Expression of recombinant rat *myo*-inositol 1,4,5-trisphosphate 3-kinase B suggests a regulatory role for its N-terminus

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We have expressed rat *myo*-inositol 1,4,5-trisphosphate (IP₃) 3-kinase B as both a full-length, recombinant, non-fusion protein and a full-length, recombinant, fusion protein with maltose-binding protein (MBP) in *Escherichia coli*. The fusion protein with MBP is soluble, binds calmodulin and is enzymically active whereas the non-fusion protein is insoluble and does not bind

calmodulin unless co-expressed with bacterial chaperone proteins (either GroES and GroEL, or DnaK, DnaJ and GrpE). However, soluble, calmodulin-binding non-fusion IP₃ 3-kinase B is enzymically inactive. The catalytic domain of the enzyme has previously been shown to reside near the C-terminus; the results we present suggest an auto-regulatory role for the N-terminus.

INTRODUCTION

myo-Inositol 1,4,5-trisphosphate (IP₃) 3-kinases are responsible for the ATP dependent phosphorylation of the Ca²⁺-mobilizing second messenger IP₃ to the putative second messenger inositol 1,3,4,5-tetrakisphosphate (IP₄) [1]. Although the role of IP₄ is not well defined, it has been suggested that it is involved in Ca²⁺ homeostasis [2,3] and vesicular trafficking [4,5]. The cloning of an IP₄-binding protein (GAP1^{IP4BP}) [6] that has Ras and Rap-GAP activity also suggests a role for IP₄ in signalling by small GTPases.

Two distinct cDNAs encoding IP₃ 3-kinase isoenzymes have been described so far. Both were isolated from a human hippocampus cDNA library, and were designated IP₃ 3-kinase A and B [7,8]. All IP₃ 3-kinases are activated to different extents by Ca²⁺/calmodulin [8–10].

Human hippocampus IP₃ 3-kinase B was initially predicted to encode a protein of 472 amino acids [11]. However, it was shown to have an incomplete N-terminus when compared with the rat orthologue [12]. The C-terminal 275 amino acids of human IP₃ 3-kinase B are required for a catalytically active domain when expressed as a fusion protein with β -galactosidase [13]. To our knowledge no IP₃ 3-kinase activity has been reported for any non-fusion full-length, recombinant IP₃ 3-kinase isoenzyme.

We report here evidence for a possible auto-inhibitory role for the N-terminus of rat IP₃ 3-kinase B. We also show that rat IP₃ 3-kinase B expressed as a full length non-fusion protein in *Escherichia coli* requires the co-expression of chaperone proteins for correct folding and for binding to calmodulin.

MATERIALS AND METHODS

Expression of recombinant rat IP₃ 3-kinase B in *E. coli*

The full-length coding sequence of rat IP₃ 3-kinase B was amplified by PCR, with the oligonucleotide primers ST10 (TTG-GTACCATATGGAAGAGGTTCCCCGGC) and ST11 (AT-AGATCTATCAGGTGAGTGGGCTGCCCT), which introduced an *Nde*I site at the 5' end and a *Bgl*II site at the 3' end of

the PCR product. The PCR product was cloned into the TA cloning vector (Invitrogen) and sequenced. The coding sequence was excised with *Nde*I/*Bgl*II and subcloned into the prokaryotic, isopropyl β -D-thiogalactoside (IPTG)-inducible expression vector pET 11a (ams biotechnology) with the *Nde*I and *Bam*HI restriction sites.

The construct was co-transformed into the *E. coli* strain BL21 (DE3) along with plasmid pRDKJG or plasmid pGroESL [14]. pRDKJG encodes the bacterial chaperones DnaJ, DnaK and GrpE under the control of an IPTG-inducible promoter, whereas pGroESL encodes the bacterial chaperones GroES and GroEL under the control of an IPTG-inducible promoter. Bacteria were grown at 37 °C on Luria–Bertani (LB) agar supplemented with 100 μ g/ml carbenicillin to select for the pET11a construct, and 25 μ g/ml kanamycin to select for pRDKJG or pGroESL. LB broth (10 ml), supplemented with 100 μ g/ml carbenicillin and 25 μ g/ml kanamycin, was inoculated with *E. coli* harbouring the two recombinant plasmids. At an *A*₆₀₀ of 0.2–0.4, IPTG was added to a final concentration of 1 mM and the culture was incubated for a further 1 h. The bacteria were harvested by centrifugation and resuspended in 1 ml of resuspension buffer [50 mM Tris/HCl, pH 8.0, containing 100 mM NaCl, 1 mM dithiothreitol, 0.1% Triton X-100 and a protease inhibitor cocktail (Pharmacia)]. Bacteria were lysed by sonication and the insoluble protein was separated by centrifugation (100 000 *g* for 20 min at 4 °C). The soluble fraction was assayed for IP₃ 3-kinase activity. The same procedure was followed in parallel in the absence of bacterial chaperones.

