Identification of the actin-binding domain of Ins(1,4,5) P_3 3-kinase isoform B (IP3K-B)

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Dewaste et al. [Dewaste, Moreau, De Smedt, Bex, De Smedt, Wuytaack, Missiaen and Erneux (2003) *Biochem. J.* **374**, 41–49] showed that over-expressed EGFP (enhanced green fluorescent protein) fused to Ins(1,4,5) P_3 3-kinase B (IP3K-B) co-localizes with the cytoskeleton, as well as with the endoplasmic reticulum and the plasma membrane. The domains responsible for these subcellular localizations are not yet identified. For the endogenous enzyme, we confirmed both actin and endoplasmic reticulum localization by employing a high affinity antibody against IP3K-B. F-actin targeting is exclusively dependent on the non-catalytic N-terminal region of IP3K-B. By expressing fragments of this N-terminal domain as EGFP-fusion proteins and inspecting transfected cells by confocal microscopy, we characterized a distinct 63-amino-acid domain comprising amino acids 108–170 of the enzyme which is responsible for F-actin targeting. A truncation of this fragment from both sides revealed that the full size of

this segment is essential for this function. Deletion of this segment in a full-length over-expressed IP3K-B-EGFP-fusion protein completely abolished F-actin interaction. Direct interaction of this actin-binding segment with only F-actin, but not with G-actin, was observed *in vitro* using a bacterially expressed, affinity-purified GST (glutathione S-transferase)–*Rattus norvegicus* IP3K (aa 108–170) fusion protein. Helix-breaking mutations within this isolated segment abolished the F-actin binding properties both *in vitro* and when over-expressed in cells, indicating that an intact secondary structure is essential for actin targeting. The segment shows sequence similarities to the actin-binding region in IP3K-A, but no similarity to other actin-binding domains.

Key words: actin-binding domain, F-actin, Ins(1,4,5) P_3 3-kinase B, subcellular localization.

INTRODUCTION

One way that metazoan cells switch off signals transmitted by the Ca^{2+} -liberating second messenger Ins(1,4,5) P_3 relies on the enzyme Ins(1,4,5) P_3 3-kinase (IP3K), which phosphorylates the inositol ring at its 3-position and generates Ins(1,3,4,5) P_4 . This product apparently serves as the main starting substrate for the biosynthesis of higher phosphorylated inositol phosphates. Previous and more recent experiments have revealed a role for Ins(1,3,4,5) P_4 in regulating Ca^{2+} signals and the frequency of Ca^{2+} oscillations in HeLa cells [1,2], as well as GAP (GTPase-activating protein) activity [3,4]. cDNAs encoding three isoforms of IP3K from vertebrates, and one alternatively spliced gene from the invertebrate *Caenorhabditis elegans* have been published [5–11]. Further isoforms, for example from the honeybee (*Apis mellifera*) and *Drosophila melanogaster*, have been predicted from their genomic sequences [12,13]. The vertebrate A isoform is mainly neuron specific, but is also found in testis and in avian erythrocytes [9,14,15]. The distribution of mRNA corresponding to the B and C isoforms has been shown in a wider range of tissues, such as brain, heart, lung, testis and kidney [14–16].

The C-terminal region of the three IP3Ks is highly conserved among all vertebrates. This conserved catalytic domain is N-terminally preceded by a calmodulin-binding domain and, further upstream, by a conserved region rich in acidic amino acids and serine/threonine residues which resembles PEST sequences

[17]. Diversity mainly appears in the N-terminal regions. The functions of the divergent N-terminal regions have not yet been sufficiently elucidated. Schell et al. [18] identified an F-actin-binding domain (F-ABD) in the N-terminus of IP3K-A, which targets the kinase to actin-rich dendritic spines in neurons. Soriano et al. [19] reported an association of the B isoform with the endoplasmic reticulum and proposed a segment in the N-terminal domain as a candidate for ER (endoplasmic reticulum) targeting. This localization was recently confirmed by Dewaste et al. [20], who found two further localizations: an over-expressed IP3K-B-EGFP (enhanced green fluorescent protein)-fusion protein appeared in the plasma membrane, cytoskeleton and in the ER.

In the present paper, the subdomain within in the N-terminal domain of IP3K-B responsible for F-actin binding was identified. The amino acids 108–170 within the N-terminal domain are shown to be sufficient and essential for F-actin localization of IP3K-B in PC12 and NRK (normal rat kidney) 52E cells *in vivo* and to be able to directly bind to F-actin *in vitro*.

EXPERIMENTAL

Materials

PC12 cells were given kindly by Dorit Arlt (Institute for Hormone and Fertility Research, Hamburg, Germany). NRK 52E cells were

Abbreviations used: ABD, actin-binding domain; DTT, dithiothreitol; ECFP, enhanced cyan fluorescent protein; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; F-ABD, F-actin-binding domain; GAP, GTPase-activating protein; GST, glutathione S-transferase; Hs, *Homo sapiens*; IP3K, Ins(1,4,5) P_3 3-kinase; NLS, nuclear localization sequence; NRK, normal rat kidney; 5'-RACE, rapid amplification of cDNA 5'-ends; Rn, *Rattus norvegicus*; RT-PCR, reverse transcriptase-PCR; TCA, trichloroacetic acid; wt, wild-type; L139P, Leu¹³⁹ → Pro.

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obtained from Cell Lines Service (Heidelberg, Germany). pEGFP-N1, pDsRed-N1 and pECFP-ER were from ClonTech (Palo Alto, CA, U.S.A.). TRITC- and FITC-labelled phalloidin was from Sigma (Deisenhofen, Germany). Texas-Red-labelled IgGs were from Dianova (Hamburg, Germany).

