Lys-197 and Asp-414 are critical residues for binding of ATP/Mg^{2+} by rat brain inositol 1,4,5-trisphosphate 3-kinase

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Rat brain inositol 1,4,5-trisphosphate ($InsP_3$) 3-kinase A was expressed in *Escherichia coli* in order to identify the amino acid residues involved in substrate ATP/Mg^{2+} binding. Two amino acid regions that are conserved in the catalytic domain of $InsP_3$ 3-kinase isoenzymes A and B had characteristics consistent with two ATP/Mg^{2+}-binding motives. Site-directed mutagenesis was performed on residues Lys-197, Lys-207 and Asp-414 to generate three mutant enzymes, referred to as C5 K197I, C5 K207I and C5 D414N. Comparison of the wild-type and mutant proteins with regard to enzymic activity revealed that C5 K197I exhibited 10% of control enzyme activity, C5 D414N was totally inactive and C5 K207I was fully active. The reduced levels of enzyme activity for C5 K197I and C5 D414N were correlated with an altered ability of the mutant enzymes to bind ATP/Mg^{2+}, as determined by ATP–agarose affinity chromatography. Neither Ca^{2+}/calmodulin binding nor $InsP_3$ binding appeared to be affected. Mutant C5 K207I showed the same characteristics as the wild-type enzyme. Taken together, these results strongly indicated (i) that amino acid residues Lys-197 and Asp-414 are necessary for $InsP_3$ 3-kinase activity and form part of the ATP/Mg^{2+}-binding domain, and (ii) that amino acid residues Lys-197, Lys-207 and Asp-414 are not involved in either $InsP_3$ binding or enzyme stimulation by Ca^{2+}/calmodulin.

INTRODUCTION

An increase in intracellular inositol 1,4,5-trisphosphate ($InsP_3$) and inositol 1,3,4,5-tetrakisphosphate ($InsP_4$) levels plays an important role in the mechanism of Ca^{2+} mobilization (Berridge and Irvine, 1989; Irvine, 1992). The enzyme $InsP_3$ 3-kinase catalyses the phosphorylation of $InsP_3$ to $InsP_4$ (Irvine et al., 1986).

The complex formed by Ca^{2+} and calmodulin (CaM) appears to be an activator of $InsP_3$ 3-kinase activity (Biden et al., 1987; Morris et al., 1987; Ryu et al., 1987; Yamaguchi et al., 1988; Takazawa et al., 1988), and this interaction has been used to purify the enzyme by affinity chromatography on CaM–Sepharose (Takazawa et al., 1989; Lee et al., 1990). cDNA clones encoding a 50 kDa rat brain $InsP_3$ 3-kinase (referred to as rat brain $InsP_3$ 3-kinase A) have been isolated and sequenced (Choi et al., 1990; Takazawa et al., 1990). The encoded protein could be expressed in *Escherichia coli* as a Ca^{2+}/CaM-sensitive β-galactosidase fusion protein (Takazawa et al., 1990). Human $InsP_3$ 3-kinase A has also been cloned (93% amino acid identity with the rat enzyme; Takazawa et al., 1991a), as has a human isoenzyme, referred to as $InsP_3$ 3-kinase B (Takazawa et al., 1991b).

Cloning and expression studies with $InsP_3$ 3-kinase have enabled us to identify protein domains and to investigate their role in substrate binding, catalysis and/or regulation (Takazawa and Erneux, 1991). It was shown that the sequence region from Ser-156 to Leu-189 is involved in CaM binding and that the catalytic domain is localized at the C-terminus. The data raised the possibility of localizing the ATP/Mg^{2+}-binding domain and of comparing it with ATP/Mg^{2+}-binding domains of other proteins.

In this paper, we report the identification by site-directed mutagenesis of two critical residues involved in ATP/Mg^{2+} binding by rat brain $InsP_3$ 3-kinase, i.e. Lys-197 and Asp-414. Our data are consistent with a decreased affinity for ATP; however, no effects on $InsP_3$ binding or the interaction with CaM were observed as a consequence of the two mutations.

MATERIALS AND METHODS

Materials

Materials for assay were as previously reported (Takazawa et al., 1989). Immune complexes were detected on blots using alkaline phosphatase-conjugated anti-(rabbit IgG) and corresponding colorimetric methods (Promega), as described previously (Takazawa et al., 1990).

ATP–agarose resin was obtained from Gibco BRL. CHAPS was from Serva. [2-^{3}H]$InsP_3$ (sp. radioactivity 3.3 Ci/mmol) was from New England Nuclear Corp. Bovine γ-globulins and poly(ethylene glycol) (8 kDa) were obtained from Sigma. Calpain inhibitors (I and II) were from Calbiochem.