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

The full-length coding region of rat IP₃ 3-kinase B was amplified by PCR, with the oligonucleotide primers ST18 (5'-T TGT CGA CAT GGA AAG AGG TTC CCC GGC-3') and ST19 (5'-ATA AAG CTT GGT GAG TGG GCT GCC CT-3'), which introduced a *Sal*I site at the 5' end and a *Hind*III site at the 3' end of the amplified fragment. The PCR product was subcloned into pCRII (Invitrogen) and confirmatory DNA sequencing was

Abbreviations used: IP₃, *myo*-inositol 1,4,5-trisphosphate; IP₄, inositol 1,3,4,5-tetrakisphosphate; IPTG, isopropyl β -D-thiogalactoside; LB, Luria–Bertani; MBP, maltose-binding protein.

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performed. The PCR product was then subcloned into the expression vector pMAL c2 (New England Biolabs) by using the *Sall* and *HindIII* restriction sites. The resulting construct was designated pMAL2.0. LB broth cultures (10 ml) of the *E. coli* strain XL1-B harbouring the plasmid pMAL2.0 were grown at 37 °C with 100 µg/ml carbenicillin. At an A_{600} of 0.5–0.6, IPTG was added to a final concentration of 1 mM and induction continued for 3 h. A soluble fraction was prepared as described previously and assayed for IP₃ 3-kinase activity.

Calmodulin-binding assay

E. coli lysate (50 µl) containing either IP₃ 3-kinase B (with or without chaperones) or IP₃ 3-kinase–MBP fusion protein, were diluted into 1 ml incubation buffer [50 mM Tris/HCl (pH 8.0)/100 mM NaCl/5 mM Ca²⁺/0.1 % Triton X-100] and incubated for 30 min with pre-equilibrated calmodulin–agarose beads (Sigma). The beads were then washed three times in washing buffer (incubation buffer without Ca²⁺) and then eluted in washing buffer with 20 mM EDTA. Aliquots were analysed by SDS/PAGE.

Assay of IP₃ 3-kinase

IP₃ 3-kinase activity was measured by the production of IP₄ from IP₃, essentially as described by Spencer et al. [14a], in a final assay volume of 15 µl in a reaction mixture containing 50 mM Hepes/NaOH (pH 7.5 at 25 °C)/1 mM MgCl₂/10 mM EGTA/24 mM 2-mercaptoethanol/1 mg/ml BSA/5 mM MgATP²⁻/20 µM IP₃ plus [³H]IP₃ (5000 d.p.m. per assay). Aliquots (5 µl) of suitably diluted bacterial supernatants were used to initiate the reaction. After incubation at 37 °C for 30 min the assays were stopped by the addition of 10 µl of 0.2 M HCl containing 300 µM IP₃ and 300 µM IP₄. The samples were then neutralized by the addition of 10 µl of 0.2 M KOH and the resultant inositol phosphates were separated on a Partisil 10 SAX HPLC column in the presence of ³²P-labelled IP₄ [15] acting as an internal indicator of the elution position of IP₄, with a gradient of 0–0.75 M NaH₂PO₄, pH 3.75, over 20 min (1 ml fractions) followed by a shallow gradient of 0.75–0.9 M NaH₂PO₄, pH 3.75, over 60 min (0.25 ml fractions). Optimal resolution of the various isomers of IP₄ is achieved under these conditions [16–19].

RESULTS

Expression of soluble, recombinant rat IP₃ 3-kinase B

We chose to express rat IP₃ 3-kinase B as a recombinant protein in *E. coli* to generate material for studies on the activity of the enzyme. Two expression constructs were made, one (pETIP33K) encoding full-length rat IP₃ 3-kinase B as a non-fusion protein, the other (pMAL2.0) encoding the full-length IP₃ 3-kinase B–MBP fusion protein (see the Materials and methods section). SDS/PAGE analysis of whole-cell lysate from transfected *E. coli* cells shows that the products of pETIP33K and pMAL2.0 (Figure 1A) were efficiently expressed after 1 h of induction with IPTG. However, after separation of soluble and insoluble material isolated from sonicated *E. coli* lysate, the majority of the non-fusion, full-length protein was present in the insoluble fraction (Figure 1B, lanes a–c). This is indicative of protein aggregation and suggests that the recombinant protein might be incorrectly folded. The observation that no IP₃ 3-kinase activity could be detected in the soluble or insoluble fractions (results not

