Calpain cleavage of the B isoform of Ins(1,4,5) P_3 3-kinase separates the catalytic domain from the membrane anchoring domain

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Inositol (1,4,5)-trisphosphate [Ins(1,4,5) P_3] is one of the key intracellular second messengers in cells and mobilizes Ca^{2+} stores in the ER (endoplasmic reticulum). Ins(1,4,5) P_3 has a short half-life within the cell, and is rapidly metabolized through one of two pathways, one of which involves further phosphorylation of the inositol ring: Ins(1,4,5) P_3 3-kinase (IP₃-3K) phosphorylates Ins(1,4,5) P_3 , resulting in the formation of inositol (1,3,4,5)-tetrakisphosphate [Ins(1,3,4,5) P_4]. There are three known isoforms of IP₃-3K, designated IP₃-3KA, IP₃-3KB and IP₃-3KC. These have differing N-termini, but highly conserved C-termini harbouring the catalytic domain. The three IP₃-3K isoforms have different subcellular locations and the B-kinase is uniquely present in both cytosolic and membrane-bound pools. As it is the N-

terminus of the B-kinase that differs most from the A- and C-kinases, we have hypothesized that this portion of the protein may be responsible for membrane localization. Although there are no known membrane-targeting protein motifs within the sequence of IP₃-3KB, it is found to be tightly associated with the ER membrane. Here, we show that specific regions of the N-terminus of IP₃-3KB are necessary and sufficient for efficient membrane localization of the protein. We also report that, in the presence of Ca^{2+} , the kinase domain of IP₃-3KB is cleaved from the membrane-anchoring region by calpain.

Key words: amphipathic helix, calpain, coiled-coil, green fluorescent protein (GFP), nucleus.

INTRODUCTION

Inositol (1,4,5)-trisphosphate [Ins(1,4,5) P_3] is a key intracellular messenger in cells, and has been well established as a mediator of Ca^{2+} release from intracellular stores [1]. Ins(1,4,5) P_3 is produced as a result of the cleavage of phosphatidylinositol (4,5)-bispophosphate [PtdIns(4,5) P_2] by phospholipase C at the plasma membrane into cytosolic Ins(1,4,5) P_3 and membrane-bound diacylglycerol. Ins(1,4,5) P_3 mobilizes Ca^{2+} stores in the ER (endoplasmic reticulum) by binding to, and concomitantly activating, the Ins(1,4,5) P_3 -receptor (IP₃R) in the ER membrane [1–4]. Upon binding of four Ins(1,4,5) P_3 molecules, the receptor (which also functions as an ion channel) opens and allows the flow of Ca^{2+} ions out of the ER. Extracellular Ca^{2+} then refills intracellular Ca^{2+} stores by a process known as capacitative Ca^{2+} entry (CCE) [2].

Like all second messengers, Ins(1,4,5) P_3 has a short half-life within the cell [5], and is rapidly metabolized through one of two pathways. Removal of the 5-phosphate from the inositol ring by inositol polyphosphate 5-phosphatases results in the production of Ins(1,4) P_2 , an inactive inositol phosphate [6]. This species is sequentially dephosphorylated to form free inositol, which can then be recycled back to form PtdIns [5].

The other pathway of Ins(1,4,5) P_3 metabolism involves further phosphorylation of the inositol ring. Ins(1,4,5) P_3 3-kinases (IP₃-3Ks) catalyse the ATP-dependent phosphorylation of the hydroxy group on the 3-carbon of inositol, resulting in the formation of inositol (1,3,4,5)-tetrakisphosphate [Ins(1,3,4,5) P_4] [7]. Ins(1,3,4,5) P_4 has also been implicated in Ca^{2+} signalling [8]. Ins(1,3,4,5) P_4 is rapidly metabolized to Ins(1,3,4) P_3 by the same inositol polyphosphate 5-phosphatase that dephosphorylates Ins(1,4,5) P_3 [6]. The exact role of Ins(1,3,4,5) P_4 remains to be

established, but specific cellular Ins(1,3,4,5) P_4 -binding proteins have been isolated [9], suggesting that this species may also act as an intracellular messenger.

To date three mammalian isoforms of IP₃-3K have been identified, designated IP₃-3KA, IP₃-3KB and IP₃-3KC. cDNA clones encoding the A and B isoforms were isolated in 1991 [10,11], although the sequence encoding the B isoform appeared to be incomplete when a longer sequence encoding rat IP₃-3KB was subsequently isolated using a strategy originally designed to isolate Golgi integral membrane proteins [12]. In 2000, a third human isoform of the enzyme (designated IP₃-3KC) was isolated [13]. A rat homologue of the human IP₃-3KC isoform has recently been identified, and was demonstrated to shuttle between the nucleus and cytoplasm of mammalian cells (AY160770; [14]). Recently, Erneux and co-workers reported the isolation of a cDNA encoding what now genuinely appears to be the full-length human B-isoform of IP₃-3KB [15]. The corresponding sequence for the rat B-isoform has also been deposited in the database [GenBank® accession no. AJ242781]. This 2841 bp sequence encodes a protein of 934 amino acids that includes a 261-amino-acid extension N-terminal to the originally published sequence [12]. {For clarity, in the present paper the full-length rat IP₃-3KB sequence (accession no. AJ242781) will be designated IP₃-3KB^{1–934} and the N-terminally truncated sequence [12] IP₃-3KB^{262–934} }.

The A, B and C isoforms of IP₃-3KB have a conserved C-terminus (≈ 80 % identity at the amino acid level) [16], which comprises the catalytic domain, and divergent N-termini, suggesting that properties of the N-terminus contribute to their regulation and/or subcellular localization. The high degree of sequence conservation within the catalytic domains of the different IP₃-3K isoforms would indicate conservation of the Ins(1,4,5) P_3 -binding site. It has been shown that the Ins(1,4,5) P_3 -binding site within

Abbreviations used: [Ca^{2+}]_i, cytosolic calcium concentration; CaM, calmodulin; CFP, cyan fluorescent protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; IP₃-3K, Ins(1,4,5) P_3 3-kinase; IP₃R, Ins(1,4,5) P_3 receptor.

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the catalytic domain of the A-kinase (residues 178 to 459) has homology with the Ins(1,4,5) P_3 -binding site of the IP₃R [17]. Lys¹⁹⁷ and Asp⁴¹⁴ have also been shown to be critical for the binding of ATP/Mg²⁺ in the A isoform [18]. All three isoforms have been shown to be sensitive to the cytosolic calcium concentration ([Ca²⁺]_i) of the cell [13,16]. The A- and B-kinases are substantially activated by increases in [Ca²⁺]_i, but the C-kinase is unique in that Ca²⁺ alone decreases the catalytic activity of the enzyme [13,16,19]. This effect on the C-kinase is reversed in the presence of CaM (calmodulin), and the binding of the Ca²⁺-CaM complex to IP₃-3KC serves to activate the enzyme [13].