Analytical procedures

$InsP_3$ 3-kinase activity was measured in 100 μl of incubation medium containing 84 mM Hepes/NaOH (pH 7.5), 1 mg of BSA/ml, 1 mM ATP, 20 mM $MgCl_2$, 12 mM 2-mercaptoethanol, 10 μM free calcium plus 0.1 μM CaM or 1 mM EGTA, [^{3}H]$InsP_3$ (1500 c.p.m./assay) and unlabelled $InsP_3$ to a final concentration of 10 μM, and finally the diluted enzyme solution (Takazawa et al., 1988). Apparent $V_{max.}$ and K_m values were measured using a non-linear least-squares curve fitting (Marquardt–Levenberg algorithm). Antibodies used in immunodetection of Western blots were affinity-purified using the recombinant fusion protein encoded by the insert of clone C5 (Takazawa et al., 1990), according to the technique described by Snyder et al. (1987). Protein concentration was measured according to Petersen (1977).

Abbreviations used: CaM, calmodulin; PMSF, phenylmethanesulphonyl fluoride.

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Oligonucleotide-directed *in vitro* mutagenesis

Mutagenesis was carried out using the method of Sayers et al. (1988) with the oligonucleotide-directed *in vitro* Mutagenesis System Version 2 (Amersham; RPN 1523). The method was modified as follows: 10 μg of single-stranded DNA template was used for the reaction, and the last polymerization–ligation reaction was carried out for 25 h. The mutated double-stranded DNAs were used to transform DH5αF′ or *E. coli* XL1-Blue competent cells.

The rat InsP_3 3-kinase cDNA (insert of clone C5 digested by *Eco*RI) was subcloned in vector M13mp19 to produce single-stranded DNA, referred to C5 M13. This was used as a template and was annealed to the oligonucleotide 5′-GGGCACACAGG-**AAGCTTC**ATAGCTGCGGGC-3′ in order to mutate Lys-197 to isoleucine (underlined). In addition, this created a new and unique *Hin*dIII restriction site (indicated in bold). We used the oligonucleotide 5′-GGCCTGATCCTGATCCG**GAGCTC**-GGAGCCT-3′ in order to mutate Lys-207 to isoleucine, and thereby created a new and unique *Sst*I restriction site in the sequence (bold). Finally, the oligonucleotide 5′-GTGTGGCT-CATCAATTTTGGCAAGAC-3′ was used to mutate Asp-414 to asparagine. This mutation resulted in the loss of a unique *Cla*I restriction site. The mutated inserts were subcloned from M13 vector to Bluescript vector for protein expression and characterization. The three mutants were referred to as C5 K197I, C5 K207I and C5 D414N respectively. Single-stranded DNA was isolated from each construction and 350 bp portions of sequence surrounding the mutations were sequenced by the Sanger dideoxy method (Sanger et al., 1977) to obtain confirmation of the expected sequence.

Expression of InsP_3 3-kinase and related mutants

To express proteins from wild-type and mutated constructs, LB medium (5 ml) containing 50 μg/ml ampicillin was inoculated for an overnight incubation at 37 °C with a single colony of *E. coli* XL1-Blue containing the Bluescript plasmid. After addition of isopropyl β-thiogalactoside (1 mM final concentration) for 4 h at 30 °C, the bacteria were harvested by centrifugation (1200 ***g***, 15 min) and resuspended in 0.5 ml of cold lysis buffer [50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 0.2 mM phenylmethane-sulphonyl fluoride (PMSF), 2.5 μM leupeptin, 5 μg/ml calpain inhibitors (I and II), 10% sucrose, 12 mM 2-mercaptoethanol and 1% Triton X-100]. After agitation for 10 min at 4 °C and centrifugation (1500 ***g***, 5 min), each supernatant was used to assay for InsP_3 3-kinase activity and to determine apparent $V_{max.}$ and K_m values for InsP_3 and ATP. Expression of related mutants was followed by Western blot and immunodetection analysis.

InsP_3 binding assay with bacterial lysate

Each *E. coli* colony containing the plasmid was incubated in 50 ml of LB medium containing 50 μg/ml ampicillin at 37 °C overnight. The incubation was continued for 3 h at 30 °C in the presence of 5 mM isopropyl β-thiogalactoside. The bacteria were harvested by centrifugation (1300 ***g***, 10 min), resuspended in 1.5 ml of buffer [50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 0.2 mM PMSF, 10% sucrose, 2.5 μM leupeptin and 12 mM 2-mercaptoethanol] at 4 °C and sonicated. A 50 μl portion of 20% CHAPS was added to the lysate, which was then centrifuged for 10 min in order to recover the supernatant for binding assays.