TCA (trichloroacetic acid) lysis of NRK 52E cells and Western blot analysis of precipitated protein

For TCA lysis, 2×10^7 to 1×10^8 NRK 52E cells were lysed with ~ 1 ml of lysis mix [900 μ l of acetone, 100 μ l of TCA (10 %, v/v), 20 μ l of DTT (dithiothreitol; 1 M)]. Proteins were precipitated for 1 h on ice. After centrifugation for 7 min at 5000 g and 4 °C, the supernatant was discarded and the pellet was washed three times with 500 μ l of acetone/DTT washing solution, containing 490 μ l of acetone and 10 μ l of 1 M DTT. The pellet was dried and re-suspended in 500 μ l of loading buffer and 50 μ l of 1 M Tris, pH 8.4. The samples (40 μ l; >100 μ g) were then subjected to electrophoresis by SDS/PAGE (12.5 % gel), transferred on to nitrocellulose for Western blotting with a monoclonal antibody against the catalytic domain of IP3K-A and IP3K-B (kin 4) generated as described previously [9]. Generation and specificity of the monoclonal antibody was described previously [21]. To control detection sensitivity of the kin 4 antibody for IP3K-A and IP3K-B, Western blots were performed with continuous dilutions of identical amounts of purified, bacterially expressed domains of IP3K-A and IP3K-B, generated in a similar manner as described previously [9,15].

5'-RACE (rapid amplification of cDNA 5'-ends) procedure and cloning of rat IP3K-B cDNA-fragments

Total RNA was prepared from adult rat brain (strain Wistar) [22]. An mRNA Purification Kit (Pharmacia Biotech, Freiburg, Germany) was used according to the manufacturer's instructions to prepare the mRNA template. Repeated 5'-RACE procedures were carried out with the Marathon kit (ClonTech) according to the manufacturer's instructions.

The primers used are listed in Table 1. The deduced full-length cDNA sequence of rat IP3K-B was deposited in GenBank® under accession number AJ242781. Internal cDNA fragments of rat IP3K-B were cloned by RT (reverse transcriptase)-PCR using the Titan RT-PCR kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions with 1 μ g or 300 ng of total RNA as template.

Non-enzymic acidic Asp-Pro cleavage of an N-terminal truncated form of IP3K-B

A truncated cDNA fragment (bp 1665–2802) for prokaryotic expression of an N-terminally truncated catalytic domain of IP3K-B, comprising aa 555–934, was cloned into pASK IBA 3 vector (IBA, Göttingen, Germany). Protein expression in *Escherichia coli* XL1 Blue and affinity purification via Strep-Tactin affinity chromatography (Strep Tag starter kit C3; IBA) were performed according to the manufacturer's recommendations.

The prokaryotically expressed N-terminal truncated form of IP3K-B (aa 555–934) in 50 μ l of buffer E (100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA and 2.5 mM desthiobiotin) was incubated with 5 μ l of 10 % TCA for 3 min at 4, 37, 60 or 95 °C. After incubation the acid was neutralized with 50 μ l of 1 M Tris buffer, pH 8.0. The probes were electrophoresed by SDS/PAGE (12.5 % gel) and transferred on to nitrocellulose for Western blotting with an anti-Strep antibody or antibody kin 4 directed against the catalytic domain of IP3K-A and IP3K-B.

Table 1 List of primers

The inserted start codons (ATG) are underlined.

Primer	Sequence
RnIP3K-B fragments	
UB186 5'	TTCATGATCACCAGGCTATTGAG
(108–583) 5'	CTCGAGATGGGCCGCTCGCGAGGGGACGCG
(108–583) 3'	CCGCGGCAGAGGCA-GGGGACCCGAGGGCT
(108–170) 5'	GGTACCGCTCGGGGCGCTTGAATAGCAG-AGGT
(170–430) 5'	CTCGAGATGAGCCGCGCTTGGTAGGGCTCGT
(170–430) 3'	GGTACCGCGGTCTGCTGGGGTCTCGGATTCC
(561–620) 5'	CTCGAGATGGGT-GCTCAGGAGGATGGAGGGG
(561–620) 3'	GGTACCGCGGTGAGTTGGGGTCCA-GAGTG
(120–148/159) 5'	CTCGAGATGGCGACCCGCATCCTCTCCCCA
(120–159) 3'	GGTACCGCGATTGCGCCTCGAACATGCCAC
(120–148) 3'	GGTACCGCCACCT-GCACATTGCAATTGCGG
QuikChange mutagenesis	
(L124A) 5'	GTGGCGACCCGCATCGCCTCCCCACCTGGGCCG
(L124A) 3'	CGGCC-AGTGGGGAGGCGATGCGGGTCGCCAC
(A132G) 5'	CCTGGGCCGAGGAGG-GCCAGAGGAAGCTGAGG
(A132G) 3'	CCTCAGCTTCTCTGGCCCTCCTCGGCCCAGG
(I138A) 5'	CAGAGGAAGCTGAGGGCCCTGCAGCGCAATTG
(I138A) 3'	CAATTGCGCTGCAGGGCCCTCAGCTTCTCTG
(Q144A) 5'	CTGCAGCGCGA-ATTGGCCAATGTGCAGGTGAACCG
(Q144A) 3'	CTGGTTCACCTGCACATTGGCCAATTGCGCTGCAG
GST-fusion construct	
(108–583) 5'	GGATCCATGGGCCGCTGCGAGGGGAC
(108–170) 3'	GAATTCCTGGGGCGCTTGAATGACAG

Construction of vectors encoding EGFP and GST (glutathione S-transferase)-tagged fusion proteins with N-terminal fragments of *Rattus norvegicus* (Rn)IP3K-B

To express tagged N-terminal segments of RnIP3K B (aa 108–583, 108–170 and 170–430) in mammalian cells, these fragments were cloned into the expression vector pEGFP-N1 (ClonTech) as described previously [15]. A GST-IP3K-B segment fusion gene was constructed by amplifying the coding sequence of residues 108–170 from the cloned cDNA by PCR. The PCR product was then cloned into the pGEMTeasy vector at *Bam*HI and *Eco*RI sites. Afterwards the cDNA fragment was subcloned into the expression vector pGEX-2T (Amersham Pharmacia Biotech, Freiburg, Germany) and transformed into *E. coli* BL21(DE3) RIL cells.