The subcellular localization of the three IP₃-3K isoforms is markedly different, and as the N-termini of the isoforms differ the most, it seems reasonable to suggest that this region of the protein may be responsible for their subcellular localization. The extreme N-terminus of the A-kinase has been shown to target the enzyme to F-actin [20]. The endogenous B-kinase is associated with the cytosolic face of the ER network [19], and is also present in the cytosol (the ratio between soluble and particulate fractions in astrocytes was shown to be 65 % to 35 % [21]). Recent results published by Erneux and co-workers provide evidence that human IP₃-3KB exists in three distinct cellular compartments, namely the ER, plasma membrane and the cytoskeleton, when expressed as a recombinant protein in COS-7 cells [22]. There are contradictory results concerning the intracellular localization of the C-kinase: Erneux and co-workers reported no membrane association or retention of the human C-kinase to any specific cellular compartment when transiently expressed in COS-7 cells [13], and concluded that the enzyme is predominantly cytosolic. In contrast, the newly isolated rat C-kinase has been demonstrated to actively shuttle between the nucleus and the cytoplasm when transiently expressed in NRK or PC12 cells [14]. The N-terminus of the rat C-kinase appears to be responsible for such localization, and contains a consensus nuclear export signal (which is sensitive to leptomycin B, a common inhibitor of nuclear export) and an as-yet-unidentified nuclear import signal (NES) [14]. The NES appears to be conserved in the human C-kinase, although this protein was not reported to behave similarly.

It is of interest to note that the sequences of IP₃-3Ks contain a number of PEST motifs [15,23–26]. [PEST motifs are regions enriched in the residues P (proline), E (glutamine), S (serine) and T (threonine).] PEST sequences are a feature common to CaM-binding proteins (such as the IP₃-3Ks). Rechsteiner and co-workers have postulated that regions rich in PEST motifs are subject to rapid limited proteolysis, potentially by calcium-activated proteases such as calpain [27]. Indeed, the A-kinase has been shown to be sensitive to calpain [23], although the physiological implications of this have yet to be investigated.

Since the N-terminus of the A- and C-kinases is responsible for their subcellular localization, we hypothesized that the same might be true for the B-kinase. Publication of the full-length sequence of the human B-kinase [15] and deposition of the full-length rat B-kinase sequence provided us with the basic material to test this hypothesis and the possibility that calpain cleavage might affect the membrane association of IP₃-3KB.

EXPERIMENTAL

Plasmids

A summary of all IP₃-3KB-GFP (green fluorescent protein) constructs made is shown in Figure 2. All PCR products were ligated into pGEM-T (Promega) and sequenced before subcloning into relevant expression vectors.

IP₃-3KB^{262–934}-pTRE

IP₃-3KB^{262–934}-pTRE has been described previously [28].

IP₃-3KB^{262–650}-pTRE

The coding region was amplified by PCR using primers TM2 (5'-TTG GCC GCG GAT GGA AAG AGG TTC CCC GGC-3') and C2/65808 (5'-GCG AGA TCT TCA TCT GCC ACA ACT AAT GCC-3') and ligated, via pGEM-T, into *SacII/BglII*-digested pTRE (BD Biosciences, Cowley, Oxford, U.K.) which had been digested with *SacII* and *BglII*.

IP₃-3KB^{1–934}-pTRE

The cDNA encoding rat IP₃-3KB^{262–934} [28] was originally isolated [12] in a construct designated GORF34.1. This construct encompasses residues 205 to 934 of IP₃-3KB. IP₃-3KB^{1–934} was constructed in a two-step process (see Figure 2): the extreme 227 bp of IP₃-3KB were amplified from rat liver cDNA using KP5 (5'-AAC CTC CGT CGG CCG CTG CGC C-3') and KP6 (5'-TAC TAT GGC TGT GTA CTG CTA TGC CC-3'), which incorporated a 5' *SacII* site and extended as far as the first internal *EagI* site (i.e. comprising residues 1 to 89). This fragment was ligated into pGEM-T Easy (Promega) and sequenced. Digestion of IP₃-3KB^{262–934}-pTRE using *SacII* and *EagI* excised a 127 bp fragment (encoding residues 262 to 318), which was replaced with the 227 bp PCR fragment. This resulted in IP₃-3KB^{Δ89–318} (lacking an internal 683 bp *EagI/EagI* fragment). This *EagI* fragment was excised from GORF34.1, and ligated into *EagI*-digested IP₃-3KB^{Δ89–318} to yield IP₃-3KB^{1–934}-pTRE.

IP₃-3KB^{1–635}-pTRE

A *BglII* site was introduced into IP₃-3KB^{1–934}-pTRE by mutation of residues 634 and 635 using the QuikChange® Site-Directed Mutagenesis kit (Stratagene) and primers KP20 (5'-GGA TCC AGC TGG CAG GAG ATC TAG GGA GCT TCA AGG CAG C-3') and KP21 (5'-GCT GCC TTG AAG CTC CCT AGA TCT CCT GCC AGC TGG ATC C-3'), according to the manufacturer's instructions. The entire C-terminus (residues 636 to 934) was then excised from IP₃-3KB^{1–934}-pTRE using *BglII* and the vector was self-ligated, resulting in IP₃-3KB^{1–635}-pTRE.

IP₃-3KB^{650–934}

The catalytic region of IP₃-3KB was amplified by PCR using TM11 (5'-CAT ATG ATG TCC TTC AAG AAG -3') and TM12 (5'-CCG CGG GGG TGA GTG GGC T-3') and the resulting 900 bp fragment was ligated into *BglII/PstI*-digested pEGFP-C1 (BD Biosciences) via pGEM-T.

PA-GFP (comprising residues Pro¹²⁷ to Ala¹⁹⁷) and GA-GFP (comprising residues Gly⁷² to Ala¹⁹⁷)

Primers KP7 (5'-GGA TCC AGC CAA GAC CCT TTC AGG AGG C-3'), KP8 (5'-AAG CTT ATG GGG GGT TGG CGC AGC GGC CG-3') and KP9 (5'-AAG CTT ATG CCT GGG CCG GAG GAG GCC C-3') were designed to amplify the relevant regions of IP₃-3KB and to incorporate *HindIII* and *BamHI* sites at the 5' and 3' ends respectively. GORF34.1 was used as a template. The amplified fragments were ligated into pGEM-T Easy, sequenced, and then ligated into pEGFP-N3 (BD Biosciences). An ER-localized cyan fluorescent protein (CFP) (BD Biosciences) was used as a marker for ER membranes.

Cell culture

pTet-Off-transfected HeLa cells (BD Biosciences) were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10 % (v/v) fetal-calf serum (Life Technologies, Inc.), 100 units/ml penicillin and 100 μ g/ml streptomycin (Life Technologies, Inc). Cells were transfected with IP₃-3KB constructs using SuperFect (Qiagen, Crawley, West Sussex, U.K.) or FuGene 6 (Roche Diagnostics, GmBH, Mannheim Germany) according to the manufacturer's instructions. For fluorescence studies, cells were seeded on to 22 mm round coverslips 24 h before transfection, and processed for microscopy 24 h post-transfection. For biochemical analysis, cells were seeded on to 90-mm-diameter dishes and grown to 40 % confluence, and then transfected with the appropriate construct. Transfected cells remained in culture for a further 48 h before membrane isolation.