A poly(ethylene glycol)precipitation method (Chadwick et al., 1990; K. Takazawa, M. Jo, T. Endo, C. Erneux and T. Onaya, unpublished work) was used to determine binding of [^{3}H]InsP_3 to the bacterial lysate. An 80 μl sample of the bacterial lysate was mixed with 10000 c.p.m. of [^{3}H]InsP_3 and different concentrations of unlabelled InsP_3 (0–45 μM) in a total incubation volume of 100 μl. After 10 min at 4 °C, 10 μl of bovine γ-globulins (50 mg/ml) and 100 μl of a 30% poly(ethylene glycol)/ 0.15 M NaCl solution were added for another 10 min incubation at 4 °C. The mixture was centrifuged at 15000 ***g*** for 10 min and the pellets were washed twice before being resuspended in 1 ml of water. The samples were mixed with 5 ml of Insta-Gel II scintillation cocktail to measure the radioactivity. The binding of [^{3}H]InsP_3 to a bacterial lysate derived from a non-recombinant plasmid was not significant (results not shown).

Elution on ATP–agarose and CaM–Sepharose columns

All steps were performed at 4 °C. ATP–agarose resin (500 μl) was rinsed thoroughly with buffer A [20 mM Tris/HCl (pH 7.5), 2 mM EGTA, 12 mM 2-mercaptoethanol, 0.2 mM PMSF, 2.5 μM leupeptin, 5 μg/ml calpain inhibitors (I and II) and 0.1% Triton X-100]. Wild-type and mutated crude bacterial extracts (500 μl) were applied to the column in the presence of 2 mM $MgCl_2$; the column was then washed with three 500 μl fractions of wash buffer. The column was eluted with buffer A containing increasing ATP concentrations (0–20 mM). Fractions of 500 μl were collected. The column was rinsed with 5 ml of buffer A, followed by 1.5 ml of buffer A containing 100 mM ATP to remove any remaining protein associated with the column. The column was regenerated by equilibration in buffer A. CaM–Sepharose affinity chromatography was performed as previously described (Takazawa et al., 1990).

RESULTS

Construction of site-directed mutants

The catalytic domain of rat brain InsP_3 3-kinase is located at the C-terminal end of the protein; it has been shown that a maximum of 275 amino acids in the C-terminal region is sufficient for the expression of a catalytically active domain. Deletion mutants also indicated that amino acid residues between Ser-185 and Ala-198 are necessary for enzymic activity. When the coding sequence was deleted at the N-terminus up to Ser-185, the protein could be expressed in bacteria; however, no InsP_3 3-kinase activity was detected (Takazawa and Erneux, 1991).

Alignment of the InsP_3 3-kinase isoenzyme sequences with ATP/Mg^{2+}- and ATP/GTP-binding site consensus sequences revealed no region of high identity (Table 1). However, the sequences Gly-191–Lys-197 and Gly-200–Lys-207 at the N-terminal and of the rat brain InsP_3 3-kinase A catalytic domain are comparable with an ATP/Mg^{2+}-binding site consensus sequence (Hanks et al., 1988), having critical lysine residues, i.e. Lys-197 and Lys-207. These two lysine residues were therefore mutated to isoleucine residues to study the effect of these mutations on InsP_3 3-kinase activity. A typical sequence Asp-Phe-Gly, which is conserved among many protein kinases as part of the ATP/Mg^{2+}-binding domain (Hanks et al., 1988), was present at the end of the C-terminal region of InsP_3 3-kinase isoenzymes; in the rat brain InsP_3 3-kinase, it comprised residues 414–416. Asp-414 was therefore mutated to asparagine.

The three mutants of rat brain InsP_3 3-kinase were referred to as C5 K197I for the mutation of Lys-197 to isoleucine, C5 K207I for the mutation of Lys-207 to isoleucine and C5 D414N for the mutation of Asp-414 to asparagine.

Influence of the mutations on InsP_3 3-kinase activity

InsP_3 3-kinase activities of expressed wild-type and mutated proteins are given in Table 2. A lysate prepared from a non-

Table 1 Alignment of rat and human brain InsP_3 3-kinase A and human brain InsP_3 3-kinase B with ATP/Mg^{2+}-binding site consensus sequences

The invariant lysine residue is represented in bold. The standard single-letter amino acid code is used, where X is any amino acid.

Protein	Sequence	Reference
ATP/Mg^{2+}-binding consensus sequence	G-X-G-X-X-G-X_{15}-**K**	Hanks et al. (1988)
ATP/GTP-binding consensus sequence	G-X-X-X-X-G-**K**	Saraste et al. (1990)
InsP_3 3-kinase A (rat)	G-H-T-G-S-F-**K** (197) G-T-S-G-L-I-L-**K** (207)	Takazawa et al. (1990)
InsP_3 3-kinase B (human)	G-H-A-G-S-F-**K** (207) A-N-G-R-I-L-**K** (216)	Takazawa et al. (1991b)

Table 2 Enzymic activities of expressed mutants of rat brain InsP_3 3-kinase A

InsP_3 3-kinase activity was determined at 10 μM InsP_3 in the presence of 0.1 μM CaM and 10 μM free calcium or of 1 mM EGTA for basal activity using crude bacterial extracts from individual clones. n.d., activity was undetectable. Results are means ± S.D. of ten determinations.