Figure 1 Expression of recombinant IP₃ 3-kinase B in *E. coli*

(A) Lysate from *E. coli* cells harbouring plasmids encoding non-fusion (lanes a and b) and MBP-fusion (lanes c and d) full-length IP₃ 3-kinase were subjected to SDS/PAGE as described in the Materials and methods section. Lysate in lanes b and d was from cells induced with IPTG; lysate in lanes a and c was from non-induced controls. (B) SDS/PAGE analysis of lysates from *E. coli* cells harbouring plasmid encoding non-fusion, full-length IP₃ 3-kinase B expressed in the absence (lanes a–c) or presence of bacterial chaperone proteins (lanes d–i, GroES/GroEL; lanes g–i, DnaJ/DnaK/GrpE). Lanes a, d and g contained material from whole cell lysates. Lanes b, e and h contained the soluble fractions, and lanes c, f and i contained the high-speed (100 000 g) pellets (i.e. insoluble material). Both panels show Coomassie Blue-stained gels. The arrow indicates the position of the recombinant IP₃ 3-kinase B; other strongly stained bands in lanes d–i correspond to the chaperone proteins.

shown) is consistent with this hypothesis. We therefore chose to express the non-fusion, full-length enzyme in *E. coli* cells in the presence of the bacterial chaperone proteins GroES and GroEL or DnaJ, DnaK and GrpE (as described in the Materials and methods section). Most of the enzyme is soluble under either of these conditions (Figure 1B, lanes d–i). Moreover, precipitation does not occur after high-speed centrifugation (results not shown), suggesting that the protein folds correctly in the presence of bacterial chaperone proteins. There seems to be a higher recovery of soluble enzyme when it is co-expressed with DnaJ, DnaK and GrpE rather than with GroES and GroEL. Therefore we used material prepared in the presence of DnaJ, DnaK and GrpE for further experiments.

Calmodulin binding by recombinant rat IP₃ 3-kinase B

IP₃ 3-kinase isoenzymes have previously been shown to bind calmodulin; this is a factor in the regulation of their activity [8–10]. Therefore, to test whether the recombinant IP₃ 3-kinase B had folded correctly in the presence of DnaJ, DnaK and GrpE, we performed binding assays on calmodulin-conjugated Seph-

Figure 2 Calmodulin-binding assays

(A) Recombinant IP₃ 3-kinase B binds calmodulin when co-expressed with bacterial chaperone proteins. Lysate from *E. coli* cells expressing full-length, non-fusion IP₃ 3-kinase B in the presence of bacterial chaperone proteins DnaJ, DnaK and GrpE was incubated in the presence of calmodulin-agarose; non-bound material was washed off before elution of bound material with EDTA as described in the Materials and methods section. The cell lysate (lys), wash (w) and EDTA-eluted (edta) fractions were subjected to SDS/PAGE. (B) Recombinant IP₃ 3-kinase B does not bind calmodulin when expressed in the absence of bacterial chaperone proteins. Lysate from *E. coli* cells expressing full-length, non-fusion IP₃ 3-kinase B in the absence of bacterial chaperone proteins was incubated with calmodulin-agarose; non-bound material was washed off and bound material was eluted with EDTA as described in the Materials and methods section. The cell lysate (lys), wash (w) and EDTA-eluted (edta) fractions were subjected to SDS/PAGE. Both panels show Coomassie Blue-stained gels; in each panel, lane m shows molecular mass markers. The arrow indicates the position of migration of recombinant IP₃ 3-kinase B; the arrowheads in (A) indicate the positions of migration of chaperone proteins DnaK (top) and DnaJ. GrpE is too small (16 kDa) to be retained in this gel. In (B) the position of migration of recombinant IP₃ 3-kinase B in the lysate and wash fractions is indicated by a white dot.

arose beads with recombinant rat IP₃ 3-kinase expressed in the presence or absence of those bacterial chaperone proteins. The results in Figure 2(A) show that soluble rat IP₃ 3-kinase B, co-expressed with DnaJ, DnaK and GrpE, binds to calmodulin and can be eluted from calmodulin-Sephadex beads with EDTA. Similar results were obtained with the MBP fusion construct and for non-fusion, full-length rat IP₃ 3-kinase B co-expressed with GroES and GroEL (results not shown). In contrast, the non-fusion, full-length rat IP₃ 3-kinase B expressed in the absence of bacterial chaperones failed to bind to calmodulin (Figure 2B).