Cloning of full-length cDNA of *Homo sapiens* (Hs)IP3K-B

cDNAs encoding the N-terminal region (bp 1–1929, aa 1–643, obtained as an expressed sequence tag clone) and the catalytic domain (bp 1851–2850 bp, aa 617–950, obtained by library screening) of the human IP3K-B were joined by fusion PCR. The amplified product was subcloned into pGEMTeasy (Promega, Mannheim, Germany) and complete confirmatory DNA sequencing was performed. To obtain a HsIP3K-B-EGFP-fusion protein the PCR product was then subcloned into the expression vector pEGFP-N1 using the *Hind*III and *Sac*II restriction sites. *E. coli* XL1 Blue was transformed with this plasmid. After DNA miniprep the plasmid was used for transient transfection of mammalian cells.

Bacterial expression and purification of wt (wild-type) and mutated ABD (actin-binding domain) of GST-RnIP3K-B

E. coli BL21 (DE3) RIL cells transformed with expression vectors pGEX-2T containing an insert encoding the wt or mutated ABD of RnIP3K-B were grown in 500 ml of Luria-Bertani medium

(plus 100 $\mu\text{g/ml}$ ampicillin and 34 $\mu\text{g/ml}$ chloramphenicol) at 37 °C. At a D_{600} of 0.5, expression was induced by adding 1.0 mM isopropyl β -D-thiogalactoside. After protein expression for 2 h, cells were harvested by centrifugation at 10000 g (Sorvall, GSA rotor) for 15 min. The cells were resuspended in 30 ml of lysis buffer (50 mM Hepes and 1 mM EDTA, pH 7.5) and lysed by addition of 1 mM PMSF, 0.5 mM benzamidine, 1 mM DTT, 0.5% (w/v) Triton X-100, followed by sonification at 4 °C. After centrifugation at 10000 g for 10 min at 4 °C, the supernatant was collected and the GST-IP3K-B-fusion protein was purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech) affinity column chromatography. Purity was assessed by SDS/PAGE. Protein concentration was determined by a Bradford assay [23]. The fusion protein was directly used in an actin-binding assay.

Helix-breaking mutations in the ABD of IP3K-B and deletion of the ABD in full-length HsIP3K-B

Double point mutations were created by using modified Quik-Change site-directed mutagenesis [24]. Either the prokaryotic expression vector pGEX-2T, carrying an insert encoding the ABD of RnIP3K-B, or the eukaryotic expression vector pEGFP-N1, carrying inserts encoding the N-terminus (aa 108–583), the ABD (108–170) of RnIP3K-B or full-length HsIP3K-B, were used as template. The following primers were used for mutagenesis of the N-terminal region or the ABD of RnIP3K-B: RnMut1 [L139P (Leu¹³⁹ → Pro), L143P] 5' primer, TGTTTCGAGGCGC-AACCCAGGCACAGCCCTCTGCTATTCAAGCG; RnMut2 (I159P, T163P) 5' primer, ATGTTTCGAGGCGCAACCCAGG-CACAGCCCTCTGCTATTCAAGCG. Mutagenesis of full-length human IP3K-B was performed with the following primer: Hs Δ ABD (deletion of aa 103–165) 5'-primer, AGCGTGAGC-AGCCCAAGTTGGAGCCCCGCCGCTTTGGGCAGGGCT. The corresponding 3' primers are complementary to the 5' primers.

Domain homology searches and structure predictions

Similarity with known protein domains was searched in the N-terminal sequence of RnIP3K-B with Pfam [25] (<http://pfam.wustl.edu/>). The local alignments were done manually. The secondary structure prediction for full-length RnIP3K-B was performed with Network Protein Sequence @analysis (<http://pbil.ibcp.fr/>).

Cell culture and transient gene expression

Cell culture media and supplements were from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.). PC12 and NRK 52E cells [26] were cultured as described previously [14]. For transfection and microscopy, cells were seeded on to chamber slides and incubated overnight. Cell transfections were performed using LIPOFECTAMINETM, Metafectene (Biotex) or fuGENE 6 (Roche), according to the manufacturers' instructions, when cells were at 60–70% confluence. Typically, 1 μg of DNA per 1 ml of transfection medium was used. For observations *in vivo*, cells were washed twice with PBS and images were obtained according to the methods described below.

Immunofluorescence and confocal fluorescence microscopy

Cells used for the fluorescence microscopy were washed twice with PBS, fixed in PBS containing 3% paraformaldehyde for 10 min and permeabilized by incubation with PBS containing 0.3% Triton X-100 for 3 min. For immunofluorescence applications cells were blocked in PBS containing 0.2% gelatine

(Merck, Berlin, Germany) for 30 min, incubated with the first antibody (kin 4) for 12 h at 25 °C, and then incubated with the second antibody for 1 h at 25 °C. F-actin was stained by incubation with TRITC-labelled phalloidin (Sigma). Every step was followed by washing three times with PBS. A competition experiment where the antibody kin 4 was pre-incubated with recombinant catalytic domain in order to prove its specificity was performed. Thereby, the above procedure was modified by the following pre-incubation step: 1.0 μg of antibody kin 4 was incubated with 0 ng, 100 ng, 500 ng, 1.0 μg , 2.5 μg or 5 μg of recombinant catalytic domain of IP3K-B (see above) in 250 μl of PBS at 25 °C for 3 h before the complex was added to NRK cells.