Clone	InsP_3 3-kinase activity (nmol/min per mg of protein)		Stimulation by CaM (fold)
	+ EGTA	+ Ca^{2+}/CaM	
Non-recombinant	n.d.	n.d.	–
C5	18.0 ± 2.5	38.0 ± 3.0	2.1
C5 K197I	1.9 ± 0.4	3.6 ± 0.3	1.9
C5 K207I	17.0 ± 3.0	33.5 ± 2.5	2.0
C5 D414N	n.d.	n.d.	–

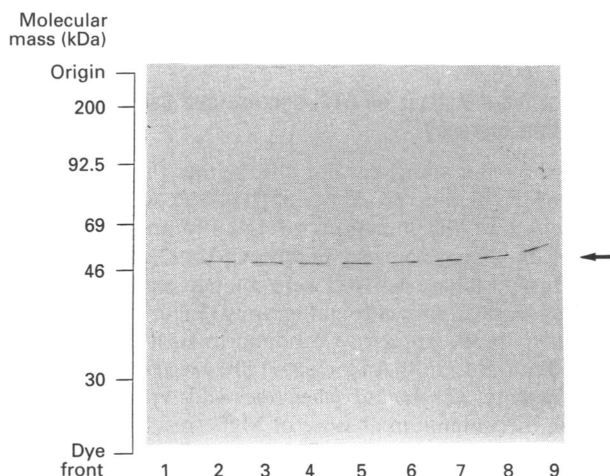

Figure 1 Western blot of expressed mutants of rat brain InsP_3 3-kinase A

Crude bacterial lysate (10 μl; about 13 μg of protein) was applied to SDS/10%-PAGE and the resolved proteins transferred to nitrocellulose. Immunoblot analysis was performed using purified antibodies to rat brain InsP_3 3-kinase. Lane 1, bacterial lysate from a non-recombinant clone (no detectable enzymic activity); lanes 2 and 3, bacterial lysates from wild-type expressed clone C5 (enzymic activity at 10 μM InsP_3 was 0.36 nmol/min); lanes 4 and 5, bacterial lysates from mutant C5 K197I (enyzmic activity at 10 μM InsP_3 was 0.04 nmol/min); lanes 6 and 7, bacterial lysates from mutant C5 K207I (enzymic activity at 10 μM InsP_3 was 0.34 nmol/min); lanes 8 and 9 are bacterial lysates from mutant C5 D414N (no detectable enzymic activity). The arrow shows the 48 kDa band of the expressed fusion protein.

Table 3 Apparent K_m values for InsP_3 and ATP for expressed wild-type and mutated proteins

The apparent K_m values for InsP_3 were estimated at 1 mM ATP and 0–20 μM InsP_3, and the apparent K_m values for ATP were at 1 μM InsP_3 and 0–1 mM ATP. Results are means ± S.D. of triplicate determinations. n.d., not detectable.

Clone	K_m (μM)	
	InsP_3	ATP
C5	7 ± 2	44 ± 5
C5 K197I	8 ± 3	190 ± 24
C5 K207I	7 ± 2	40 ± 6
C5 D414N	n.d.	n.d.

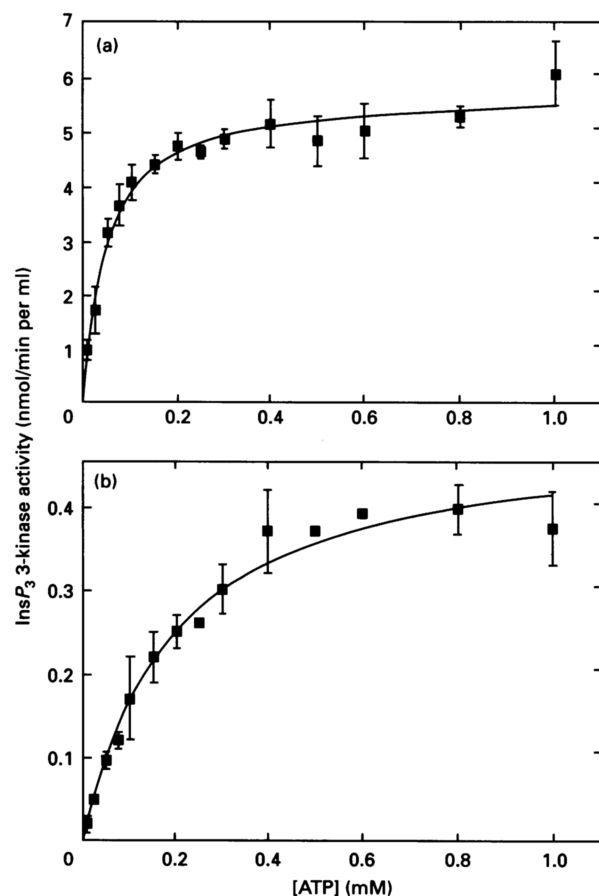

Figure 2 Substrate–velocity relationships of C5 and mutant C5 K197I

The data are shown as a direct plot in the 0–1 mM ATP range for the wild-type protein C5 (**a**) and the mutant C5 K197I (**b**). Assays were performed at 1 μM InsP_3 in the presence of 0.1 μM CaM plus 10 μM free Ca^{2+}.

recombinant clone did not have InsP_3 3-kinase activity after expression (Takazawa et al., 1990). The activity of mutant C5 K197I was about 10% of the wild-type activity. It was, however, still Ca^{2+}/CaM-sensitive, as for the wild-type protein. In contrast, InsP_3 3-kinase activity determined with or without Ca^{2+}/CaM was not affected by the mutation of Lys-207 to isoleucine. The mutant C5 D414N was totally inactive.

