here (see Table 1), has enriched the enzyme approximately 1000-fold. In the preparation gained from gel filtration, the 200 kDa band which has so far been identified as type 3 PtdIns 4-kinase, represents on average 3% of the protein as determined by SDS-PAGE. Thus, the enzyme has been purified to an extent of approximately 30000-fold. Indeed, this protein band could still be composed of more than one polypeptide. However, all of the peptide sequences found and reported here originate from one protein which was confirmed by cDNA sequencing. Thus, this band seems to represent a homogeneous protein, namely the type 3 PtdIns 4-kinase.

The cDNA sequence determined until now yields a molecular weight of 166,742 for the deduced amino acid

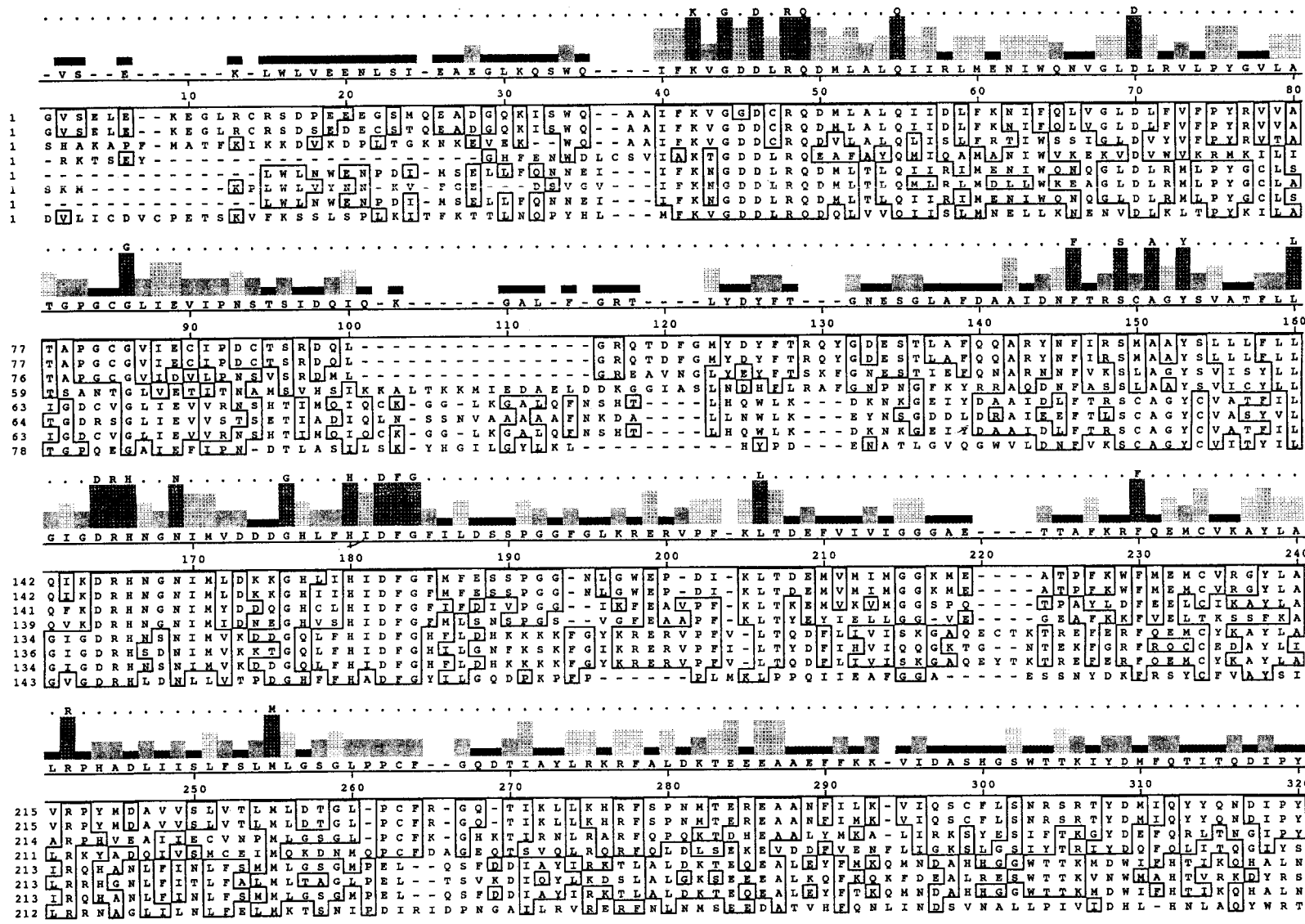

Fig. 7. Alignment of PtdIns kinases. The first line shows invariant amino acids in all primary structures, the second line a histogram of the consensus strength and the third line the consensus sequence for the PtdIns kinases. Boxed residues in the alignment match the consensus exactly. The alignment was carried out with the clustal algorithm and default programme parameters (see Section 2).

sequence, which is lower than the experimentally determined molecular weight of 200 kDa. Indeed, no Kozak consensus sequence has been found upstream of the uppermost methionine [47]. The N-terminus is blocked (not shown), so that no unequivocal assignment of the start codon is possible. Thus a small part of the sequence might still be missing.