Quasi-confocal cell microscopy was performed using a monochromator-based imaging system (Improvision, Heidelberg, Germany) built around a Leica DM IRBE fluorescence microscope at $\times 100$ magnification. Images were taken with a 12-bit grey-scale charge-coupled-device camera (type C4742-95-12 NRB; Hamamatsu, Enfield) on 15 consecutive horizontal (z) planes, each 0.5 μm , from the bottom to the top of the cell. The spatial resolution in the horizontal plane (x – y plane) of the camera was 0.067 $\mu\text{m/pixel}$. To obtain digital confocal images, mathematical deconvolution of raw data based upon the point-spread algorithm was carried out using the Openlab Confocal Imaging software module [27] (Improvision). Usually, deconvolution was carried out using 2 or 3 neighbours on each side of the central horizontal plane of the cell.

Epi-fluorescence microscopy was carried out as previously described [28].

Actin preparation and *in vitro* actin-binding assays

Rabbit skeletal muscle actin was prepared according to Rees and Young [29]. Part of the protein was modified with *N*-ethyl-maleimide at Cys³⁷⁴ and subsequently with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBT-Cl) at Lys³⁷³ to produce fluorescently labelled monomeric actin [30]. The actin concentration was determined photometrically at 290 nm using an absorption coefficient of 24900 $\text{M}^{-1} \cdot \text{cm}^{-1}$ [31]. The protein was applied to a Sephacryl S-200 column (2.5 $\text{cm} \times 70 \text{ cm}$) equilibrated with buffer A (0.5 mM ATP, 0.2 mM CaCl_2 , 200 mg/l NaN_3 , and 5 mM triethanolamine/HCl, pH 7.5).

Labelled monomeric actin (10%; 1 μM) was co-polymerized with unmodified actin (9 μM) in buffer A (above) supplemented with 1 mM MgCl_2 and 100 mM KCl at 37 °C for 1 h [29]. For the F-actin-binding assays various concentrations of IP3K-B(ABD)–GST-fusion proteins (1 μM –20 μM) were incubated with 1 μM F-actin. The association of fusion proteins was monitored for 20 min by the fluorescence increase. For the G-actin-binding assays various concentrations of IP3K-B(ABD)–GST-fusion protein (12 μM and 24 μM) or 10 mM Tris/HCl were incubated with labelled G-actin at a fixed concentration of 1 μM , and the fluorescence intensity was monitored for 20 min. Fluorescence was measured by using a PerkinElmer LS-50 fluorimeter. The excitation wavelength was 480 nm, and the fluorescence intensity was measured at 540 nm. All measurements were performed at 37 °C in buffer A supplemented with 1 mM MgCl_2 and 100 mM KCl.

RESULTS

Immunofluorescence analysis reveals co-localization of endogenous RnIP3K-B in NRK 52E cells with the ER and actin filaments

To investigate whether the localization of endogenous IP3K-B is equal to the localization of strongly over-expressed enzyme

Figure 1 Endogenous RnIP3K-B detected by immunofluorescence in NRK 52E cells

Fixed NRK 52E cells were incubated with anti-IP3K-B antibody (directed against the catalytic domain; kin 4) overnight to detect endogenous RnIP3K-B and stained by incubation with Alexa Fluor 568 (red)-labelled goat anti-mouse IgG (**B**, **E**). F-actin was stained with FITC-labelled phalloidin (**A**). Endogenous RnIP3K-B co-localized with stained F-actin appears yellow in the merged image (**C**). ER was detected by over-expressed ER-specific protein labelled with ECFP (**D**). (**F**) is the merge of (**D**) and (**E**). Confocal images were recorded at $\times 100$ magnification. Scale bar represents 10 μm .

described by Dewaste et al. [20], we determined the subcellular localization of RnIP3K-B by confocal immunofluorescence microscopy employing the above characterized high-affinity antibody kin 4, obtained as monoclonal antibody by screening against the catalytic domain of IP3K-B [9]. Only two localizations could be confirmed for the endogenous enzyme: at filamentous actin and at ER membranes. Figure 1(B) shows the staining pattern of NRK 52E cells incubated with kin 4 and red-labelled anti-mouse secondary antibody. Actin was stained by FITC-labelled phalloidin (Figure 1A). Merging of Figures 1(A) and 1(B) shows the targeting of endogenous RnIP3K-B to F-actin (Figure 1C). ER localization could also be confirmed by investigating the co-localization of the endogenous RnIP3K-B (Figure 1E) with an over-expressed ER-specific protein labelled with ECFP (enhanced cyan fluorescent protein) (Figure 1D). Merging of Figures 1(D) and 1(E) shows the targeting of endogenous RnIP3K-B to the ER (Figure 1F). Specificity of kin 4 was further confirmed by a competition experiment. After pre-incubation of kin 4 with recombinant catalytic domain of RnIP3K-B, specific labelling was lost (results not shown).

Western blot analysis of IP3K isoform expression in NRK 52E cells

The high affinity monoclonal antibody (kin 4) was employed for detection of endogenous IP3K. This antibody binds the bacterially expressed catalytic domain of rat IP3K-B (aa 570–934) at the $\text{Ins}(1,4,5)\text{P}_3$ binding site with a $K_d < 1 \text{ nM}$, as determined from ELISA assays (results not shown). This antibody exhibits no binding to IP3K-C, but also detects rat IP3K-A with a $K_d \sim 0.1 \text{ nM}$. Therefore, we had to determine the level of expression of the latter isoform in the NRK 52E cells that were mainly used in immunofluorescence studies. For these cells, only the presence of IP3K-B has been reported previously [19].

The IP3K isoform expression profile was determined by Western blot analyses of cell lysates using the antibody kin 4.

