Membrane association, localization and topology of rat inositol 1,4,5-trisphosphate 3-kinase B: implications for membrane traffic and Ca^{2+} homoeostasis

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We previously reported the isolation of a rat cDNA clone encoding a protein with significant sequence homology to the B isoform of human *myo*-inositol 1,4,5-trisphosphate 3-kinase (IP_3 3-kinase B); this protein was thus designated rat IP_3 3-kinase B [Thomas, Brake, Luzio, Stanley and Banting (1994) *Biochim. Biophys. Acta* 1220, 219–222]. However, no IP_3 kinase isoform had been shown to generate the physiologically important isoform of inositol tetrakisphosphate, i.e. inositol 1,3,4,5-tetrakisphosphate. We now present direct evidence that the putative rat IP_3 3-kinase B is genuinely an IP_3 3-kinase. We also show that the enzyme exists both as a peripheral membrane

protein tightly associated with the cytosolic face of the extended endoplasmic reticulum network, and as a cytosolic protein. Association of the IP_3 3-kinase with membranes is not affected by treatment with brefeldin A, Na_2CO_3 (pH 11.5), 2 M NaCl, or alteration of $[\text{Ca}^{2+}]$. However, treatment of isolated membranes with 4 M urea leads to dissociation of the kinase from the membrane, implying that membrane association involves specific, conformation-dependent protein–protein interactions. The fact that IP_3 3-kinase B is localized exclusively to membranes of Ca^{2+} stores, is consistent with a model where this kinase plays a role in IP_3 -dependent Ca^{2+} release.

INTRODUCTION

The pivotal role that *myo*-inositol trisphosphate (IP_3) plays as an intracellular signalling molecule has been self-evident since the initial demonstration that it mediates the release of Ca^{2+} from intracellular Ca^{2+} stores [1]. IP_3 is generated at the cytosolic face of the plasma membrane as a result of the phospholipase C-catalysed hydrolysis of membrane-associated phosphatidylinositol bisphosphate to soluble IP_3 and membrane-associated diacylglycerol. Phospholipase C itself is activated following the binding of ligand to specific, phospholipase C-associated integral membrane receptors in the plasma membrane [2]. Thus binding of specific ligands to cognate receptors in the plasma membrane leads to the release of IP_3 into the cytosol. Various classes of IP_3 receptor have been identified and localized to different membranes within different cell types (see [3] for a recent review). The 'classic' IP_3 receptor is a tetrameric integral membrane protein localized to the endoplasmic reticulum (ER) membrane. This protein acts as an IP_3 -activatable Ca^{2+} channel, releasing Ca^{2+} from the ER into the cytosol upon binding of IP_3 (see [2,4] for reviews).

The metabolism of IP_3 is an area that has been less well studied than its role in intracellular signalling. However, it is clear that IP_3 acts as substrate for the generation of inositol tetrakisphosphate (IP_4) by IP_3 3-kinase [5]. The role played by IP_4 is the subject of much discussion [6–10], but it is of note that the recently cloned IP_4 binding protein ($\text{GAP}^{\text{IP}_4\text{BP}}$; GAP = GTPase activating protein) has both Ras and Rap-GAP activity [11]. The mechanism by which IP_3 is converted into IP_4 , the

intracellular location of that conversion, and the means by which it is regulated, are thus all important areas of study. The previously identified and cloned rat and human A isoforms of IP_3 3-kinase were shown to be cytosolic and to use IP_3 as substrate for conversion into IP_4 [12–17]. However, the IP_4 produced was not shown to be the physiologically important I(1,3,4,5) P_4 isomer. The same is true for the published data concerning the B isoform of human IP_3 3-kinase [18]. The apparent cytosolic location of the B isoform of human IP_3 3-kinase [19] was of interest since we had isolated cDNA clones encoding the B isoform of rat IP_3 3-kinase using a strategy designed to isolate integral membrane or tightly associated peripheral membrane proteins. Experiments were therefore performed (i) to determine the precise enzymic activity of the protein encoded by the cDNA clone that we had previously isolated and provisionally identified as an IP_3 3-kinase (based on comparison with published sequences) [20], and (ii) to address the intracellular location of this protein.

We now show that (i) rat IP_3 3-kinase B genuinely is an IP_3 3-kinase [i.e. it converts I(1,4,5) P_3 into I(1,3,4,5) P_4], (ii) the enzyme exists in two intracellular pools, one tightly bound to the extended network of the ER (nuclear membrane, ER, ER-to-Golgi intermediate compartment; ERGIC), the other cytosolic, (iii) the catalytic domain of the enzyme faces the cytosol, and (iv) membrane association is dependent upon protein–protein interactions. These observations have important implications for intracellular signal transduction, membrane traffic within eukaryotic cells and Ca^{2+} homoeostasis, supporting a role for IP_3 3-kinase in IP_3 -dependent Ca^{2+} release and providing a possible explanation for quantal release of Ca^{2+} in many cell types.