The extracts assayed in Table 2 were subjected to SDS/PAGE and immunoblotting (Figure 1). Purified antibodies recognized a unique 48 kDa band in lysates derived from expressed C5 and

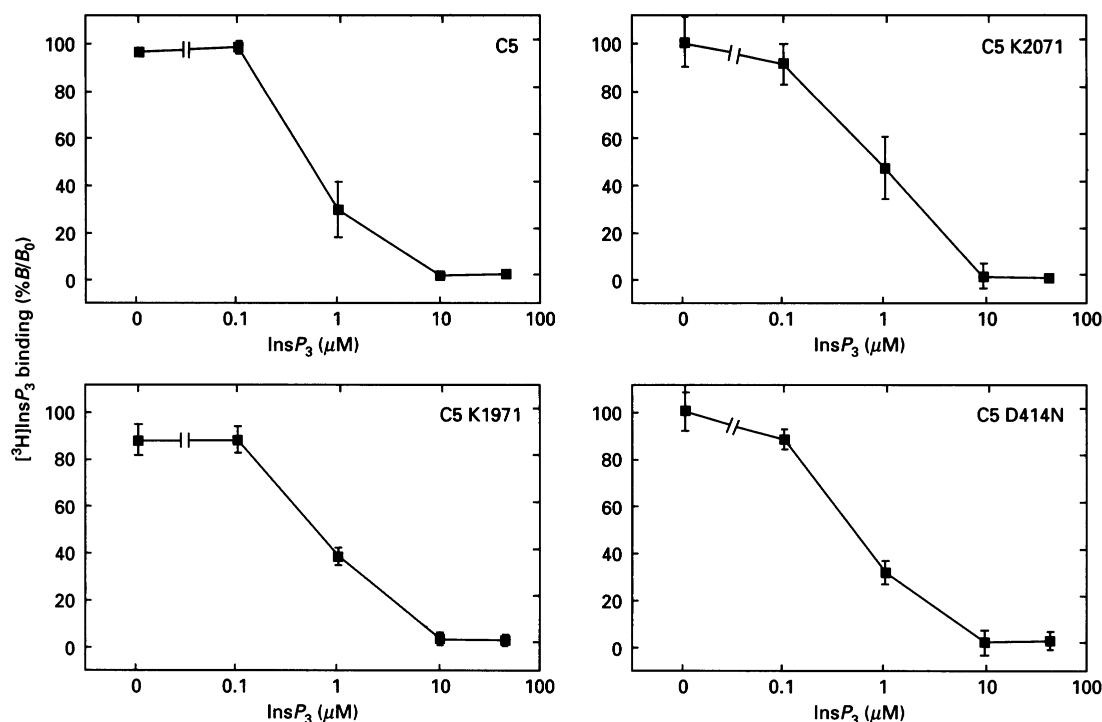

Figure 3 Displacement of [^{3}H]InsP_3 binding by unlabelled InsP_3 for expressed mutants of rat brain InsP_3 3-kinase A

Bacterial lysates were prepared for clones C5, C5 K197I, C5 K207I and C5 D414N and used in the InsP_3 binding assay. [^{3}H]InsP_3 (10000 c.p.m.) was at 2.1 nM. Displacement was conducted with added unlabelled InsP_3 (0–45 μM); 100% corresponds to 1150 $\pm$ 65 c.p.m. of bound [^{3}H]InsP_3. Results are means $\pm$ S.D. of triplicate determinations.

expressed mutants. The lysate prepared from a non-recombinant clone which had no InsP_3 3-kinase activity did not show any immunoreactive signal (Figure 1, lane 1). The expression levels were similar for the mutants and the wild-type proteins (Figure 1, lanes 2–9), suggesting that the differences in InsP_3 3-kinase activities in Table 2 are not due to changes in the protein expression level or to the expression of more or less proteolysed protein products.