Neutral lysis techniques, e.g. with hot or cold SDS buffer, always led to a complex pattern of proteolytic IP3K bands of low reproducibility and thus were unsuitable for these experiments. Only TCA lysis led to reproducible, distinct bands of IP3K-B without proteolysis. As shown in Figure 2(A), Western blots of extracts from these cells almost exclusively exhibited bands specific for isoform B. But intriguingly, only a small amount (3 %) of uncleaved full-length protein was detected as a band slightly below 100 kDa, whereas most of the protein (97 %) was non-enzymically cleaved by the TCA treatment at Asp–Pro sites. Cleavage at Asp–Pro clusters has been reported in many proteins and can occur under mildly acidic conditions [32,33]. Occurrence of Asp–Pro cleavage of IP3K-B was verified with a purified prokaryotically expressed N-terminally truncated form of IP3K-B (aa 555–934) including three Asp–Pro clusters at aa 610, 617 and 765. Incubation of the enzyme for only 3 min at pH 2–3 and temperatures of 4, 37, 60 and 95 °C, in fact led to the expected specific bands at about 18 and 22 kDa (see Figure 2B) in Western blot analyses employing antibody kin 4. Strong cleavage was already seen at a temperature of 37 °C (results not shown). Cleavage of the full-length enzyme at one or several of the four predicted sites (Figure 2B) can lead to seven fragments, 82.3, 58.5, 39.1, 37.5, 36.8, 18.1 and 17.4 kDa in size, all containing the epitope detected by antibody kin 4. In fact, the predicted 82.3 and 58.5 kDa fragments, as well as the 37.5 kDa and 36.8 kDa fragments, the latter appearing together in one broader band, are found in the blot (Figure 2A). The 39.1 kDa band and those below 20 kDa were not detected, probably due to very limited cleavage at more than one Asp–Pro site during the TCA treatment at between 4 °C and 25 °C. A band which might be a very small amount of intact IP3K-A of about 55 kDa could be detected too, but to a much lesser extent. IP3K-A contains one Asp–Pro site at aa 285 corresponding to the site at aa 765 in IP3K-B. Therefore, a fragment of 31.5 kDa should also be formed when IP3K-A was present. Since such a fragment was never detected, it is unlikely that the 55 kDa band

Figure 2 Western blot analysis of IP3K-B and IP3K-A in TCA-treated lysates from NRK 52E cells

(A) Washed TCA lysate prepared from NRK 52E cells was subjected to SDS/PAGE and transferred on to nitrocellulose. Immunoblotting was performed using anti-IP3K antibody (catalytic domain; kin 4) and AP-conjugated goat anti-mouse secondary antibody. (B) Predicted fragment pattern of IP3K-B obtained by acidic cleavage at Asp-Pro sites. Asp-Pro clusters are located at aa 408, 610, 617 and 765. Cleavage at these sites leads to seven predicted fragments detectable by an antibody against the catalytic domain of IP3K-A and -B. The sizes of the fragments detected in the blot are 82.3, 58.5, 37.5 and 36.8 kDa (marked by large arrows). A very small amount of a band at 55 kDa (marked by a small arrow) is also evident.

represents IP3K-A. It rather represents a trace of a proteolytic cleavage fragment of IP3K-B which is formed in high amounts upon neutral extraction (see above). From independent analyses of the minimal detection limit of kin 4 for purified recombinant catalytic domains of IP3K-A and IP3K-B, we confirmed that also under our blotting conditions the lower limit of IP3K-A detection is at least 5-fold better than for traces of IP3K-B. Based on a

densitometric band analysis and taking into account this higher detection sensitivity of kin 4 for IP3K-A than for isoform B, IP3K-A would be at most 1 % of the amount of IP3K-B. Furthermore, several attempts to detect IP3K-A mRNA in NRK 52E cells by RT-PCR were performed, but all were negative. In conclusion, an expression level of IP3K-A of between 0 % and 1 %, which cannot be ruled out by these controls, should not influence the immunofluorescence data on localization of IP3K-B in this work.

N-terminal region of rat IP3K-B co-localizes with the cellular actin cytoskeleton

The N-terminal region of IP3K-B was further investigated as the main candidate structure for the F-actin-targeting function on the basis of (i) the findings of Schell et al. [18], who identified an F-ABD in the N-terminus of IP3K-A, (ii) its targeting function when over-expressed as a fusion protein with EGFP observed by Dewaste et al. [20], and (iii) our own findings of a homogeneous distribution of EGFP-fusion proteins comprising the calmodulin-binding plus catalytic domains of rat IP3K-B when over-expressed in PC12 and A431 cells (results not shown). The coding sequence of the N-terminus of rat IP3K-B (GenBank® accession no., AJ242781) was completed by 5'-RACE, using RNA from rat brain as template. To further examine the substructure of rat IP3K-B responsible for actin-targeting, NRK 52E and PC12 cells were transfected with an expression construct coding for the N-terminal region of rat IP3K-B (aa 108–583) and C-terminally fused EGFP. The first 107 amino acids were omitted due to solubility problems of this fusion protein upon over-expression. Confocal microscopy of fixed NRK 52E (Figures 3A–3C) and PC12 cells (results not shown) clearly showed a co-localization of IP3K-B(108–583)-EGFP with F-actin labelled by TRITC-phalloidin. No co-localization with the ER was found, indicating that for ER binding the N-terminal domain alone may not be sufficient or not even necessary. A certain amount of nuclear fluorescence was always observed with this fragment, which may be due to the presence of a predicted monopartite NLS (nuclear localization sequence) Arg-Lys-Leu-Arg at aa 134–137.

A fragment of the N-terminal region of IP3K-B comprising 63 amino acids is able to promote co-localization with F-actin *in vivo* depending on the correct secondary structure

To define in more detail the amino acids mediating actin binding, secondary structure prediction for full-length RnIP3K-B was

Figure 3 Co-localization of a long N-terminal IP3K-B (aa 108–583) fragment with F-actin in fixed NRK 52E cells

NRK 52E cells were transfected with the IP3K-B(108–583)-EGFP construct. For F-actin staining, fixed cells were incubated with TRITC-labelled phalloidin. IP3K-B(108–583)-EGFP (A), F-actin (red) (B), Overlay between IP3K-B(108–583)-EGFP and F-actin (C). Confocal micrographs were recorded at $\times 100$ magnification.