Abbreviations used: ER, endoplasmic reticulum; ERGIC, ER-to-Golgi intermediate compartment; GAP, GTPase activating protein; HA, haemagglutinin; IP_3 , *myo*-inositol trisphosphate; IP_4 , inositol tetrakisphosphate; MBP, maltose binding protein; MHV, mouse hepatitis virus; NRK, normal rat kidney; SLO, streptolysin O; TGN, trans-Golgi network.

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EXPERIMENTAL

Materials

Brefeldin A was purchased from Epicentre Technologies (Madison, WI, U.S.A.), stored as a 5 mg/ml stock solution in methanol at -20°C and used at a final concentration of 5 $\mu\text{g}/\text{ml}$ in culture medium. Streptolysin O (SLO) was kindly given by Dr. Sucharit Bhakdi (Institute of Medical Microbiology, Mainz, Germany). It was stored at 0.1 mg/ml in PBS at -20°C and used at a final concentration of 250 ng/ml in incubation buffer [BSA (1 mg/ml)/1 mM dithiothreitol in PBS].

Expression of IP₃ 3-kinase/maltose binding protein (MBP) fusion protein

An 1844 bp fragment was excised from GORF 34.1 [20] using the restriction enzyme *Xba*I and subcloned into the *Xba*I site of pMAL 1c (New England Biolabs), resulting in the plasmid construct pMAL1.8x. Expression from this construct is predicted to give a MBP/IP₃ 3-kinase B fusion protein that lacks the 141 N-terminal amino acids of rat IP₃ 3-kinase B. Luria broth cultures (10 ml) of the *Escherichia coli* strain XL1-B harbouring the plasmid pMAL1.8x were grown with 100 $\mu\text{g}/\text{ml}$ carbenicillin at 37°C . At an A_{600} of 0.5–0.6, isopropyl β -D-thiogalactoside was added to a final concentration of 0.2 μM and induction continued for 3 h. A soluble fraction was prepared as described elsewhere [21] and assayed for IP₃ 3-kinase activity. Recombinant, full-length non-fusion IP₃ 3-kinase B was expressed in *E. coli* and purified as described elsewhere [21].

Assay of IP₃ 3-kinase was performed as previously described [21].

Preparation and subcellular fractionation of rat liver membranes

This was performed as described by Howell et al. [22] with the modifications of Cartwright and Higgins [23], for the isolation of rat liver Golgi. In total, seven fractions were isolated and characterized: total liver homogenate (H); nuclear fraction (N); total membrane (TM) (all cellular membranes except those isolated in the previous fraction); Fraction 1, material at the bottom of the sucrose density gradient (F1); Fraction 2, material at the interface between the 1.5 M and 1.15 M sucrose solutions (F2); Fraction 3, material at the interface of the 1.15 M and 0.86 M sucrose solutions (F3); Fraction 4, material between the 0.86 M and 0.25 M sucrose solutions (F4). Once isolated, the fractions were placed on ice and used immediately for enzyme analysis or frozen and stored at -70°C for subsequent use in immunoblot analysis. Protein concentrations were determined according to the method of Bradford [24].

Analysis of subcellular fractions

This was performed using the following enzyme assays: neutral α -glucosidase assay [25] as a marker of the ER; galactosyl-transferase [26] as a marker of the Golgi stack; alkaline phosphatase [27] as a marker of the plasma membrane; β -hexosaminidase [28] as a lysosomal marker.

Antibodies

Polyclonal antisera against rat IP₃ 3-kinase were raised using the peptide sequence MERGSPASPRC, which corresponds to the N-terminal 12 amino acids of the protein. The peptide was coupled to BSA or thyroglobulin via its C-terminal cysteine residue using an MBS (*m*-maleimidobenzoyl-*N*-hydroxy-succinimide ester) bifunctional cross-linker (Sigma) according to previously published procedures [29]. The coupled peptide was

used to immunize rabbits according to published procedures [30]. Purification of the resulting polyclonal antiserum was as follows: immunoglobulins from 2 ml of serum were precipitated using $(\text{NH}_4)_2\text{SO}_4$ (BDH; enzyme grade) at 0.29 g/ml, dialysed extensively against PBS and incubated overnight at 4°C with 1 ml of Sepharose 4B beads (Sigma) coupled to the peptide antigen. After extensive washing in PBS, antibodies were eluted with 100 mM glycine, pH 2.3, and the pH was neutralized immediately with 2 M Tris, pH 8.0. Aliquots with $A_{280} > 0.1$ were pooled and concentrated to 1 mg/ml with a micro-concentrator (Amicon). This antiserum was designated '52'. The specificity of this antiserum in immunofluorescence and immunoblot assays was demonstrated by competition with the immunizing peptide. Monoclonal antibody 12CA6, against the haemagglutinin (HA) sequence YPYDVPDYA, was generously provided by Dr. T. Nilsson (Imperial Cancer Research Fund, London, U.K.) as ascites fluid. Monoclonal antibody 2F7.1 has been described previously [31]. Fluorescein-labelled rabbit anti-mouse IgG was from Molecular Probes and fluorescein-labelled sheep anti-mouse IgG was from Amersham. Rhodamine-labelled swine anti-rabbit IgG was from Dako (High Wycombe, Bucks., U.K.) as were alkaline phosphatase- and horseradish-conjugated mouse anti-rabbit and sheep anti-mouse IgGs; Texas red- and fluorescein-labelled donkey anti-rabbit IgGs were from Amersham.