Fluorescence imaging of transfected cells

Cells, seeded on to coverslips, were treated 24 h post-transfection. Cells were washed once in PBS, fixed with PFA (2 % paraformaldehyde/0.02 % glutaraldehyde) for 20 min at room temperature ($\approx 22^\circ\text{C}$), and then permeabilized with 0.1 % (v/v) Triton X-100 for 4 min at room temperature, followed by a further wash in PBS. Coverslips were finally mounted on to microscope slides with Mowiol^{TM}. Images were obtained by confocal laser-scanning microscopy using a Leica AOBs Sp2 confocal imaging system attached to a Leica DM IRE2 inverted epifluorescence microscope with an argon (488 nm) excitation laser (Leica Microsystems). An oil-immersion objective lens 63 $\times$, N.A. 1.4 was used, and imaging parameters were selected to optimize confocal resolution. Images were processed for presentation using Adobe Photoshop 6.0.

Membrane preparations of mammalian cells

Lysates of isolated membranes were prepared as described previously [29]. In brief, transfected cells were lysed in a hypotonic lysis buffer, and the post-nuclear supernatant was subjected to centrifugation at 100 000 g to pellet membranes. Cytosolic proteins were precipitated by incubation with an equal volume of 25 % (w/v) trichloroacetic acid for 10 min on ice followed by centrifugation at 1000 g for 5 min. Precipitated proteins were washed twice in acetone, pellets were dried, and then resuspended in sample buffer. Samples were lysed in SDS/PAGE sample buffer prior to SDS/PAGE and immunoblot analysis. Bands were visualized using an enhanced chemiluminescence detection system (Roche Diagnostics, GmBH) following antibody probing as described below.

Antibodies

Nitrocellulose membranes were firstly probed with an anti-EGFP antibody (Roche Diagnostics GmBH) in order to visualize the localization of GFP-tagged IP₃-3KB constructs. Membranes were then stripped by incubation with stripping buffer (100 mM 2-mercaptoethanol/2 % SDS in 62.5 mM Tris/HCl, pH 6.8) for 30 min at 55 $^\circ\text{C}$ [30], then re-probed with an antibody raised against the ER protein calreticulin (Affinity BioReagents). A specific antibody SS1 was raised against the peptide TLQH-DVPWQEGNREDGYC, corresponding to amino acid residues 897–913 of rat IP₃-3KB, as described previously [19]. This antibody recognizes a glutathione S-transferase (GST) fusion protein encompassing residues 650–934 of IP₃-3KB, but not GST alone.

In vitro calpain cleavage of recombinant IP₃-3KB expressed in HeLa cells

HeLa cells expressing IP₃-3KB^{262–934}, (designated C6) [28], were lysed in the presence of either 5 mM Ca²⁺ or 5 mM EGTA. In the case of cells lysed in the presence of ionomycin, cells were incubated with 1.3 μ M ionomycin for 20 min prior to lysis in Ca²⁺-free buffer. In this case, the extracellular medium had a [Ca²⁺] of 1.8 mM. C6 lysate (60 μ l) was incubated with 10 μ g of m-calpain (from rabbit skeletal muscle, 30 units $\cdot$ mg⁻¹; Sigma) in 120 μ l of buffer [50 mM NaCl/20 mM Hepes (pH 7.5)/5 mM CaCl₂/1 mM dithiothreitol]. Cleavage reactions were performed at 27 $^\circ\text{C}$. At various time points, 20 μ l aliquots of the reaction mix were removed to Microfuge tubes containing 5 μ l of 50 mM EGTA to terminate the reaction. Following the final termination, each sample was separated into two aliquots and all samples were then snap-frozen in liquid nitrogen and stored at -80°C . From each reaction, 10 μ l was subjected to SDS/PAGE on a 10 % (w/v) polyacrylamide gel. The gel was blotted on to nitrocellulose and the blot was probed with primary antibody SS1, used at a dilution of 1:1000. Parallel samples were assayed for IP₃-3K activity as described previously [28]. Assays were performed at 37 $^\circ\text{C}$ for 8 min, and each 15 μ l assay contained 1 μ l of the calpain cleavage reaction mix.

In vitro calpain cleavage of recombinant IP₃-3KB expressed in *Escherichia coli* in the presence of CaM

Lysate was prepared from *E. coli* expressing a series of constructs encoding N-terminally truncated forms of IP₃-3KB (IP₃-3KB^{262–934}, IP₃-3KB^{328–934}, IP₃-3KB^{465–934}, IP₃-3KB^{562–934} and IP₃-3KB^{649–934}): an overnight culture of transformed *E. coli* was diluted 1:50 and grown at 37 $^\circ\text{C}$ with vigorous shaking until the exponential growth phase was reached. Protein expression was induced by the addition of isopropyl β -D-thiogalactoside (to a final concentration of 1 mM). Following growth for a further hour, the culture was centrifuged at 3500 g for 10 min at 4 $^\circ\text{C}$ in a Sorvall H-6000A rotor. The supernatant was discarded and the pellet was resuspended in 10 ml of ice cold lysis buffer [10 mM Tris/HCl (pH 7.5)/100 mM NaCl/0.1 % (v/v) Triton X-100/1 mM dithiothreitol] and the centrifugation was repeated. The supernatant was discarded, and the pellet was resuspended in 1 ml of lysis buffer supplemented with protease inhibitors (1 μ g $\cdot$ ml⁻¹ leupeptin, 2 μ g $\cdot$ ml⁻¹ antipain, 10 μ g $\cdot$ ml⁻¹ benzamidin, 10 units $\cdot$ ml⁻¹ aprotinin, 1 μ g $\cdot$ ml⁻¹ chymostatin and 1 μ g $\cdot$ ml⁻¹ pepstatin A). Cells were then lysed by sonication. The lysate was centrifuged at 17 000 g for 20 min at 4 $^\circ\text{C}$, the supernatant was then separated into aliquots, snap-frozen in liquid nitrogen and stored at -80°C . Aliquots of the lysate (5 μ l) were diluted 1:3 in lysis buffer and incubated with 4 μ g of m-calpain in 75 μ l of buffer [50 mM NaCl/20 mM Hepes (pH 7.5)/5 mM CaCl₂/1 mM dithiothreitol] in the presence of 30 μ M CaM or BSA. Cleavage was then performed at 27 $^\circ\text{C}$. At various time points, 7.5 μ l aliquots of the reaction were terminated by removal into Microfuge tubes containing 2.5 μ l of 40 mM EGTA. Following the final termination, all samples were snap-frozen in liquid nitrogen and stored at -80°C . Aliquots of each sample (0.25 μ l) were subjected to SDS/PAGE on a 10 % gel, which was then blotted on to nitrocellulose. The blot was blocked and probed with antibody SS1.

IP₃-3K assays

IP₃-3K assays were performed using a procedure based on that described by Spencer et al. [31].