Enzyme activity of recombinant rat IP₃ 3-kinase B

The soluble non-fusion, full-length rat IP₃ 3-kinase B produced in the presence of bacterial chaperones was used in an assay of IP₃ 3-kinase activity (see the Materials and methods section). No enzyme activity was detected (Figure 3A). However, enzyme activity was detected in lysate from *E. coli* cells expressing the full-length MBP-IP₃ 3-kinase B fusion protein (Figure 3B).

Figure 3 Assay of IP₃ 3-kinase activity

□, HPLC profiles of ³H-labelled compounds from bacterial extracts containing (A) recombinant, full-length IP₃ 3-kinase B expressed as a non-fusion protein and (B) recombinant, full-length IP₃ 3-kinase B expressed as a C-terminal fusion to MBP. ◇, HPLC profiles of a ³²P-labelled IP₄ marker. The region of each HPLC trace corresponding to elution of the ³²P-labelled IP₄ marker (◇), and any ³H-labelled IP₄ generated as a result of enzymic activity in the bacterial lysate (□), is shown in enlarged format in an inset in each panel. Enzyme assays were performed as described in the Materials and methods section. Neutralized assays were applied to a Partisil 10 SAX HPLC column and eluted as described in the Materials and methods 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]IP₃. The subsequent peaks contained [³H]IP₄ (□) and [³²P]IP₄ (◇). The specific activity of recombinant, full-length IP₃ 3-kinase B expressed as a C-terminal fusion to MBP was calculated as 10 nmol/min per mg of *E. coli* protein lysate; no specific activity could be calculated for the recombinant, full-length IP₃ 3-kinase B expressed as a non-fusion protein because no enzyme activity was detectable.

DISCUSSION

We have shown that recombinant rat IP₃ 3-kinase B can be expressed as a full-length, soluble, calmodulin-binding, non-fusion protein in *E. coli* cells. The solubility and calmodulin-binding characteristics of the recombinant protein suggest that it is correctly folded; however, it is devoid of enzyme activity under conditions where an MBP-IP₃ 3-kinase B fusion protein is active. One interpretation of this observation is that sequences within the non-fusion enzyme are in some way involved in inhibition of its catalytic activity. The most obvious candidate for such an inhibitory role is the N-terminal 200 amino acid residues of the

enzyme, because this region is not present in the A isoform [12]. There is some evidence in favour of this hypothesis. The B isoform of rat IP₃ 3-kinase exists in both cytosolic and membrane-bound pools (S. Soriano, Thomas, S., High, S., Griffiths, G., D'Santos, C. and Banting, G., unpublished work), and because this feature is unique among IP₃ 3-kinases, it is reasonable to suppose that the 200 N-terminal residues (absent from all other isoforms) are responsible for membrane attachment in mammalian cells. Moreover, although most non-recombinant IP₃ 3-kinase activities described so far are cytosolic [8,20–24], they are associated with low-molecular-mass molecules, potentially arising owing to proteolysis and 'release' of the catalytic domain of IP₃ 3-kinase B into the cytosol during sample preparation or because of different isoforms of the enzyme.

It could be argued that the lack of activity from the full-length recombinant enzyme in the presence of bacterial chaperones is due to misfolding of the enzyme, but this is unlikely for two reasons. First, misfolding would imply precipitation after prolonged high-speed centrifugation, which does not occur. Secondly, a misfolded form of the enzyme would be unlikely to bind reversibly to calmodulin; however, the full-length recombinant enzyme does. Therefore the most likely explanation for our results is that the enzyme is correctly folded and locked in an inactive conformation. When the N-terminus of the protein is forced to adopt a different conformation (when fused to MBP), the enzyme is constitutively active.

In summary, we show that co-expression with bacterial chaperone proteins is necessary for the production of soluble, full-length recombinant rat IP₃ 3-kinase B, and for binding to calmodulin. This strongly suggests that the enzyme folds correctly when co-expressed with bacterial chaperone proteins. However, this recombinant protein is enzymically inactive. In contrast, full-length rat IP₃ 3-kinase B fused to MBP does not require bacterial chaperones for its solubility: it binds calmodulin and possesses IP₃ 3-kinase activity. Taken together, these results are consistent with a self-regulatory mechanism for rat IP₃ 3-kinase B, possibly involving the N terminus of the enzyme.

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