Figure 4 IP3K-B secondary structure prediction

Sketch of the predicted secondary structure of IP3K-B. High content of helical structure is predicted for the region aa 108–170. The four different constructs used for transfection experiments are represented by bars. IP3K-B Fragment I (aa 108–170); II (aa 170–430); III (aa 561–620); IV (aa 108–583); V is the catalytic domain represented by the broken line.

Figure 5 Fragment I of IP3K-B (aa 108–170) co-localizes with F-actin in fixed NRK 52E and PC12 cells and disrupts F-actin binding of endogenous RnIP3K-B

NRK 52E (A–C) and PC12 (D–F) cells were transfected with the IP3K-B(108–170)–EGFP construct. For F-actin staining, cells were treated with TRITC-labelled phalloidin. Confocal micrographs were recorded at $\times 100$ magnification. IP3K-B(108–170)–EGFP (A and D), F-actin (B and E), fusion protein co-localizing with stained F-actin appearing yellow in the merged images is shown in (C and F). Scale bar represents 10 μm . The overlay (I) of (G) and (H) shows that over-expressed ABD (G) disrupts F-actin targeting of endogenous IP3K-B, stained by kin 4 (H).

performed with Network Protein Sequence @analysis [http://pbil.ibcp.fr/] (Figure 4). Because of its high density of α -helical secondary structure, the segment between amino acids 108–170 was hypothesized as a candidate abd. This segment showed similarities to the F-ABD of IP3K-A identified by Schell et al. [18]. Confocal microscopy of fixed NRK 52E (Figures 5A–5C) and PC12 cells (Figures 5D–5F) transfected with a vector encoding fragment I (aa 108–170) of IP3K-B fused to EGFP, in fact, showed a clear co-localization with F-actin labelled by TRITC-phalloidin.

When present in high cellular concentration, this fragment is furthermore able to disrupt endogenous RnIP3K-B localization to F-actin. Endogenous RnIP3K-B detected by kin 4 (Figures 5H

Figure 6 Localization of different ABD mutants fused to EGFP

Non-confocal micrographs were recorded at $\times 100$ magnification. Scale bar represents 10 μm . Two truncated forms of the ABD of RnIP3K-B described in text show an equal distribution in the NRK 52E cells similar to the distribution of EGFP (C). (A) shows a fragment comprising aa 120–148, and (B) a fragment composed of aa 120–159. Likewise, helix-breaking mutations in either the first (RnMut1, L139P, L143P) (D) or the second α -helix (RnMut2, I159P, T163P) (E) of the ABD lead to evenly distributed fusion protein, with some remaining F-actin localization observed in (D). RnMut2 present in the long N-terminal region (aa 108–583) of RnIP3K-B (F) leads also to an evenly distributed fusion protein with no detectable F-actin binding. The F-actin localized wt EGFP-fusion protein of this N-terminal region fragment is shown in (G).

and 5I) was no longer co-localized with F-actin fibres after over-expression of fragment I (Figures 5G and 5I). Beside a granular fluorescence in the cytosol and some fluorescence in the nucleus, indicating persistent ER and nuclear localization respectively, only a faint fluorescence near the plasma membrane remained visible.

Truncation of the discovered ABD both at its N- and C-terminal flank by about 11 residues led to fragments without actin-binding potential. Fragments comprising aa 120–148 or 120–159 C-terminally fused to EGFP were equally distributed in the cytosol and the nucleus in transfected NRK 52E cells, similar to the EGFP control (Figures 6A–6C). Helix-breaking mutations in the first α -helix (L139P, L143P) lead to a marked loss of F-actin-binding ability, as shown in Figure 6(D), with only a slight binding to some filaments remaining. Point mutations in the second α -helix (I159P, T163P) lead to equally distributed fusion proteins (Figure 6E), with F-actin targeting no longer detectable. These experiments show that the fragment ranging from aa 108 to 170 is necessary in its whole length and its native structure to be able to promote F-actin localization. A comparison with Figure 5 indicates that somewhat more of the fusion protein might be located in the nucleus when F-actin targeting is abolished.

Putative ABD comprising aa 108–170 is the only actin-binding region in the N-terminal domain of IP3K-B

In order to further prove that the segment comprising aa 108–170 is the only ABD in the N-terminal domain of IP3K-B and to show that the secondary structure of this segment is essential for actin binding, helix-breaking mutant RnMut 2 was also generated in the RnIP3K-B(108–585)–EGFP-fusion construct. This mutant, when over-expressed in NRK 52E cells, in fact was no longer detectably targeted to F-actin (Figure 6F), but was homogeneously distributed in the cells, whereas the non-mutated fusion protein (aa 108–585) was strongly targeted to F-actin (Figure 6G). Since such a discrete mutation within the discovered ABD nearly completely abolished F-actin binding of the fusion protein comprising most of

Figure 7 Disruption of F-actin localization by deletion of the identified ABD in over-expressed full-length IP3K-B

Non-confocal fluorescence micrographs were recorded at $\times 100$ magnification. Scale bar represents $10\ \mu\text{m}$. NRK cells were transfected with a vector encoding full-length HsIP3K-B-EGFP-fusion protein (A), F-actin was stained with red-labelled phalloidin (B). In (C) a vector carrying a deletion removing aa 103–165 was transfected.

the N-terminal domain of IP3K-B, this experiment increases the evidence that it is this distinct domain and its special secondary structure which are mainly responsible for F-actin binding of IP3K-B.

Detected ABD in the N-terminal domain is the only functional domain in full-length IP3K-B

The two types of specific localization observed for endogenous IP3K-B, at F-actin and the ER, were clearly confirmed by over-expression of full-length HsIP3K-B in NRK cells. After different unsuccessful approaches to clone the full-length cDNA of rat IP3K-B, the human full-length cDNA of IP3K-B was cloned by fusion PCR and employed for this experiment.