Figure 1 Structural-prediction analysis of IP₃-3KB: a region at the N-terminus is predicted to form a coiled-coil that may be an amphipathic helix

An online program (<http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html>) was used to demonstrate how residues 124 to 141 of IP₃-3KB have the potential to form an amphipathic helix in which one face of the helix is non-polar and the other face is charged.

RESULTS AND DISCUSSION

Structural-prediction analysis of rat IP₃-3KB

Since we had hypothesized that the N-terminus of rat IP₃-3KB may be responsible for its membrane association and subcellular localization, the complete sequence of IP₃-3KB^{1–934} was analysed using programs designed to identify potentially conserved structural elements. No known membrane-targeting motifs were identified. The sequence was also analysed using the COILS program (http://www.ch.embnet.org/software/COILS_form.html) and a region at the N-terminus (residues 120–150) was predicted to form a coiled-coil domain. This observation was supported by both JPred [32] and Lupas [33] prediction programs. This region is possibly too small to be a classical coiled-coil domain (involved in the interaction between two proteins) [34], but such a region of hydrophobic, polar and charged residues could form an amphipathic helix with the potential to bind membranes [34] (Figure 1).

Constructs encoding different regions of IP₃-3KB fused to GFP (summarized in Figure 2) were made in order to provide tools to directly address which region(s) of IP₃-3KB is involved in membrane association. These constructs include the full-length and isolated N-terminus of both the complete (residues 1–934; accession no. AJ242781) and partial (residues 262–934; accession no. X74227) IP₃-3KB sequences, and the isolated kinase domain (C-terminus; residues 650–934). Two short N-terminal constructs, encoding the putative helical region were also made as GFP fusions. The shorter of the two, designated PA (since it comprises residues Pro¹²⁷ to Ala¹⁹⁷), encodes just the helical region, whereas the longer construct, GA (Gly⁷² to Ala¹⁹⁷) encompasses the whole of PA and the extended serine-rich region upstream of the predicted helix.

Localization of IP₃-3KB constructs in mammalian cells

Full-length IP₃-3KB (IP₃-3KB^{1–934}) was expressed in HeLa cells as a GFP fusion protein (Figure 3A), and its subcellular localization was compared with that of an ER-localized CFP (ER-CFP; BD Biosciences) (Figure 3B). It is clear that IP₃-3KB^{1–934} is localized to intracellular membranes (see the inset to Figure 3A), as evidenced by the distinct reticular appearance of the cytosol. This is reminiscent of the ER as visualized by ER-CFP (see inset to Figure 3B) [this CFP is targeted to the lumen of the ER, and so is, by definition, reticular (see www.clontech.com)].

The other IP₃-3KB constructs were also expressed in mammalian cells. Most of the constructs did not appear to be distinctly ER-localized. The full-length N-terminal construct (IP₃-3KB^{1–635}; Figure 3F) appears to have some localization to the plasma membrane and reticular regions (see the arrows). Such a distribution is not observed for either of the constructs containing a truncated N-terminus [i.e. IP₃-3KB^{262–934} (Panel C) or IP₃-3KB^{262–650}; Figure 3D] or for the isolated C-terminus (IP₃-3KB^{650–934}; Figure 3E). Of the short N-terminal truncations, the putative helical region alone (PA; Figure 3G) does not appear to be membrane-associated, whereas the helical region along with the upstream extended serine-rich region (GA; Figure 3H) does localize GFP to some reticular structures, a pattern reminiscent of the ER-localized CFP (Figure 3B). As a comparison, cells expressing GFP alone (Figure 3I) were also imaged, and show a homogeneous distribution throughout the cytosol.

Figure 2 IP₃-3KB constructs

Schematic diagram of IP₃-3KB constructs used in this study. The extension at the N-terminus and the minimal catalytic domain are shaded differently. All constructs incorporated a C-terminal GFP tag, except the isolated kinase domain (IP₃-3KB^{650–934}), which has an N-terminal tag.

Figure 3 Fluorescence images of mammalian cells expressing different IP₃-3KB constructs

HeLa cells were transfected with DNA 24 h prior to live cell imaging using the FuGene6 transfection system, according to the manufacturer's instructions. Arrowheads indicate regions of IP₃-3KB association with the plasma membrane. (A), IP₃-3KB¹⁻⁹³⁴; (B), ER-CFP; (C), IP₃-3KB²⁶²⁻⁹³⁴; (D), IP₃-3KB²⁶²⁻⁶⁵⁰; (E), IP₃-3KB⁶⁵⁰⁻⁹³⁴; (F), IP₃-3KB¹⁻⁶³⁵; (G) PA-GFP; (H), GA-GFP; (I) GFP.

There is a significant GFP signal from the nucleus in cells transfected with a number of IP₃-3KB constructs [IP₃-3KB¹⁻⁹³⁴ (Figure 3A), IP₃-3KB²⁶²⁻⁹³⁴ (Figure 3C), IP₃-3KB²⁶²⁻⁶⁵⁰ (Figure 3D), PA-GFP (Figure 3G) and GA-GFP (Figure 3H)]. The longer IP₃-3KB constructs all contain a C-terminal GFP tag (see Figure 2), and IP₃-3KB is known to be a substrate for the Ca²⁺-activated proteases, i.e. the calpains [23]. Calpain-mediated cleavage of IP₃-3KB N-terminal to the GFP tag could potentially release GFP alone into the cytosol, from where it may enter the nucleus (since GFP is small enough to translocate through nuclear pores in the nuclear membrane [35]).

Thus imaging of cells expressing different GFP-tagged IP₃-3KB constructs indicates that the full-length IP₃-3KB is targeted to intracellular membranes. Furthermore, isolated regions of the protein (including the putative helical region at the N-terminus) appear to be capable of targeting GFP to intracellular membranes, albeit with different efficiencies.

Membrane versus cytosol localization of IP₃-3KB

The membrane association of each IP₃-3KB construct was also addressed biochemically. HeLa cells growing in 90 mm dishes were transfected with constructs encoding the relevant GFP fusion protein, and membranes were isolated. Cytosolic proteins were also precipitated. Cytosolic and membrane proteins were resolved by SDS/PAGE, transferred to nitrocellulose and assayed for the presence of the IP₃-3KB construct using anti-GFP antibodies

(Figure 4). For comparison, HeLa cells were transfected with constructs encoding either GFP (which should be only in the cytosol fraction) or ER-CFP (which should be predominantly in the membrane fraction), and analysed in the same way. The nitrocellulose membranes were also probed with an anti-calreticulin antibody to demonstrate efficient isolation of internal membranes.

As expected, when expressed alone, GFP was detected only in the cytosolic fraction (Figure 4, lanes 3 and 4). In addition, the ER-CFP construct is totally absent from the cytosolic fraction, and only appears in the membrane fraction (Figure 4, lanes 1 and 2), demonstrating that a protein localized to ER membranes does not contaminate the cytosolic fraction.