Cell culture and immunofluorescence microscopy

Normal rat kidney (NRK) cells were grown as previously described [32]. Monkey COS-7 cells were grown in RPMI 1640 medium (GIBCO, Life Technologies, Paisley, Scotland), 2 mM glutamine (GIBCO), 100 $\mu\text{g}/\text{ml}$ streptomycin (Evans Medical, Langhurst, Horsham, U.K.) and 60 $\mu\text{g}/\text{ml}$ penicillin (Glaxo Laboratories, Greenford, Middx., U.K.) at 37°C in 5% (v/v) CO_2 . For immunofluorescence, cells were fixed by incubating with PBS/3% (v/v) paraformaldehyde for 15 min, washed three times with PBS and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 4 min. Cells were then washed in PBS/0.2% (w/v) BSA and incubated for 1 h with antibody 52 (diluted 1:50 in PBS/0.2% BSA). After washing with PBS/0.2% BSA, cells were incubated for 1 h with Rhodamine-coupled or fluorescein-coupled anti-rabbit IgG (diluted 1:1000 in PBS/0.2% BSA), washed and mounted in Mowiol (Calbiochem, San Diego, CA, U.S.A.). Epifluorescence microscopy was performed on a Universal microscope (Zeiss, Oberkochen, Germany) with a $63\times$, 1.4 oil immersion objective.

Electron microscopy

Cryosections of paraformaldehyde-fixed cells were prepared and labelled as described previously [33–35].

Cell permeabilization

Cells were permeabilized with purified SLO as previously described [36]. Immunofluorescence analysis was then performed as described above.

Subcloning of rat IP₃ 3-kinase B cDNA for expression in COS-7 cells

The full-length coding sequence of rat IP₃ 3-kinase B was amplified by PCR using oligonucleotide primers that introduced a *Sal*I site immediately before the initiation codon and converted the sequence flanking the 'stop' codon (TGA) into TGGCCC-GCGG. This procedure introduced a *Sac*II site at the 3' end of the PCR product and converted the C-terminus of the encoded protein from GSPLT* into GSPLTWPA. The *Sac*II site in the

PCR product is in frame with the triple HA tag of the eukaryotic expression vector pCMUIV-HA [37] generously provided by Dr. T. Nilsson (ICRF). The PCR product was cloned into pCRII (Invitrogen), sequenced and subcloned into pCMUIV-HA using the *SalI* and *SacII* restriction sites. This procedure generated a cDNA encoding a modified form of rat IP₃ 3-kinase B with the following C-terminal sequence: GSPLTWPAGPGFSLKFGGR IFYPYDVPDYAGYPYDVPDYAGSYDVPDYAAQCGR*. The added sequence is underlined, the triple HA epitope is indicated in bold, and represents the stop codon. Large-scale preparations of the pCMUIV-rat IP₃ 3-kinase B plasmid construct for transfections were made using Qiagen columns (Diagen). Transfections were carried out using the Transfectam method (Promega). Each transfection was carried out in a 75 cm² tissue culture flask (Falcon) with cells at 50% confluency and according to the manufacturer's instructions.

In vitro transcription/translation and product analysis

Plasmids encoding rat IP₃ 3-kinase B and the invariant chain of the MHC class II complex were used to prime a TNT reticulocyte lysate-coupled transcription/translation system as described by the manufacturer (Promega). Translation products were labelled with [³⁵S]methionine and canine pancreatic microsomes were added to a final concentration of 2.4 A₂₈₀ units/ml. After incubation at 30 °C for 90 min, membranes were isolated by centrifugation through a high salt/sucrose cushion [50 mM Hepes/KOH (pH 7.6)/5 mM Mg(OAc)₂/1 M KOAc/0.25 M sucrose] for 10 min at 100000 g. The membrane pellet was resuspended in the buffer just described and split into four equal fractions. One fraction was incubated in the presence of 0.1 M Na₂CO₃, pH 11.5, for 15 min on ice and the membranes recovered by centrifugation for 10 min at 100000 g. The remaining samples were incubated with 0.23 mg/ml proteinase K or 0.23 mg/ml proteinase and 1% Triton X-100 or buffer alone for 30 min on ice and precipitated by the addition of trichloroacetic acid to 10% final w/v. Following extraction and proteinase treatment, samples were solubilized in sample buffer and analysed by SDS/PAGE [12% (w/v) gels].