The apparent K_m values for ATP and InsP_3 for C5 versus C5 K197I and C5 K207I are given in Table 3 and Figure 2. Apparent K_m values for InsP_3, measured at 1 mM ATP, were the same for the wild-type protein and the mutants. The apparent $V_{max.}$ values were 33.0 $\pm$ 5.0 nmol/min per mg for C5, 3.4 $\pm$ 0.4 nmol/min per mg for C5 K197I and 32.0 $\pm$ 4.0 nmol/min per mg for C5 K207I. The apparent K_m values for ATP, measured at 1 μM InsP_3, were the same in mutant C5 K207I and in C5. However, the mutant C5 K197I had an apparent K_m of 190 $\pm$ 24 μM for ATP which is increased by a factor of 4–5 compared with that of C5. The apparent $V_{max.}$ values were 5.6 $\pm$ 0.2 nmol/min per mg for C5 and 0.49 $\pm$ 0.02 nmol/min per mg for C5 K197I. Figure 2 shows typical substrate–velocity relationships for C5 and C5 K197I. It was shown that the apparent K_m for ATP was 44 $\pm$ 5.0 μM and 190 $\pm$ 24 μM for C5 and C5 K197I respectively.

Influence of the mutations on [^{3}H]InsP_3 binding

To examine directly the influence of the different mutations on InsP_3 binding, the displacement of [^{3}H]InsP_3 binding by increasing concentrations of unlabelled InsP_3 was studied (Figure 3). Non-specific binding was determined in the presence of 45 μM InsP_3; this was the same for each expressed protein, and was equal to the [^{3}H]InsP_3 binding measured for a non-recombinant clone (i.e. 1.0 $\pm$ 0.3% of the total radioactivity present per assay tube; results not shown). C5 and each mutant had the same specific [^{3}H]InsP_3 binding (i.e. 1150 $\pm$ 65 c.p.m. in the experiment shown in Figure 3); this binding was displaced by unlabelled InsP_3 with an IC_{50} of about 0.9 $\pm$ 0.1 μM (Figure 3).

Influence of the mutations on ATP–agarose and CaM–Sepharose affinity chromatography

Since the three mutations did not affect either the apparent K_m for InsP_3 or the binding properties of [^{3}H]InsP_3, we considered a possible effect of the mutations of Lys-197 and Asp-414 on ATP/Mg^{2+} binding. Expressed mutants C5 K197I and C5 K207I, that had InsP_3 3-kinase activity, were compared after elution on an ATP–agarose affinity chromatography (Figure 4). The InsP_3 3-kinase activity of expressed C5 bound to such a column and was eluted in the 15 mM ATP eluate (70% recovery). No InsP_3 3-kinase activity was bound when the wild-type enzyme was applied on this column in absence of Mg^{2+} (results not shown). In contrast, about 75% of the InsP_3 3-kinase activity of the mutant C5 K197I was in the flow-through, and only 15% in the 15 mM ATP eluate. We conclude, therefore, that C5 K197I did not bind to immobilized ATP. Expressed C5 K207I was eluted in 15 mM ATP eluate, as for the wild-type protein. The elution of expressed C5 D414N was followed by immunodetection of eluted fractions after ATP–agarose or CaM–Sepharose affinity chromatography to show if the mutated protein was able to bind ATP/Mg^{2+} or Ca^{2+}/CaM respectively (Figure 5). Mutant C5 D414N could be adsorbed on to CAM–Sepharose and eluted in Ca^{2+}-free buffer: a strong signal corresponding to a 48 kDa band was present only in the 1 mM EGTA eluate (Figure 5, lane 3). In contrast, after elution on an ATP–agarose column, a 48 kDa