The IP₃-3KB¹⁻⁹³⁴ and IP₃-3KB¹⁻⁶³⁵ (i.e. with the complete N-terminus) constructs were found to be exclusively present in the membrane fraction [Figure 4, lanes 5 and 6 (IP₃-3KB¹⁻⁹³⁴) and lanes 7 and 8 (IP₃-3KB¹⁻⁶³⁵)]. In contrast, the N-terminal truncation mutants IP₃-3KB²⁶²⁻⁹³⁴ and IP₃-3KB²⁶²⁻⁶⁵⁰, although present in both cytosol and membrane fractions, were primarily present in the cytosol [Figure 4, lanes 9 and 10 (IP₃-3KB²⁶²⁻⁹³⁴) and lanes 11 and 12 (IP₃-3KB²⁶²⁻⁶⁵⁰)]. The isolated catalytic domain, IP₃-3KB⁶⁵⁰⁻⁹³⁴ (lanes 13 and 14) appears to be present in both cytosolic and membrane fractions, suggesting that this region is not efficiently targeted to membranes. Of the short N-terminal fragments of IP₃-3KB, the putative helical region alone (PA) is present in approximately equal amounts in membrane and cytosol fractions (Figure 4, lanes 17 and 18). However, GA (the helical region along with the upstream extended serine-rich

Figure 4 Membrane preparations of IP₃-3KB-transfected cells

HeLa cells were grown in 90 mm dishes and transfected with relevant plasmid constructs 48 h before experimentation using the SuperFect transfection system, according to the manufacturer's instructions. Membranes were isolated and cytosolic proteins were precipitated, and then resolved by SDS/PAGE (10% gels) and transferred to nitrocellulose. Membranes were loaded into odd-numbered lanes; cytosolic proteins were loaded into even-numbered lanes. Upper panels show the location of GFP-tagged fragments of IP₃-3KB (using an α -GFP antibody) and lower (control) panels show the presence of the ER-membrane marker calreticulin. Lanes 1 and 2, ER-CFP; lanes 3 and 4, GFP; lanes 5 and 6, IP₃-3KB¹⁻⁹³⁴; lanes 7 and 8, IP₃-3KB¹⁻⁶³⁵; lanes 9 and 10, IP₃-3KB²⁶²⁻⁹³⁴; lanes 11 and 12, IP₃-3KB²⁶²⁻⁶⁵⁰; lanes 13 and 14, IP₃-3KB⁶⁵⁰⁻⁹³⁴; lanes 15 and 16, GA-GFP; lanes 17 and 18, PA-GFP; lanes 19 and 20, untransfected HeLa cells. The presence of multiple bands in some lanes is due to non-specific cleavage of the GFP fusion proteins resulting in the release of smaller fragments that still retain the epitope recognized by the α -GFP antibody. The band corresponding to the relevant IP₃-3KB protein is marked by an arrowhead in each case. Results are typical of at least three separate experiments.

region) is almost exclusively present in the membrane fraction (lanes 15 and 16), indicating that this stretch of 125 amino acids is sufficient to direct membrane association of GFP (or the rest of IP₃-3KB). Bands corresponding to lower-molecular-mass proteins in these lanes represent cleavage products of the GFP fusion proteins, in which the epitope recognized by the antibody is retained. Arrowheads show the bands that correspond to PA-GFP (approx. 35 kDa) and GA-GFP (approx. 40 kDa). The α -GFP antibody does not recognize any endogenous proteins, as shown by the absence of cross-reactive bands in Figure 4, lanes 19 and 20 (untransfected HeLa cells).

Calpain cleavage of IP₃-3KB

In support of the hypothesis that it is the N-terminal region of IP₃-3KB that is required for its subcellular localization, the isolated C-terminal kinase domain appears to be present predominantly in the cytosol fraction (although some protein appears in the membrane fraction). The kinase domain is highly conserved between isoforms, and neither the A-kinase nor C-kinase (both with significantly shorter N-termini than the B-kinase) have been shown to be membrane-associated. It is of interest to note that the complete human IP₃-3KB sequence contains five PEST motifs [15], four of which are retained in the rat sequence [as predicted by the program PESTfind (<http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind/>); see Figure 5D, ii]. PEST sequences are a feature common to CaM-binding proteins, such as the IP₃-3KBs. Rechsteiner and colleagues [27] have postulated that regions rich in PEST motifs are subject to rapid limited proteolysis, potentially by calcium activated proteases such as calpain.

The positions of the PEST regions in rat IP₃-3KB (see Figure 5D, ii) led us to speculate that calpain-mediated cleavage of

IP₃-3KB might separate the C-terminal catalytic domain of the protein from what we have demonstrated to be the N-terminal, membrane-anchoring domain of the protein. In order to address this possibility, the following experiments were performed. HeLa cells expressing IP₃-3KB²⁶²⁻⁹³⁴ (designated C6 [28]) were lysed in the presence of Ca²⁺ or EGTA, proteins were resolved by SDS/PAGE and transferred to nitrocellulose, and then probed with antibody SS1. In addition to bands corresponding to IP₃-3KB²⁶²⁻⁹³⁴, both lanes were found to possess a series of faint bands corresponding to proteins with molecular masses lower than that of IP₃-3KB²⁶²⁻⁹³⁴ (Figure 5A, lanes 1 and 2). These lower bands are likely to correspond to C-terminal fragments of IP₃-3KB, since antibody SS1 was raised against a sequence close to the C-terminus of IP₃-3KB. It was found that when lysis was performed in the presence of Ca²⁺ (as opposed to EGTA), three additional bands were observed. These correspond to proteins with molecular masses of approx. 80, 55 and 35 kDa (Figure 5A, lane 2). This suggests that the presence of Ca²⁺ specifically stimulates the cleavage of IP₃-3KB at three discrete sites within the protein. In order to establish whether an increase in [Ca²⁺]_i could stimulate IP₃-3KB cleavage in intact cells, ionomycin (to a final concentration of 1.3 μ M) was added to the growth medium ([Ca²⁺]_i of 1.8 mM) of C6 cells prior to lysis in a Ca²⁺-free buffer. Ionomycin causes cellular membranes to become permeable to Ca²⁺, and consequently ionomycin treatment results in equilibration of [Ca²⁺]_i with the extracellular medium [36]. Lysate was prepared from the ionomycin-treated cells in parallel with untreated cells and analysed by SDS/PAGE. The gel was then blotted and probed with antibody SS1. Lysate from ionomycin-treated cells was found to possess IP₃-3KB-cleavage products, with molecular masses of 80 and 55 kDa (Figure 5A, lanes 3 and 4). These were not present in lysate prepared from untreated cells (Figure 5A, lane 3). The band corresponding to a protein of 35 kDa identified in the previous experiment was not observed.