Membrane association of rat IP₃ 3-kinase

For studies on the endogenous rat IP₃ 3-kinase, NRK cells were trypsinized, washed in homogenization buffer [250 mM sucrose/10 mM Tris (pH 7.4)/0.1 mM MgCl₂/0.1 mM CaCl₂] and resuspended in homogenization buffer (10⁷ cells/ml) containing a protease inhibitor cocktail (Boehringer Mannheim). Cells were then homogenized by passing them (10–15 times) through a 23-gauge needle attached to a 1 ml syringe. By this procedure, 60–70% of cells were lysed, as judged by light microscopy. The cell homogenate was then centrifuged at 2500 rev./min (SS-34 rotor) for 15 min and the post-nuclear supernatant further centrifuged at 100000 g for 1 h. The supernatant (cytosol fraction) was collected and the pellet (membrane fraction) was washed (4 times) with 0.5 ml of deionized water. Cytosol (50 µg of protein) and membrane fractions (corresponding to 5 × 10⁶ cells) were run on an SDS/PAGE (8% gel) and immunoblotted as described by Brake et al. [38]. Blots were probed with antibody 52 or 12CA6, and bound antibody was detected with alkaline phosphatase-conjugated secondary antibody and a chromogenic substrate, or by enhanced chemiluminescence (Amersham). Identical procedures were followed for studies on recombinant rat IP₃ 3-kinase B expressed in transiently transfected COS-7 cells. Treatments [4 M urea/0.2 M Na₂CO₃ (pH 11.5)/5 mM

Figure 1 Recombinant rat IP₃ 3-kinase can convert IP₃ into IP₄

HPLC profile of ^3H -labelled compounds ($\square$) from bacterial extracts containing the recombinant C-terminal fragment of IP₃ 3-kinase B expressed as an MBP fusion protein (A) and an MBP control (B). The HPLC profile of a ^{32}P -labelled I(1,3,4,5)P₄ marker ($\diamond$) is also shown. Enzyme assays were carried out as described in the Experimental section. Neutralized assays were applied to a Partizal 10 SAX HPLC column and eluted as described in the Experimental section. Fractions were collected and counted in a final solution containing 2 ml of methanol and 4.5 ml of scintillation fluid. The peak that eluted between 11 and 14 min contained [^3H]I(1,4,5)P₃. The subsequent peaks contained [^3H]I(1,3,4,5)P₄ ($\square$) and [^{32}P]I(1,3,4,5)P₄ ($\diamond$). Panel (C) presents data from a repeat of the experiment shown in (A), with the relevant region of the HPLC profile enlarged.

EDTA/5 mM EGTA] were carried out on the post-nuclear supernatant fractions. Samples were left on ice for 30 min before centrifugation at 100 000 *g*.

RESULTS

Demonstration of the IP₃ 3-kinase activity of rat IP₃ 3-kinase B

Previous studies demonstrated that sonicates of *E. coli* cultures expressing β -galactosidase fusion proteins bearing the catalytic domains of the rat A isoform, the human A isoform, or the human B isoform of presumptive IP₃ 3-kinase possess enzyme activity capable of converting IP₃ into IP₄ [15,18,39]. However, no data have been published to show that the I(1,3,4,5)P₄ generated is the physiologically important isoform IP₄. Sonicates from *E. coli* cultures expressing either MBP alone, or an MBP fusion protein bearing the predicted catalytic domain of the putative rat IP₃ 3-kinase B were used in an assay of IP₃ kinase activity using [³H]IP₃ as substrate (see the Experimental section). The products of the reaction were analysed by HPLC, under conditions that discriminate between different IP₄ isomers (see the Experimental section), along with a ³²P-labelled I(1,3,4,5)P₄ marker.

Sonicates from the *E. coli* culture expressing MBP alone failed to convert any of the [³H]IP₃, whereas sonicate from the *E. coli* culture expressing the MBP-IP₃ 3-kinase B fusion protein was capable of converting [³H]IP₃ into [³H]I(1,3,4,5)P₄ (as shown by the co-migration of the tritiated reaction product with ³²P-labelled I(1,3,4,5)P₄ marker in the HPLC trace; Figure 1). These data demonstrate that the recombinant enzyme activity is that of an IP₃ 3-kinase converting I(1,4,5)P₃ into I(1,3,4,5)P₄. The specificity of this activity was confirmed by the demonstration that the product generated by the recombinant enzyme, when using I(1,4,5)P₃ as a substrate, binds to the IP₄ binding protein RasGAP^{IP4BP} [11] with the same affinity as I(1,3,4,5)P₄. RasGAP^{IP4BP} has an exquisite specificity for the I(1,3,4,5)P₄ isomer [11]. The specificity of RasGAP^{IP4BP} for I(1,3,4,5)P₄ unequivocally establishes the isomeric configuration of the kinase product.

The recombinant enzyme phosphorylated I(1,4,5)P₃ with an apparent *K_m* of 0.7 μ M and a *V_{max}* of 8.5 μ mol/min per mg.

Immunolocalization of rat IP₃ 3-kinase B

A polyclonal antiserum was raised in rabbits against a peptide corresponding to the isoform-specific extreme N-terminus of rat IP₃ 3-kinase B and purified as described in the Experimental section. In immunoblot analysis, this antibody (52) recognized bacterially expressed, full-length non-fusion recombinant rat IP₃ 3-kinase B (Figure 2, lane 1), and a faint single band of the same apparent electrophoretic mobility in whole cell lysate prepared from NRK cells (Figure 2, lane 2). Antibody binding was completely inhibited by the immunizing peptide. Immunoblot analysis of whole cell lysate prepared from several cell lines of rat, mouse, monkey and human origin showed that the protein was not present in detectable amounts in any of them (results not shown). The original cDNA clone encoding rat IP₃ 3-kinase B had been identified using a strategy designed to identify Golgi integral membrane proteins present in rat liver. Therefore rat liver membranes were isolated and fractionated on a sucrose gradient before processing for immunoblot analysis using antibody 52 as probe (Figure 2, lanes 3–6). Rat IP₃ 3-kinase B is detectable only in the ER-enriched fraction (Figure 2, lane 3).