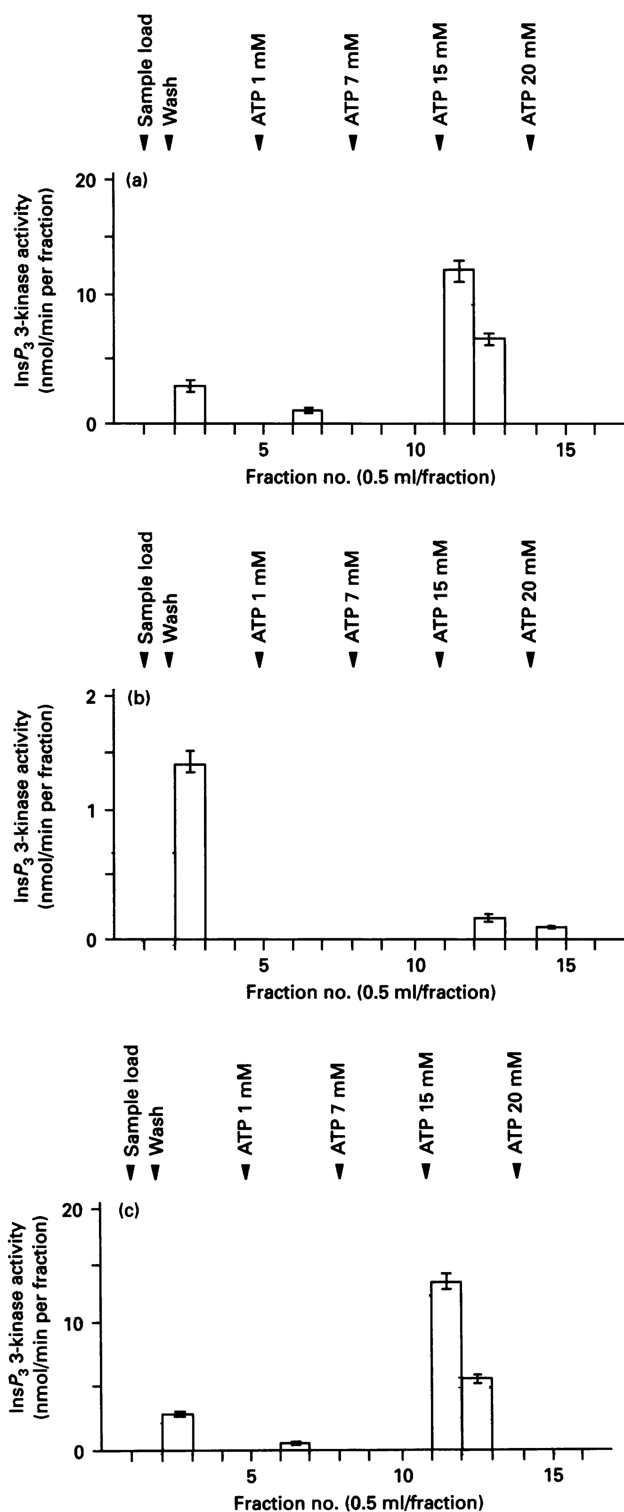

Figure 4 ATP–agarose chromatography of expressed wild-type C5 and mutants C5 K197I and C5 K207I

A crude lysate of each expressed protein (500 μl) was applied to an ATP–agarose column (500 μl) and elution was performed in buffer containing increasing ATP concentrations (0–20 mM). Fractions were assayed at 10 μM InsP_3 in the presence of 10 μM free Ca^{2+} and 0.1 μM CaM. (**a**) Elution profile of C5: protein applied, 675 μg; activity applied, 24.5 nmol/min; activity recovery in wash fractions, 15%; activity recovery in 15 mM ATP eluate, 70%. (**b**) Elution profile of C5 K197I: protein applied, 675 μg; activity applied, 2.0 nmol/min; activity recovery in wash fractions, 75%; activity recovery in 15 mM ATP eluate, 15%. (**c**) Elution profile of C5 K207I: protein applied, 675 μg; activity applied, 24.0 nmol/min; activity recovery in wash fractions, 15%; activity recovery in 15 mM ATP eluate, 80%. The elution profiles shown are from one representative experiment out of three.

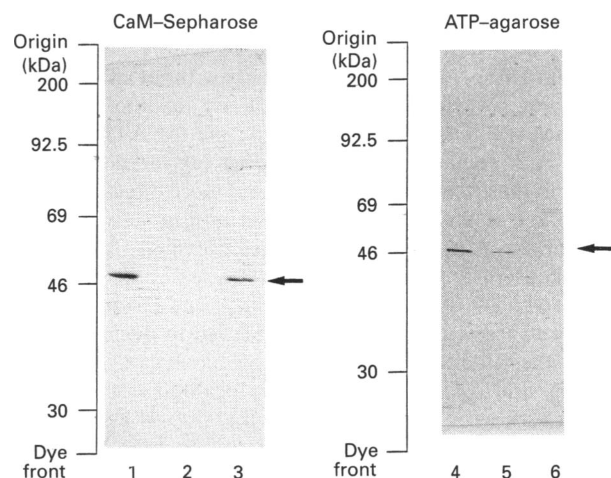

Figure 5 Western blot of expressed mutant C5 D414N after CaM–Sepharose and ATP–agarose chromatography

A crude bacterial lysate of expressed clone C5 D414N (8 μl; lanes 1 and 4) was applied to SDS/10%-PAGE. Lanes 2 and 5 contain 50 μl of the wash eluate from a CaM–Sepharose affinity column and from an ATP–agarose affinity column respectively; lane 3, 50 μl of the EGTA eluate from the CaM–Sepharose column; lane 6, 50 μl of the 15 mM ATP eluate from the ATP–agarose column. The arrows show the immunoreactive 48 kDa band.

band was observed only in the flow-through wash and not in the 15 mM ATP eluate (Figure 5, lane 6).