The activity profile following SDS-gel electrophoresis which identifies the 200 kDa protein as a type 3 PtdIns 4-kinase agrees with the localization of a catalytic domain on this protein. The approximately 300 C-terminal amino acids not only share a high degree of similarity with the human type 2 PI4Kα, as was expected, but surprisingly they are nearly completely identical. As has already been reported, this domain shows a high degree of sequence identity to the corresponding yeast counterparts and, to a lesser degree, to the catalytic domains of the PtdIns 3-kinases (not shown). Furthermore, sequence motifs characteristic of protein kinases can clearly be identified in the catalytic domain of lipid kinases. In fact, there is a conserved aspartate-phenylalanine-glycine motif, as well as aspartate, asparagine and lysine residues located 11, 18 and 122 residues upstream, respectively. Most of these conserved positions were shown to be essential for expressing catalytic activity of lipid kinases, too [48].

The part of the sequence (aa 600-1100) preceding the catalytic domain of the human PI4Kα is also nearly identical to that of the bovine sequence. The surprisingly high degree of conservation indicates that an important function is also residing in this part of the molecule. The difference in properties of the type 3 and type 2 isoforms can now be assigned to the N-terminal part of the type 3 PtdIns 4-kinase which is not present in the corresponding human type 2 PI4Kα [23]. A Northern blot of human tissue reveals two transcripts of 7.5 and 3.2 kb. Indeed, a stronger signal is found for the 7.5 kb band than for the 3.2 kb material [23]. Alternative splicing does not necessarily occur identically in two different species [49], however, the almost identical amino acid sequences of human type 2 PI4Kα and bovine type 3 PI4K200 strongly indicate that these two forms might have been produced in human tissue by alternative splicing. Enzymologically a 97 kDa form corresponding in size to the human PI4Kα cannot be detected in bovine tissue (compare Figs. 1 and 5); moreover, no cDNA clone containing a sequence similar to the 5' non-coding region of PI4Kα was found. Thus, if indeed alternative splicing occurs in bovine as in human tissue, the product corresponding to PI4Kα is most probably a very minor component.

The N-terminal part present in type 3, but not in type 2 PtdIns 4-kinase, may play a modulatory role in this type 3 enzyme or is responsible for localization in special compartments. Interestingly, one of two leucine zipper motifs is located in this N-terminal part which exceeds the type 2 PI4Kα. Both zipper motifs can clearly be identified in the sequence. Leucine zippers have been found in a number of eukaryotic DNA binding proteins that act as transcription factors [50]. Binding to DNA requires dimerization via the two helices which expose leucine side chains on one side and the presence of adjacent basic regions. However, the adjacent stretches in the PI4K200 sequence only contain a few basic residues. Similarly, a helix loop helix motif-also identified in the PI4K200 sequence-binds to DNA as a dimer via adjacent basic regions. On the other hand, these basic regions are absent in the PI4K200 sequence. A whole class of proteins contains the helix loop helix motif but lacks an adjacent basic domain and is thought to inhibit transcriptional activation by forming heterodimers with proteins containing the basic helix loop helix motif [51]. However, whether this enzyme is indeed transported into the nucleus, as suggested by the presence of the two nuclear localization signals, remains to be shown.

Cells overexpressing a growth factor receptor stimulate PtdIns 4-kinase when treated with an agonist, suggesting that this enzyme is potentially regulated by tyrosine phosphorylation. This observation is in line with several other reports, indicating tyrosine phosphorylation of the type 3 isoform. An analysis of the sequence reveals four potential tyrosine phosphorylation sites for which a fairly good consensus sequence has been found. Further studies are clearly required to clarify this conundrum.

The situation regarding other protein kinases is similar. It has been suggested that both, cAMP-dependent protein kinase and protein kinase C, are involved in regulating the type 3 PtdIns 4-kinase activity (for review, see Ref. [4]). In the sequence reported here, several potential target sites for these two protein kinases can be identified (see Fig. 7). Furthermore, there are several putative phosphorylation sites for casein kinase II. The identification of potential phosphorylation sites should facilitate the localization of actual phosphorylation sites employed in the living cell.

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