When used in immunofluorescence analysis of paraformal-

Figure 2 Antibody 52 recognizes rat IP₃ 3-kinase B

Recombinant rat IP₃ 3-kinase B expressed as a recombinant full-length non-fusion protein in *E. coli* (100 ng; lane 1), NRK cell lysate (100 μ g; lane 2), or different rat liver membrane fractions (100 μ g; lanes 3–6) were electrophoresed on 8% polyacrylamide gel, transferred to nitrocellulose and immunoblotted with antibody 52 (detection of bound antibody is described in the Experimental section). Lanes are numbered 1–6 from left to right. The positions of migration of molecular mass standards are indicated in kDa. Primary antibody binding was completely inhibited by the immunizing peptide.

Figure 3 Subcellular localization of rat IP₃ 3-kinase B by immunofluorescence

NRK cells were processed for immunofluorescence analysis as described in the Experimental section before incubation with antibody 52, the binding of which was detected with a Rhodamine-labelled swine anti-rabbit IgG. Primary antibody binding was completely inhibited by the immunizing peptide. Both juxtanuclear staining and faint reticular staining throughout the cell can be observed.

dehydrate-fixed Triton X-100-permeabilized NRK cells, antibody 52 showed a predominantly juxtanuclear pattern of reactivity with some diffuse staining throughout the rest of the cell (Figure 3). This signal was completely inhibited by competition with the immunizing peptide (results not shown). The juxtanuclear pattern of staining is reminiscent of that observed with antibodies to integral membrane proteins of the intracellular membrane system, and implied that rat IP₃ 3-kinase B might be a membrane-associated protein, if not an integral membrane protein, of the intracellular membrane system, as predicted from

(a)

Figure 4 For legend see part (b) of this Figure on page 584

(b)

Figure 4 Subcellular localization of rat IP₃ 3-kinase B by electron microscopy

(a) Thawed cryosections of L-cells infected with MHV were permeabilized with SLO before fixation. The sections were labelled with antibody 52 [anti-(IP₃ 3-kinase B)] and Protein A gold. In both panels A and B, the gold is specifically associated with the membranes of the ER (arrowheads), as well as the nuclear envelope (N = nucleus). In panel A labelling is also evident on the periphery of the Golgi stack (G), although the main part of the Golgi stack is unlabelled. Another view of the Golgi stack is shown in panel C in which the Golgi region is unlabelled. The large arrowheads in all panels indicate the MHV particles. Bars = 200 nm. (b) Preparation as in (a). In panel A the arrowheads point to many of the gold particles that are associated with ER membranes. The Golgi region, including the membranes around the budding virions (V), are unlabelled in this example, as is the mitochondrion (M) and putative endocytic structures (E). In panel B an area is shown where small, but significant, amounts of gold labelling are associated with the intermediate compartment, defined by the presence of budding and budded virions (V). The small Golgi stack (G), centriole (C) and endosome structure (E) are all labelled. Bars = 100 nm.

Figure 5 Hydropathy plot of rat IP₃ 3-kinase B

This was performed using the 'TopPredII' programme developed by M.-G. Claros and based on the algorithm of Claros and von Heijne [42] with GES hydropobicity parameters, a critical length of 60 amino acids, a full window of 21 amino acids and a core window of 11 amino acids.

the strategy used to isolate the cDNA clone. In order to accurately localize IP₃ 3-kinase B in the cell, Immunogold labelling was performed on thawed cryosections of SLO-permeabilized, mouse hepatitis virus (MHV)-infected L-cells using antibody 52 as primary antibody. It has previously been shown that SLO treatment before cryofixation leads to an improvement of the definition of membranes within the cell following Immunogold labelling, and that budding profiles of MHV are an excellent marker for the ERGIC [35]. Gold particles can be seen to decorate an extensive series of membranes, notably the nuclear membrane, ER and ERGIC (the latter being defined by the presence of MHV profiles; Figure 4a). There is a distinct lack of gold particles associated with Golgi membranes (Figures 4a and 4b). Thus the electron microscopy data are in accord with the immunoblot data that show that rat IP₃ 3-kinase B is detectable only in fractions enriched in nuclear and ER membranes. In addition, dual-label immunofluorescence analysis demonstrated co-localization of rat IP₃ 3-kinase B with rab2, a peripheral membrane protein previously shown to associate specifically with ERGIC membranes [40] and with type I IP₃ receptor (results not shown).