DISCUSSION

Molecular cloning and expression of two isoenzymes of InsP_3 3-kinase has provided the means for identifying residues that are essential for enzymic activity and binding of Ca^{2+}/CaM, as well as allowing structural comparisons with other kinases and CaM-sensitive enzymes. Previous characterization of deletion mutants of the rat brain enzyme showed that residues His-164–Leu-174 constituted the main CaM-binding site of InsP_3 3-kinase A and that the catalytic domain was located at the C-terminus (Takazawa and Erneux, 1991). Two consensus motifs are well conserved among the catalytic domains of many ATP/Mg^{2+}-binding enzymes (Walker et al., 1982; Hanks et al., 1988). The first consists of the Gly-Xaa-Gly-Xaa-Xaa-Gly-Xaa_{15}-Lys ATP/Mg^{2+}-binding sequence (where Xaa is any amino acid), where the invariant lysine appears to be directly involved in the binding of the phosphate groups of ATP during the phosphotransfer reaction (Kamps and Sefton, 1986). The second region consists of Asp-Phe-Gly, where the key residue, aspartate, is suspected to bind Mg^{2+} ions complexed with ATP and so might participate in enzymic catalysis (Brenner, 1987). This motif Asp-Phe-Gly is present and conserved in the catalytic domain of isoenzymes A (from rat and human brain) and isoenzyme B (from human brain). However, the first motif is absent from the catalytic domain of InsP_3 3-kinase and from the catalytic domain of hexokinases (Griffin et al., 1991). Nevertheless, the sequence of amino acids between Gly-191 and Lys-207 shows potential similarities with this motif. Furthermore, the region between Gln-188 and Ala-199 is conserved in both InsP_3 3-kinase isoenzymes. Three mutants were prepared (C5 K197I, C5 K207I and C5 D414N), with which we aimed to assay bacterial lysates for enzymic activity and InsP_3 binding and to directly characterize the ATP/Mg^{2+}-binding domain by ATP-agarose affinity chromatography.

The results of the present study clearly demonstrate that Lys-197 and Asp-414 of InsP_3 3-kinase are critical residues for

ATP/Mg^{2+} binding. The evidence in support of this hypothesis includes the following. (1) Mutation of Lys-197 to isoleucine resulted in a 90 % loss of enzymic activity without affecting either the binding of InsP_3 or the sensitivity of the enzyme to Ca^{2+}/CaM. (2) The affinity of the enzyme for ATP/Mg^{2+} was affected as a result of the mutation, as suggested by a 5-fold increase in the apparent K_m for ATP and more directly shown by the inability of the mutant to interact and be adsorbed on to ATP–agarose. A similar result was also obtained after mutation of the conserved lysine residue in the catalytic domain of thymidine kinase of Vaccinia virus (Black and Hruby, 1990). (3) Direct measurement of InsP_3 binding failed to demonstrate any effect of the mutation on InsP_3 binding activity, suggesting that the InsP_3- and ATP-binding sites are localized in two separate domains within the protein. Evidence that Asp-414 is involved in ATP/Mg^{2+} binding is provided by the observation that mutant C5 D414N was totally inactive, even in the presence of very high concentrations of Mg^{2+} (up to 80 mM; results not shown). C5 D414N was nevertheless expressed, as shown by immunoblotting and in the InsP_3 binding assay. The mutation does not modify either Ca^{2+}/CaM binding or InsP_3 binding activities. It does, however, prevent the protein from binding strongly to an ATP–agarose affinity column, suggesting that the residue is involved in ATP/Mg^{2+} binding. Our data also indicate an additional effect of the mutation of Lys-197 on the apparent $V_{max.}$ of the enzyme (assuming that the expression did not vary between wild-type and mutant C5 K197I, as shown in Figure 1). This was shown at saturating ATP and using variable InsP_3 concentrations.

Using the method of Chou and Fasman (1978) for secondary structure predictions for InsP_3 3-kinase A, the region Gly-191–Ala-199 is comprised of a β-sheet–α-helix interface (results not shown) which is susceptible to forming a flexible loop structure in which the positive charge of Lys-197 might interact with the negatively charged phosphoryl groups of ATP, as suggested for pig adenylate kinase (Fry et al., 1986). Furthermore, structure predictions could be compared with *E. coli* adenylate kinase, in which Asp-84, which is involved in ATP/Mg^{2+} binding, lies at the end of a β-sheet followed by a β-turn (Rose et al., 1991), or with the cyclic AMP protein kinase-α catalytic domain, in which Asp-184 was shown to be involved in ATP/Mg^{2+} binding (Brenner, 1987). In contrast, Lys-207 did not seem to play any role in the enzymic activity of InsP_3 3-kinase or in its regulation by Ca^{2+}/CaM. Altogether, our data suggest that residues Lys-197 and Asp-414 are necessary for InsP_3 3-kinase activity and form part of the ATP/Mg^{2+}-binding domain located in the 275-amino-acid-long catalytic domain of isoenzyme A.

Sim et al. (1990) identified several potential phosphorylation sites for protein kinase C in the amino acid sequence of InsP_3 3-kinase A and showed that *in vitro* phosphorylation of purified native InsP_3 3-kinase A (from rat brain) by protein kinase C induced a dramatic loss of enzymic activity. One of the phosphorylation sites was Ser-185, which is located just at the beginning of the catalytic domain, suggesting that the introduction of a negative charge after phosphorylation of this serine by protein kinase C might directly affect the ATP/Mg^{2+}-binding domain, including Lys-197, and would therefore influence enzyme activity.

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