Comparison of cells stained with TRITC-labelled phalloidin (Figure 7B) and over-expressing full-length HsIP3K-B-EGFP-fusion protein (Figure 7A) shows that the over-expressed enzyme is mainly located at the ER and at F-actin. No direct plasma membrane localization could be shown by inspecting over 100 transfected cells, but, in contrast with the endogenous enzyme, a fraction of the over-expressed fusion protein was clearly localized inside the nucleus at a higher extent than as over-expressed control EGFP. This again indicates the activity of weak NLS in IP3K-B.

In order to rule out that an additional ABD is either present in the catalytic domain or is generated in addition to the identified one only in the context of N-terminal and catalytic domain, aa 103–165 of the sequence encoding for fragment I in the HsIP3K-B, were deleted in the full-length HsIP3K-B-EGFP-fusion vector. This deletion mutant (Figure 7C), when over-expressed in NRK cells, does no longer bind to F-actin, but still binds to the ER. Furthermore, it is exclusively located in the cytoplasm, confirming the now deleted activity of the putative NLS consisting of aa 128–131 in HsIP3K-B.

Binding measurements of IP3K-B fragment I (aa 108–170) to F-actin *in vitro*

Since all experiments performed hitherto could show a co-localization with F-actin, but were unsuitable to confirm a direct molecular interaction between fragment I and F-actin. F-actin-binding assays were performed *in vitro* with bacterially expressed wt actin-binding fragment I (aa 108–170) of RnIP3K-B, and with mutants again carrying the helix-breaking mutations (L139P, L143P) or (I159, T163). All fragments were expressed as GST-fusion proteins and purified to homogeneity by glutathione-Sepharose affinity chromatography. After incubation of the wt fusion protein with F-actin, the fluorescence intensity of the labelled

Figure 8 Actin-binding measurements of IP3K-B ABD-fragment I (aa 108–170) and its mutants 1 and 2 to F-actin *in vitro*

Normalized fluorescence intensity after incubation of different concentrations of fusion protein GST/RnIP3K/ABD (wt-ABD, $\blacklozenge$), mutant 1 ($\blacksquare$) and mutant 2 ($\blacktriangle$), (0–20 μM) with 1 μM F-actin (A) or of wt/GST/RnIP3K/ABD with 1 μM G-actin (B). A binding curve calculated for $K_d = 10.1\ \mu\text{M}$ (continuous line) was the best fit for the measured fluorescence intensity versus ABD-fusion protein concentration changes.

pre-polymerized filaments increased, indicating a direct protein binding. From the concentration-dependent final fluorescence increases a $K_{d(\text{app})}$ of about $10\ \mu\text{M}$ was derived for the binding of the GST-IP3K-B-fusion protein bound to F-actin. The two ABD mutants did not show any increase of fluorescence intensity (Figure 8A).

No concentration dependent increase of fluorescence intensity could be detected when wt GST-IP3K-B(108–170)-fusion protein was incubated with fluorescently labelled G-actin, (Figure 8B), indicating that the IP3K-B fragment I does not bind to G-actin.

DISCUSSION

It has previously been shown that endogenous or over-expressed IP3K-B is localized at the ER and actin [19,20,34], but the domains responsible for these localizations have not yet been identified. Dewaste et al. [20] theoretically suggested a domain consisting of the amino acids 231–309, because of its helical and proline-rich character, as a putative ABD. We showed that a fragment comprising aa 170–430, i.e. this segment, does not bind to actin. However, we proved by a number of complementary approaches that the amino acids 108–170 include the necessary and essential domain for the targeting of RnIP3K-B to F-actin. Firstly, only fragments (over-expressed as EGFP-fusion proteins) including all of these amino acids do bind to F-actin. Secondly, helix-breaking mutants of the N-terminal domain (aa 108–583)-EGFP-fusion protein show a strong decrease of actin binding ability up to the total loss of binding in the case of the destroyed second α -helix, indicating that an intact secondary structure of this ABD is necessary and essential for F-actin binding. Thirdly, the F-actin localization of endogenous enzyme could be specifically disrupted by over-expressing a fragment of the N-terminal domain of IP3K-B only containing the identified ABD.

The possibility that (a) further F-ABD(s) reside somewhere else in the full-length enzyme protein (in the sequence 1–108 or in the C-terminal part beyond amino acid 583) could be ruled out by investigating the localization of full-length IP3K-B lacking only the segment identified here. This no longer exhibited F-actin localization, but retained ER localization. Intriguingly, this deletion also completely abolished the nuclear localization of part of the over-expressed protein, which thus is likely to be due to the monopartite NLS with sequence RKLR residing within the ABD. This experiment also showed that the identified ABD is not an artefact occurring in expressed fragments of the N-terminal domain, but a functional ABD present in the full-length enzyme protein. Finally, the displacement of endogenous IP3K-B by over-expressed ABD fragment proves its reversible actin-binding potential in intact cells.

The direct binding of the discovered ABD to F-actin *in vitro* reveals that no adapter proteins are apparently involved in the targeting of IP3K-B to F-actin, but the ABD itself interacts with actin filaments.

In comparison with other F-actin-binding proteins the binding affinity found in our *in vitro* binding experiments ($K_{d,app} \sim 10 \mu\text{M}$) is relatively high: plectin binds to F-actin with an apparent K_d of $0.3 \mu\text{M}$ [35], Abp1p with a $K_d < 0.1 \mu\text{M}$ [36], the Arp2/3 complex with a K_d of $2 \mu\text{M}$ [35], and WASp with a $K_d \approx 1 \mu\text{M}$ [37]. The $K_{d,app}$ of our bacterially expressed ABD–GST-fusion protein is not necessarily the one of the intact ABD in the full-length enzyme, since fusion with GST, potential oligomerization of this fusion protein as sometimes mediated by GST in fusion proteins, and the absence of the sequence context of the full N-terminal domain of IP3K-B may all negatively influence the correct 3D-structure formation of the ABD and thus the binding affinity. Although apparently unable to exhibit F-actin binding by themselves, further segments within the N-terminus of IP3K-B may still contribute to F-actin binding by stabilizing the correct three-dimensional structure of the ABD or by contributing further contact sites with F-actin.