Topology of rat IP₃ 3-kinase B

The immunoblot, immunofluorescence and immuno-electron microscopy data implied that rat IP₃ 3-kinase B is a membrane protein. Since hepatic IP₄ 3-phosphatase has been shown to reside within the lumen of the ER [41] it was necessary to ask the following questions. If rat IP₃ 3-kinase B is a membrane protein, is it a peripheral or integral membrane protein, and on which face of the nuclear/ER/ERGIC membrane is the catalytic domain exposed? A hydropathy plot of the predicted amino acid sequence of the protein reveals a stretch of hydrophobic residues near its N-terminus that might act as a transmembrane domain (Figure 5). However, this plot provides no indication of the topology of such an integral membrane protein in the membrane.

In order to address the question of topology we initially chose to perform an *in vitro* transcription/translation assay, using a full-length IP₃ 3-kinase transcript, in the presence of canine pancreas microsomes. The translation product was tightly associated with the membranes (not extracted by a pH 11.5 Na₂CO₃ wash; Figure 6, lane 4). Protease treatment of the membranes at the end of the *in vitro* transcription/translation

Figure 6 Membrane association of rat IP₃ 3-kinase B

cDNAs encoding full-length rat IP₃ 3-kinase B or the invariant chain of the MHC complex were transcribed and translated *in vitro* in the presence of canine pancreatic microsomes and [³⁵S]methionine. Membrane-associated translation products were recovered and processed by extraction with alkaline Na₂CO₃ solution (lanes 4 and 8) or treatment with proteinase K (lanes 1–3 and 5–7). Samples were analysed by SDS/PAGE on a 12% polyacrylamide gel, detected by autoradiography. Lanes are numbered 1–9 from left to right. Molecular mass (mol wt.) markers are myosin (220 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa). TX-100 = Triton X-100.

reaction led to the complete digestion of the membrane-associated translation product (Figure 6, lane 2). In contrast, the large luminal domain of the invariant chain was completely protected against protease digestion under these conditions (Figure 6, lane 6) and only became accessible to added protease when detergent was present (Figure 6, lane 7). These results suggested that the vast majority, if not all, of the *in vitro*-translated IP₃ 3-kinase B was on the cytosolic face of the membranes.

Figure 7 Immunofluorescence analysis of SLO-permeabilized NRK cells

NRK cells were permeabilized with SLO before fixation with paraformaldehyde alone, (b) and (d), or with paraformaldehyde followed by Triton X-100 treatment (4 min; 0.1% in PBS), (a) and (c). Immunofluorescence analysis was carried out using antibody 52 [anti-(IP₃ 3-kinase B)] (a) and (b) or 2F7.1 [anti-(rat TGN38 mature N-terminus)] (c) and (d) before incubation with appropriate tetramethyl Rhodamine isothiocyanate- and fluorescein isothiocyanate-conjugated secondary antibodies. Bar = 10 μ m.

One interpretation of these results is that the extreme N-terminus of the protein is translocated, with the short hydrophobic stretch identified by the hydropathy plot acting as a transmembrane domain, leaving the rest of the protein (including the catalytic domain of the C-terminus) in the cytosol. The protease-resistant part of a protein with such a topology would be predicted to have a molecular mass of approx. 4 kDa, which is too small to be retained on the gel represented in Figure 6. An alternative interpretation of the data would be that rat IP₃ 3-kinase B is not an integral membrane protein, but rather a protein tightly associated with the cytosolic face of the membrane. A similar association has been demonstrated for the α subunit of the SRP receptor [43]. We chose to perform the following experiment in order to discriminate between these two possibilities. The plasma membrane of NRK cells was permeabilized with the pore-forming toxin SLO before fixation of the cells in paraformaldehyde (leaving intracellular membranes intact). These cells were then incubated with antibody 52 (which recognizes the extreme N-terminus of rat IP₃ 3-kinase B). Bound antibody was detected with a Rhodamine-conjugated species-specific second antibody. A distinct pattern of staining can be observed (Figure 7b). The same cells were also incubated with a monoclonal antibody that recognizes an epitope at the extreme N-terminus of trans-Golgi network (TGN)38 [31] (Figure 7d). Binding of this antibody was detected with a species-specific second antibody conjugated to fluorescein. TGN38 is a type I integral membrane protein predominantly localized to the TGN of NRK cells [44]. Its N-terminus is therefore within the lumen of the TGN and should not be accessible to the monoclonal antibody added in this experiment: indeed, it is not (Figure 7d),

thus demonstrating that intracellular membranes remained intact following SLO permeabilization of the plasma membrane. This experiment was repeated with the modification that Triton X-100 was added following paraformaldehyde fixation in order to permeabilize intracellular membranes. Both antibodies were able to bind to their respective antigens under these conditions (Figures 7a and 7c). These data indicate that the N-terminus of rat IP₃ 3-kinase B is exposed on the cytosolic face of the ER membrane. In combination with the data from *in vitro* transcription/translation experiments (Figure 6), they indicate that both N- and C-termini of the protein are exposed on the cytosolic face of the ER membrane. Thus the most likely topology for this protein is that of a tightly membrane-associated molecule exposed on the cytosolic face of the nuclear/ER/ERGIC membrane system.