Figure 5 Calpain cleavage of IP₃-3KB^{262–934}

(A) Ca²⁺-dependent degradation of IP₃-3KB. Lysates prepared from C6 HeLa cells were separated on SDS/8 % polyacrylamide gels, which were then blotted to nitrocellulose. Blots were probed with antibody SS1 recognizing the C-terminus of IP₃-3KB. Lane 1, lysis performed in the presence of 5 mM EGTA; lane 2, lysis performed in the presence of 5 mM CaCl₂. Cells incubated in the absence (lane 3) or presence (lane 4) of 1.3 mM ionomycin for 20 min immediately prior to lysis. (B) Time course for cleavage of IP₃-3KB by purified calpain. (i) Lysates from C6 cells were incubated with calpain for the indicated time periods, then separated by SDS/PAGE (10 % gels), followed by blotting on to nitrocellulose. The blot was then probed with antibody SS1. (ii) In parallel with the blot, IP₃-3K activity assays were performed on equivalent samples. The y-axis indicates activity as a percentage of the activity prior to calpain treatment. (C) Identification of the location of calpain cleavage sites in IP₃-3KB. (i) Size comparison of IP₃-3KB calpain-cleavage products with recombinant IP₃-3KB truncations of known size. Samples were separated on an SDS/10 % polyacrylamide gel, which were then blotted on to nitrocellulose. The blot was probed with antibody SS1. Lane 1, IP₃-3KB^{262–934}; lane 2, IP₃-3KB^{262–934} partially digested with calpain; lane 3, Recombinant N-terminally truncated IP₃-3KB constructs. Arrows indicate the position of migration of each construct. (ii) Cartoon illustrating the relative sizes of the N-terminal truncations and cleavage fragments of IP₃-3KB shown on the blot in (i). (D) Effect of CaM on calpain cleavage of IP₃-3KB. (i) *E. coli* lysates expressing IP₃-3KB were incubated with calpain for indicated time periods. Cleavage reactions were separated on SDS/10 % polyacrylamide gels, and the gels were then blotted on to nitrocellulose. Blots were probed with antibody SS1. Cleavage was performed in the presence of CaM (lanes 1–4) or BSA (lanes 5–8). (ii) Cartoon showing location of PEST motifs, the CaM-binding domain and proposed locations of calpain-cleavage sites.

These data suggest that IP₃-3KB is a substrate for a protease that is activated when cytosolic [Ca²⁺] is elevated. Calpain is such a protease.

In order to test the hypothesis that calpain might cleave IP₃-3KB, lysate from C6 cells was incubated with purified m-calpain in the presence of CaCl₂. Cleavage reactions were terminated at different time points by chelation of free Ca²⁺ by addition of EGTA. Samples were subjected to SDS/PAGE, blotted on to nitrocellulose and the blot was probed with antibody SS1. It was found that calpain rapidly cleaved IP₃-3KB, resulting in the formation of discrete fragments of 55 and 35 kDa (Figure 5D, i). Because the antibody used on this blot was raised against a sequence 21 residues from the C-terminus of IP₃-3KB, the bands on the blot represent C-terminal cleavage fragments. After calpain addition (0.5 min), the majority of IP₃-3KB^{262–934} had been cleaved to give the 55 kDa C-terminal fragment. Subsequently, 2 min after calpain addition, the 35 kDa form was the most prevalent C-terminal fragment, and after 4 min essentially all the IP₃-3KB present had been processed to the 35 kDa form. These data suggest that calpain can cleave IP₃-3KB to give a 55 kDa

C-terminal fragment, which is subsequently cleaved again to give a 35 kDa C-terminal fragment. When an antibody recognizing the N-terminus of IP₃-3KB^{262–934} was used in place of SS1, the band corresponding to IP₃-3KB^{262–934} was rapidly lost, but not replaced by lower-molecular-mass forms (results not shown). This suggests that an initial cleavage event occurs very close to the N-terminus of this construct, such that the fragment containing the epitope is too small to be retained on an SDS/10 % polyacrylamide gel. The 80 kDa C-terminal fragment observed in Figure 5(A), lanes 2 and 4 could result from such a cleavage. During the time course of cleavage *in vitro*, all the IP₃-3KB had been cleaved to yield the 55 kDa C-terminal fragment by the first time point (30 s). It is conceivable that an 80 kDa C-terminal fragment existed transiently prior to the 30 s time point. These data demonstrate that IP₃-3KB is a substrate for calpain, and also suggest that the Ca²⁺-dependent cleavage events described above are likely to be mediated by calpain.

The samples used for the blot shown in Figure 5(D), part i, were also assayed for IP₃-3K activity. The overexpressed IP₃-3KB has previously been shown to account for the majority (~95 %) of

IP₃-3K activity in the cell extract [21]. It was found that the total IP₃-3K activity had fallen by approx. 15 % after 0.5 min of calpain treatment, but then changed little over the next 3.5 min (Figure 5B, ii). It is notable that, despite the fact that all the recombinant IP₃-3KB^{262–934} present had been cleaved into several fragments after 4 min of calpain treatment, 82 % of the original IP₃-3K activity remained. These data indicate that the C-terminal 35 kDa fragment of IP₃-3KB retains the majority of catalytic activity of the enzyme. Consistent with this observation is the finding that a truncated version of IP₃-3KB, consisting of amino acids 649–934, can be expressed in *E. coli* and possesses catalytic activity (results not shown). This truncation is similar in size to the 35 kDa cleavage fragment, and encompasses the same region of the protein.

Attempts were then made to identify the location of the calpain-cleavage sites within IP₃-3KB^{262–934}. The *in vitro* calpain-cleavage time course was repeated. A sample corresponding to a time point at which the 35 kDa and 55 kDa cleavage products were both present was blotted alongside a series of N-terminal truncations of IP₃-3KB (Figure 5C). The 35 kDa fragment was found to be slightly larger than the Δ 649–934 truncation, whereas the 50 kDa fragment was slightly smaller than the Δ 465–934 truncation. Thus the calpain-cleavage sites can be assigned to specific regions of IP₃-3KB (as indicated in Figure 5D, ii). These assignments are made on the assumption that the cleavage products have intact C-termini. We have made this assumption because the epitope recognized by the antibody used is only 21 residues from the C-terminus of IP₃-3KB, and it is unlikely that there is an additional cleavage site C-terminal to the epitope. The two cleavage sites giving rise to the 50 kDa and 35 kDa fragments are therefore likely to be approximately at residues 476 and 641 of IP₃-3KB^{1–934} (Figure 5D, ii). These cleavage sites are approximate, since there is no known consensus sequence for calpain cleavage.

As mentioned above, PEST-rich proteins such as IP₃-3KB often bind CaM. The CaM-binding domain of IP₃-3KB has been mapped to residues 634–661 [26]. The calpain-cleavage time-course experiments described above were repeated in the presence of CaM using IP₃-3KB expressed in *E. coli*. Calpain cleavage of IP₃-3KB^{262–934} was performed in the presence of 30 μ M CaM or 30 μ M BSA as a control. It was found that in the presence of CaM, IP₃-3KB^{262–934} was cleaved to yield the previously identified 55 kDa fragment, but this was inefficiently converted into the 35 kDa fragment (Figure 5D, part i, lanes 1–4). This suggests that CaM protects IP₃-3KB^{262–934} from cleavage specifically at the residue-641 cleavage site. This provides good evidence that this cleavage site is within, or close to, the CaM-binding site (amino acids 624–661; see Figure 5D, ii). This is similar to the situation observed following ionomycin treatment of C6 HeLa cells, where the 55 kDa fragment was observed, but the 35 kDa fragment was not (Figure 5A). As CaM would be expected to bind IP₃-3KB when [Ca²⁺]_i is elevated by ionomycin treatment, the lack of the 35 kDa fragment could result from CaM protection of the cleavage site. This interaction between calpain cleavage of IP₃-3KB and CaM binding could well be of physiological significance, with CaM acting to modulate the cleavage of IP₃-3KB by calpain.