Also an isolated N-terminal domain of IP3K-B, perhaps cleaved *in vivo* from the catalytic domain by calpain cleavage at the strongest PEST sequence according to the proposal of Pattni et al. [38], would remain or become bound to F-actin, as seen in our findings of a strong F-actin binding of a long N-terminal fragment comprising aa 108–583. This might give the N-terminal domain of IP3K-B a cellular function distinct from the catalytic domain.

Although a variety of actin-binding proteins is known, only a few typical ABDs have been identified: the CH domain (calponin homology domain), the gelsolin homology domain, the WH2 domain (WASP homology domain 2), the ADF-H (ADF homology) domain and the I/LWEQ module [39–41]. The ABD identified in the present study shows some sequence similarity to the block I sequence of an I/LWEQ module. However, further investigations led to the conclusion that aa 108–170 build an ABD different to the block I of an I/LWEQ module or any other known ABD. All I/LWEQ modules identified up to now are localized in the C-terminus of the respective proteins, whereas the ABD of IP3K-B is localized in the N-terminus. I/LWEQ modules only bind to actin if all four blocks comprising the module are in a certain close sequence context and are undisrupted. In the IP3K-B only three amino acid segments resembling blocks I, III and IV of the I/LWEQ module can be found in the N-terminus of the protein, and the sequence between block III and IV is much too long. Furthermore, the domain comprising the aa 107–180, which only shows weak similarities to block I of the I/LWEQ module, was sufficient to promote co-localization *in vivo* with actin filaments alone. Forms truncated to the size of a block I segment of such an I/LWEQ module lost their actin-binding ability (see the Results

Figure 9 Comparison of the ABDs of IP3K-A and IP3K-B

Secondary structure prediction and alignment of the ABDs of IP3K-A (upper, amino acids 1–66) and IP3K-B (lower, amino acids 108–170). Prolines are indicated by arrowheads, β -strands by a light grey box and α -helices by dark grey boxes. Identical residues are indicated by black background.

section). Our conclusion from these discrepancies, and from ineffective mutants of residues in the ABD critical in block I in I/LWEQ modules for F-actin binding (results not shown), is that the resemblance is random or a result of convergent evolution.

Interestingly, the identified ABD of IP3K-B shows about 20% identity with the ABD of IP3K-A described by Schell et al. [18] (Figure 9). The characteristic segment of the ABD of IP3K-A is a proline-rich region including six prolines followed by a predicted α -helix. The ABD of IP3K-B includes three prolines followed by two predicted α -helices. Two motifs within both sequences (boxed in Figure 9) are well conserved: the first one in the proline-rich N-terminal part contains five identities and several conservative exchanges within a segment of 9 amino acids, the second one is located within the α -helical C-terminal part and contains the sequences LRLI and LRIL in IP3K-A and IP3K-B respectively. Our experimental data and the sequence comparison indicate that the F-ABDs of IP3K isoforms A and B are members of a novel type of F-ABDs.

The targeting of the endogenous IP3K-B to the ER and to filamentous actin, the latter in the form of stress fibres, the cortical actin mesh, and worm-like fibres in NRK 52E cells (see Figure 1B), as well as the low concentration of soluble endogenous enzyme, make it likely that these localizations influence both the fate of $\text{Ins}(1,4,5)\text{P}_3$ when released from the plasma membrane upon phospholipase C stimulation and the duration of action of $\text{Ins}(1,4,5)\text{P}_3$ on InsP_3 receptors and thus on Ca^{2+} release. Targeting of IP3K-B to cortical actin might 'channel' part of the released $\text{Ins}(1,4,5)\text{P}_3$ towards higher phosphorylated inositols, $\text{Ins}(1,3,4,5)\text{P}_4$ and finally InsP_6 , before being able to diffuse away from the plasma membrane and bind to the InsP_3 receptors at the ER. The high local concentrations of $\text{Ins}(1,3,4,5)\text{P}_4$ thus generated near the plasma membrane could more efficiently regulate $\text{Ins}(1,3,4,5)\text{P}_4$ -sensitive GAP proteins located at this compartment [3,42–44]. They also might interfere with PtdInsP_3 -binding receptor proteins, some of which appear to also bind $\text{Ins}(1,3,4,5)\text{P}_4$ *in vitro* [45,46]. Binding of IP3K-B to ER structures also comprising $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores would help to terminate the $\text{Ins}(1,4,5)\text{P}_3$ -induced Ca^{2+} signals by more efficiently guiding $\text{Ins}(1,4,5)\text{P}_3$ released from InsP_3 receptors towards phosphorylation in addition to dephosphorylation. Since IP3K-B is a strongly Ca^{2+} /calmodulin-activated enzyme, both the latter type of targeting near the cellular site of Ca^{2+} release and the former type of targeting near the sites of Ca^{2+} influx via store-operated or otherwise agonist-gated plasma membrane Ca^{2+} channels should synergistically act in driving more of the totally released $\text{Ins}(1,4,5)\text{P}_3$ towards further phosphorylation instead of dephosphorylation. The much lower K_m of IP3K-B for $\text{Ins}(1,4,5)\text{P}_3$, as compared with that of InsP_3 -5-phosphatases (reviewed in [47,48]), would further guarantee this switch in the metabolic fate. Finally, the intriguing finding of a weak NLS within the ABD responsible for uptake of part of the over-expressed IP3K-B into the nucleus might also allow some endogenous enzyme to enter the nucleus when cellular F-actin depolymerizes to a larger extent.

Further experiments will be necessary to investigate in more detail the dynamics, regulation and functional role of the

F-actin–IP3K-B interaction in the above discussed context, as well as to find out the domains in IP3K-B responsible for the observed ER interaction.

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