Cytosolic versus membrane-bound rat IP₃ 3-kinase B

We argued that if rat IP₃ 3-kinase B exists as a tightly membrane-associated molecule exposed on the cytosolic face of the ER, it might also exist as a soluble, cytosolic molecule. Several proteins involved in the mechanics of vesicle-mediated membrane traffic are precedents for such a dual localization, e.g. adaptins (e.g. [45–47]), rab proteins (e.g. [48]), ADP-ribosylation factor proteins [45,46,49] and other coatamer components [50–52]. In addition, initial assays of IP₃ 3-kinase enzyme activity had indicated that the activity is cytosolic [53], and rat IP₃ 3-kinase A enzyme activity in transfected COS cells had been detected in cytosolic fractions [39]. Due to the difficulties in detecting endo-

Figure 8 Immunofluorescence analysis of transiently transfected COS-7 and HeLa cells

COS-7 (**a**) and HeLa (**b**) cells were transiently transfected with expression constructs encoding HA epitope-tagged rat IP₃ 3-kinase B (pCMUIV-rat IP₃ 3-kinase B) and processed for immunofluorescence analysis using monoclonal antibody 12CA6 [anti-(HA epitope)] and an appropriate fluorescein isothiocyanate-conjugated secondary antibody. Bar = 10 μ m. Juxta-nuclear staining is clearly evident in both COS-7 and HeLa transfected cells.

genous IP₃ 3-kinase B in cell lines in culture, we chose to address the question of cytosolic versus membrane-bound pools of IP₃ 3-kinase B in cells transfected with plasmid constructs encoding recombinant enzyme. COS-7 and HeLa cells were transfected

with the full-length rat IP₃ 3-kinase B cDNA cloned into the plasmid-based eukaryotic expression vector pCMUIV (see the Experimental section). This construct was designed to express full-length rat IP₃ 3-kinase B, tagged at its C-terminus with three copies of the HA epitope, which is recognized by monoclonal antibody 12CA6. Figure 8 shows the pattern of staining of COS-7 cells (panel a) and HeLa cells (panel b) transiently transfected with pCMUIV-IP₃ 3-kinase B expression construct and processed for immunofluorescence analysis using monoclonal antibody 12CA6 [anti-(HA tag)]. The recombinant protein can be seen to be present in the ER membrane system, including the nuclear membrane, showing no gross mis-localization at this level of resolution, compared with the endogenous protein. Similar results were obtained using antibody 52 [anti-(rat IP₃ 3-kinase)]; results not shown]. Therefore immunoblots were performed on homogenates from transiently transfected COS-7 cells, using antibodies 12CA6 [anti-(HA epitope tag)] or 52 [anti-(rat IP₃ 3-kinase)]. Different treatments were applied to separate aliquots of cell homogenate in order to determine the requirements for membrane association of rat IP₃ 3-kinase B (see the Experimental section). The enzyme is resistant to extraction with pH 11.5 carbonate buffer, but can be released by treatment with 4 M urea (Figure 9), implying that conformation-specific protein-protein interactions are required for membrane association of the protein. TGN38 (an integral membrane protein resident in the TGN) was detected in the membrane fraction (results not shown) showing that *bona fide* membrane proteins remain in the membrane after urea extraction. Membrane association of the kinase was not affected by treatment of cell homogenates with 1 M or 2 M NaCl, and was Ca²⁺- and Mg²⁺-independent as determined by chelation of these ions from homogenates or their addition to homogenates before immunoblot analysis (Figure 9; results not shown). It should be noted that the difference in apparent electrophoretic mobility between the endogenous rat IP₃ 3-kinase B (Figure 2) and the recombinant protein encoded by the pCMUIV-rat IP₃ 3-kinase B expression construct is due to the presence of the HA epitope tag on the recombinant protein.

These data demonstrate the existence of a soluble, cytosolic pool of rat IP₃ 3-kinase B, in addition to the membrane-bound pool. There is no detectable difference in apparent molecular

Figure 9 Rat IP₃ 3-kinase B exists as a cytosolic and membrane-bound protein

Total membrane and cytosol samples isolated from COS-7 cells that had been transiently transfected with the pCMUIV-rat IP₃ 3-kinase B expression construct were electrophoresed on 8% polyacrylamide gels, transferred to nitrocellulose and immunoblotted with antibody 52 (detection of bound antibody is described in the Experimental section). Whole-cell lysates were incubated under the indicated conditions before separation of membrane and cytosol fractions (as described in the Experimental section). The positions of migration of molecular mass standards are indicated in kDa. The difference in apparent electrophoretic mobility between the endogenous rat IP₃ 3-kinase B (Figure 2) and the recombinant protein encoded by the pCMUIV-rat IP₃ 3-kinase B expression construct is due to the presence of the HA epitope tag on the recombinant protein.

mass between the cytosolic and membrane-bound forms of the enzyme.

DISCUSSION

The data presented show that the cDNA clone previously described by Thomas et al. [20] is an inositol trisphosphate 3-kinase that uses $I(1,4,5)P_3$ as its substrate to generate $I(1,3,4,5)P_4$.