Thus we have evidence that the N-terminus of IP₃-3KB is involved in the membrane association of the enzyme, and that the C-terminal catalytic region of the enzyme can be released from that membrane-binding domain by cleavage of the protein by calpain. We were therefore intrigued to know what would happen to the localization of the IP₃-3KB catalytic domain in cells in which [Ca²⁺]_i had been elevated (i.e. calpain-activated). This question was addressed by monitoring the localization of GFP-tagged IP₃-3KB^{1–934} in HeLa cells following addition of ionomycin to those cells. The GFP tag is at the C-terminus of IP₃-

3KB in this construct (see Figure 2), and so would provide an indicator of the location of the IP₃-3KB catalytic domain following calpain cleavage of IP₃-3KB^{1–934}. Membranes from HeLa cells transfected with IP₃-3KB^{1–934}, IP₃-3KB^{262–934}, IP₃-3KB^{1–635} or IP₃-3KB^{650–934} were isolated following a 20 min incubation with 1.3 μ M ionomycin, as in Figure 5(A). The localization of C-terminal fragments of these constructs were identified using the a-GFP antibody as before. However, we were unable to observe any significant translocation and cleavage of IP₃-3KB (results not shown).

Thus elevation of [Ca²⁺]_i can lead to cleavage of IP₃-3KB, which in turn can potentially lead to release of the catalytic domain from the ER membrane, i.e. removal from the immediate vicinity of the IP₃R. CaM and calpain have been reported to work in conjunction in the modulation of cyclic nucleotide phosphodiesterase activity [37]. We have previously shown that elevated expression of IP₃-3KB in HeLa cells leads to abrogation of IP₃-induced release of Ca²⁺ from intracellular stores [28]. At that time, we speculated that, among other things, ER-associated IP₃-3KB may have a role in regulating Ins(1,4,5)P₃ levels in the vicinity of the IP₃R, and thereby serve to modulate Ca²⁺ release from the ER. The data we now present suggest that if [Ca²⁺]_i reaches a level sufficiently high to activate calpain, then the catalytic domain of IP₃-3KB can be released from the ER membrane. This clearly has (at least) two significant consequences. It removes the catalytic domain of the enzyme from being in proximity to the IP₃R, and thereby releases the IP₃R from the regulation imposed by the local conversion of Ins(1,4,5)P₃ into Ins(1,3,4,5)P₄. Thus the 'brake' imposed on Ca²⁺ release from the ER by conversion of Ins(1,4,5)P₃ into Ins(1,3,4,5)P₄ at the cytosolic face of the ER membrane by IP₃-3KB would be released, and [Ca²⁺]_i would rise. The presence of CaM would also affect the cleavage of IP₃-3KB by calpain. We have mapped two potential calpain-cleavage sites that would give rise first to a 50 kDa, and, upon further cleavage, to a 35 kDa C-terminal fragment. However, in the presence of CaM *in vitro*, the 35 kDa fragment (most likely the isolated catalytic domain) does not accumulate, presumably because the binding of CaM inhibits binding of (and therefore cleavage by) calpain. This may represent a more complicated *in vivo* situation, in which CaM may serve to prevent accumulation of this smaller fragment until it has diffused away from the localized high [Ca²⁺]_i. Once this CaM–IP₃-3KB complex reaches lower [Ca²⁺]_i levels, CaM will no longer be able to bind IP₃-3KB efficiently, and the complex will dissociate. Further cleavage of the resulting 50 kDa fragment by calpain will depend on whether the [Ca²⁺]_i is still sufficiently high to allow calpain activity.

Much has been written about the spatiotemporal regulation of Ca²⁺ mobilization within cells. We suggest that the localization, cleavage and relocation of IP₃-3KB we describe here adds another layer of complexity to the mechanisms underlying the spatiotemporal regulation of Ca²⁺ mobilization.

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REFERENCES

- Berridge, M. J. (1993) Inositol trisphosphate and calcium signalling. *Nature* (London) **361**, 315–325.
- Irvine, R. F. (1990) 'Quanta' Ca²⁺ release and the control of Ca²⁺ entry by inositol phosphates – a possible mechanism. *FEBS Lett.* **263**, 5–9.
- Mezna, M. and Michelangeli, F. (1996) The effects of inositol 1,4,5-trisphosphate (InsP₃) analogues on the transient kinetics of Ca²⁺ release from cerebellar microsomes. InsP₃ agonists act as partial agonists. *J. Biol. Chem.* **271**, 31818–31823.