The enzyme is present as a protein of 90 kDa in NRK cells, and in fractions enriched in ER and nuclear membranes of rat liver (Figure 2). The membrane localization is in apparent contradiction with published data, since the vast majority of activities described to date are present in cytosolic fractions [12,13,15–17,19]. However, these activities are associated with low-molecular-mass molecules, some of which might have arisen due to proteolysis and 'release' from membranes into the cytosol, possibly during the course of sample preparation. Also, these studies do not address the possibility of a membrane-associated activity in the tissues or cell lines examined. When membrane fractions have been analysed, IP_3 3-kinase activity has been detected in turkey erythrocytes [54] and bovine iris sphincter smooth muscle [55]. Taken together, these data present strong evidence in favour of an IP_3 3-kinase activity being present in intracellular membranes.

As for the topology of rat IP_3 3-kinase, the hydropathy plot (Figure 5) shows the presence of three putative transmembrane domains. However, the results from the *in vitro* transcription/translation assay (Figure 6) indicate that only the stretch from amino acids 30–48 can possibly act as a transmembrane domain, leaving the catalytic domain on the cytosolic face of the membrane. The other possibility is that the enzyme is a peripheral protein on the cytosolic face of the extended ER network. Immunofluorescence analysis of SLO-permeabilized cells (Figure 7) clearly shows that rat IP_3 3-kinase B is not an integral membrane protein, but rather associated with membranes on the cytosolic face of specific intracellular membranes.

The data discussed so far leave open the question of a possible cytosolic pool of IP_3 3-kinase B. This was originally studied by immunoblot analysis of membrane and cytosolic fractions isolated from NRK cells. Both fractions show the presence of the enzyme, but its detection was difficult (presumably due to the sensitivity of the assay), and the 90 kDa band was not sufficiently intense to analyse the conditions required for membrane association of the kinase. Therefore we chose to tag IP_3 3-kinase B at the C-terminus with an HA epitope tag. The epitope-tagged enzyme was then expressed in different cell lines and the pattern of expression examined. Membrane and cytosolic fractions were immunoblotted using the monoclonal antibody 12CA6 (against the HA tag), and the data presented in Figure 9 show the presence of the protein in both fractions, indicating the existence of a cytosolic pool of rat IP_3 3-kinase B. The same band was recognized using antibody 52 (against the N-terminus of IP_3 3-kinase; results not shown). Although it can be argued that all the protein is membrane-bound in intact cells and homogenization causes release into the cytosol, this is unlikely, since 4 M urea is needed to release the membrane-bound pool into the soluble fraction (Figure 9). The requirement of 4 M urea for membrane dissociation also implies that membrane binding is via high-affinity conformation-dependent protein–protein interactions. It is possible that the cytosolic pool of recombinant IP_3 3-kinase B detected in transiently transfected cells arises as a result of saturation of receptor sites in the appropriate membranes.

It is of note that the product of IP_3 3-kinase, namely $I(1,3,4,5)P_4$, has been suggested to play a role in membrane

trafficking. It binds to synaptotagmin II [56], a synaptic vesicle protein thought to be involved in vesicular fusion and docking and also to function as a Ca^{2+} sensor [57]. $I(1,3,4,5)P_4$ also binds to Golgi coatamer [8], a coat protein complex that participates in vesicular traffic between Golgi cisternae (see [58] for a review). Binding of $I(1,3,4,5)P_4$ and other inositol polyphosphates blocks the K^+ -selective channel activity of the coatamer in reconstituted planar bilayers [8]. $I(1,3,4,5)P_4$ also binds to AP-2 [59], a clathrin assembly protein complex that promotes clathrin lattice formation [60]. The binding of $I(1,3,4,5)P_4$ inhibits reconstitution of clathrin cages [59]. Clearly, a possible role for $I(1,3,4,5)P_4$ in the regulation of membrane trafficking via interaction with AP-2, coatamer and synaptotagmins is suggested from these data. However, it should be noted that, in those cases where IP_4 has been shown to influence coat assembly, IP_5 and IP_6 are more potent inhibitors of that process. If IP_4 were to play a role in regulating coat assembly during membrane traffic, a logical site for the controlled generation of IP_4 would be the cytosolic face of the membrane, which gives rise to the initial transport vesicles in the secretory pathway, i.e. the ER membrane, which is the location of IP_3 3-kinase B.

In summary, the data presented show that IP_3 3-kinase B indeed produces the physiologically relevant $I(1,3,4,5)P_4$. It is cytosolic and is also present as a peripheral protein on the cytosolic face of the extended ER network. We have also shown that IP_3 3-kinase B binds to membranes through high-affinity protein–protein interactions. Moreover, the fact that the membranes involved are those corresponding to Ca^{2+} stores, i.e. the extended ER network, provides an invaluable insight into the role of $I(1,3,4,5)P_4$ in IP_3 -induced Ca^{2+} release. It also raises the intriguing possibility that the Ca^{2+} -regulated generation of IP_4 at the cytosolic face of membranes at the start of the secretory pathway may provide a mechanism for the regulation of coat assembly and thus vesicular transport itself.

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