- 4 Michelangeli, F., Mezna, M., Tovey, S. and Sayers, L. G. (1995) Pharmacological modulators of the inositol 1,4,5-trisphosphate receptor. *Neuropharmacology* **34**, 1111–1122
- 5 Michell, R. H. (1975) Inositol phospholipids and cell surface receptor function. *Biochim. Biophys. Acta* **415**, 81–147
- 6 Erneux, C., Govaerts, C., Communi, D. and Pesesse, X. (1998) The diversity and possible functions of the inositol polyphosphate 5-phosphatases. *Biochim. Biophys. Acta* **1436**, 185–199
- 7 Irvine, R. F., Letcher, A. J., Heslop, J. P. and Berridge, M. J. (1993) The inositol tris/tetrakisphosphate pathway—demonstration of Ins(1,4,5) P_3 3-kinase activity in animal tissues. *Nature (London)* **320**, 631–634
- 8 Irvine, R. F., McNulty, T. J. and Schell, M. J. (1999) Inositol 1,3,4,5-tetrakisphosphate as a second messenger – a special role in neurones? *Chem. Phys. Lipids* **98**, 49–57
- 9 Cullen, P. J., Hsuan, J. J., Truong, O., Letcher, A. J., Jackson, T. R., Dawson, A. P. and Irvine, R. F. (1995) Identification of a specific Ins(1,3,4,5) P_4 -binding protein as a member of the GAP1 family. *Nature (London)* **376**, 527–530
- 10 Takazawa, K., Perret, J., Dumont, J. E. and Erneux, C. (1991) Molecular cloning and expression of a human brain inositol 1,4,5-trisphosphate 3-kinase. *Biochem. Biophys. Res. Commun.* **174**, 529–535
- 11 Takazawa, K., Perret, J., Dumont, J. E. and Erneux, C. (1991) Molecular cloning and expression of a new putative inositol 1,4,5-trisphosphate 3-kinase isoenzyme. *Biochem. J.* **278**, 883–886
- 12 Thomas, S., Brake, B., Luzio, J. P., Stanley, K. and Banting, G. (1994) Isolation and sequence of a full length cDNA encoding a novel rat inositol 1,4,5-trisphosphate 3-kinase. *Biochim. Biophys. Acta* **1220**, 219–222
- 13 Dewaste, V., Pouillon, V., Moreau, C., Shears, S., Takazawa, K. and Erneux, C. (2000) Cloning and expression of a cDNA encoding human inositol 1,4,5-trisphosphate 3-kinase C. *Biochem. J.* **352**, 343–351
- 14 Nalaskowski, M. M., Bertsch, U., Fanick, W., Stockebrand, M. C., Schmale, H. and Mayr, G. W. (2003) Rat inositol 1,4,5-trisphosphate 3-kinase C is enzymatically specialized for basal cellular inositol trisphosphate phosphorylation and shuttles actively between nucleus and cytoplasm. *J. Biol. Chem.* **278**, 19765–19776
- 15 Dewaste, V., Roymans, D., Moreau, C. and Erneux, C. (2002) Cloning and expression of a full-length cDNA encoding human inositol 1,4,5-trisphosphate 3-kinase B. *Biochem. Biophys. Res. Commun.* **291**, 400–405
- 16 Woodring, P. J. and Garrison, J. C. (1997) Expression, purification, and regulation of two isoforms of the inositol 1,4,5-trisphosphate 3-kinase. *J. Biol. Chem.* **272**, 30447–30454
- 17 Togashi, S., Takazawa, K., Endo, T., Erneux, C. and Onaya, T. (1997) Structural identification of the *myo*-inositol 1,4,5-trisphosphate-binding domain in rat brain inositol 1,4,5-trisphosphate 3-kinase. *Biochem. J.* **326**, 221–225
- 18 Communi, D., Takazawa, K. and Erneux, C. (1993) Lys-197 and Asp-414 are critical residues for binding of ATP/Mg²⁺ by rat brain inositol 1,4,5-trisphosphate 3-kinase. *Biochem. J.* **291**, 811–816
- 19 Soriano, S., Thomas, S., High, S., Griffiths, G., D'Santos, C., Cullen, P. and Banting, G. (1997) Membrane association, localization and topology of rat inositol 1,4,5-trisphosphate 3-kinase B: implications for membrane traffic and Ca²⁺ homeostasis. *Biochem. J.* **324**, 579–589
- 20 Schell, M. J., Erneux, C. and Irvine, R. F. (2001) Inositol 1,4,5-trisphosphate 3-kinase A associates with F-actin and dendritic spines via its N-terminus. *J. Biol. Chem.* **276**, 37537–37546
- 21 Communi, D., Dewaste, V. and Erneux, C. (1999) Calcium/calmodulin-dependent protein kinase II and protein kinase C-mediated phosphorylation and activation of *D-myo*-inositol 1,4,5-trisphosphate 3-kinase B in astrocytes. *J. Biol. Chem.* **274**, 14734–14742
- 22 Dewaste, V., Moreau, C., De Smedt, F., Bex, F., De Smedt, H., Wuytack, F., Missiaen, L. and Erneux, C. (2003) The three isoenzymes of human inositol 1,4,5-trisphosphate 3-kinase show specific intracellular localization but comparable Ca²⁺ responses upon transfection in COS-7 cells. *Biochem. J.* **374**, 41–49
- 23 Choi, K. Y., Kim, H. K., Lee, S. Y., Moon, K. H., Sim, S. S., K-im, J. W., Chung, H. K. and Rhee, S. G. (1990) Molecular cloning and expression of a complementary DNA for inositol 1,4,5-trisphosphate 3-kinase. *Science* **248**, 64–66
- 24 Lee, S. Y., Sim, S. S., Kim, J. W., Moon, K. H., Kim, J. H. and Rhee, S. G. (1990) Purification and properties of *D-myo*-inositol 1,4,5-trisphosphate 3-kinase from rat brain. Susceptibility to calpain. *J. Biol. Chem.* **265**, 9434–9440
- 25 Takazawa, K., Lemos, M., Delvaux, A., Lejeune, C., Dumont, J. E. and Erneux, C. (1990) Rat brain inositol 1,4,5-trisphosphate 3-kinase. Ca²⁺-sensitivity, purification and antibody production. *Biochem. J.* **268**, 213–217
- 26 Bertsch, U., Haefs, M., Moller, M., Deschermeier, C., Fanick, W., Kitzerow, A., Ozaki, S., Meyer, H. E. and Mayr, G. W. (1999) A novel A-isoform-like inositol 1,4,5-trisphosphate 3-kinase from chicken erythrocytes exhibits alternative splicing and conservation of intron positions between vertebrates and invertebrates. *Gene* **228**, 61–71
- 27 Rogers, S., Wells, R. and Rechsteiner, M. (1986) Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364–368
- 28 Millard, T. H., Cullen, P. J. and Banting, G. (2000) Effects of elevated expression of inositol 1,4,5-trisphosphate 3-kinase B on Ca²⁺ homeostasis in HeLa cells. *Biochem. J.* **352**, 709–715
- 29 Walker, J., Watson, J., Holmes, C., Edelman, A. and Banting, G. (1995) Production and characterization of monoclonal and polyclonal antibodies to different regions of the cystic fibrosis transmembrane conductance regulator (CFTR): detection of immunologically related proteins. *J. Cell Sci.* **108**, 2433–2444
- 30 Patel, S., McLauchlin, J. and Casemore, D. P. (1997) A simple SDS-PAGE immunoblotting technique using an enhanced chemiluminescence detection system to identify polyclonal antibody responses to complex cryptosporidial antigen preparations following a monoclonal antibody retest and image overlay technique. *J. Immunol. Methods* **205**, 157–161
- 31 Spencer, C. E. L., Stephens, L. R. and Irvine, R. F. (1990) in *Methods in Inositolide Research*, pp. 39–43, Raven Press Ltd
- 32 Cuff, J., Clamp, M., Siddiqui, A., Finlay, M. and Barton, G. (1998) JPred: a consensus secondary structure prediction server. *Bioinformatics* **14**, 892–893
- 33 Lupas, A., Van Dyke, M. and Stock, J. (1991) Predicting coiled coils from protein sequences. *Science* **252**, 1162–1164
- 34 Skehel, J. J. and Wiley, D. C. (1998) Coiled coils in both intracellular vesicle and viral membrane fusion. *Cell* **95**, 871–874
- 35 Vasu, S. K. and Forbes, D. J. (2001) Nuclear pores and nuclear assembly. *Curr. Opin. Cell Biol.* **13**, 363–375
- 36 Toeplitz, B. K., Cohen, A. I., Funke, P. T., Parker, W. L. and Gougoutas, J. Z. (1979) Structure of ionomycin – a novel diacidic polyether antibiotic having high affinity for calcium ions. *J. Am. Chem. Soc.* **101**, 3344–3353
- 37 Kakkar, R., Raju, R. V. and Sharma, R. K. (1999) Calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1). *Cell. Mol. Life Sci.* **55**, 1164–1186

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