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Identification of multifunctional ATP-citrate lyase kinase as the α-isoform of glycogen synthase kinase-3

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Multifunctional ATP-citrate lyase kinase (ACLK) exhibits several properties that are similar to glycogen-synthase kinase-3 (GSK-3). The molecular cloning of two distinct mammalian GSK-3 cDNAs and a *Drosophila melanogaster* (fruitfly) homologue, *zeste-white3*^{sgg}, has established the existence of a GSK-3 subfamily. A multifunctional protein kinase first identified as an ACLK has recently been shown to exhibit several similarities to the α- and β-forms of GSK-3. Here we have used immunological and biochemical analyses to directly compare these enzymes. Thus purified preparations of ACLK isolated from brain and liver preferentially cross-react with anti-GSK-3α antisera and phosphorylate previously defined substrates of GSK-3 at identical sites. Conversely, both α- and β-forms of GSK-3 phosphorylated ATP-citrate lyase at the same site(s) targeted by ACLK. These, and other similarities, demonstrate ACLK to be identical with, or highly related to, GSK-3α, the implications of which are discussed.

INTRODUCTION

One of the rapid intracellular effects of insulin is the stimulation of phosphorylation and dephosphorylation of certain proteins [1–3]. The former category includes ribosomal protein S6, and two key proteins involved fatty acid metabolism, namely acetyl-CoA carboxylase and ATP-citrate lyase [ATP citrate (*pro*-3*S*)-lyase, EC 4.1.3.8] [4–8]. Insulin treatment of rat adipocyte tissue increases phosphorylation of one ATP-citrate lyase peptide (termed peptide A) while decreasing phosphorylation of a second peptide (peptide B), with dephosphorylation of the latter apparently stimulating phosphorylation of the former [9]. A protein kinase that specifically phosphorylates peptide B, termed ATP-citrate lyase kinase (ACLK) and subsequently renamed multifunctional protein kinase (MFPK) to emphasize both its spectrum of substrates and its F_A activity, has been purified from rat liver and brain ([10,11]; S. Ramakrishna and W. B. Benjamin, unpublished work). *In vitro*, ACLK also phosphorylates acetyl-CoA carboxylase, and glycogen synthase [12,13]. The activity of ACLK has been demonstrated to decrease in response to insulin, which may underlie the effect of the hormone on ATP-citrate lyase [14].

Recently, the residues in peptide B phosphorylated by ACLK have been identified [15]. The sequence of this peptide is reminiscent of a peptide from glycogen synthase that, like peptide B, is also dephosphorylated in response to insulin (albeit in skeletal muscle) [16]. The glycogen synthase peptide is specifically phosphorylated by a protein-serine kinase termed glycogen-synthase kinase-3 (GSK-3) [17–20]. The molecular cloning of cDNAs for this enzyme revealed the existence of two closely related polypeptides termed GSK-3α and GSK-3β [21]. These enzymes are both highly homologous to a *Drosophila melanogaster* (fruitfly) homoeotic gene termed *zeste-white3*^{sgg} that is required during embryogenesis [22–24]. Purification of these kinases in baculovirus-infected insect cells and from tissue has enabled comparison of their properties and specificities and the generation of immunological reagents ([25]; K. Hughes, J. R. Woodgett, M. Bourouis & P. Simpson, unpublished work).

In view of the similarities between ACLK and the two forms of GSK-3 and their potential importance in insulin action, we have directly compared these enzymes using immunological and biochemical reagents specific to each protein kinase. These data demonstrate that ACLK is part of the GSK-3 subfamily.

METHODS

Purification of ATP-citrate lyase and ACLKs

ATP-citrate lyase was purified as described in [10]. A more complete description of the purification procedures for both liver and brain ACLK used in this study follows. Rat liver (3.5 kg) was homogenized in 3 vol. of 20 mM-Tes/KOH (pH 7.5)/5 mM-potassium phosphate/5 mM-EDTA/5 mM-EGTA/0.5 mM-phenylmethanesulphonyl fluoride (PMSF)/1 mM-dithiothreitol (DTT)/aprotinin (1 μg/ml)/leupeptin (1 μg/ml)/100 mM-KCl (buffer A). Solid poly(ethylene glycol) was added to 6% (w/v) and, after centrifugation, further poly(ethylene glycol) was added to the supernatant (to 13%, w/v). After centrifugation the pellet was dissolved in buffer A and applied to a DEAE-Sephadex column. The flow-through and wash fractions were loaded on to a phosphocellulose (P-11) column equilibrated in 50 mM-Tris/HCl (pH 7.5)/2 mM-DTT/0.25 mM-PMSF/5% glycerol (v/v)/proteinase inhibitors (buffer B). ACLK was eluted from the column with a linear gradient of 0.075–0.6 M-KCl in buffer B. The pool of activity, as judged using ATP-citrate lyase as substrate, was diluted with 3 vol. of buffer B and applied to an S-Sepharose Fast Flow column. After elution with 0.3 M-KCl, the material was diluted 3-fold with buffer B and loaded on to a phosvitin–Sepharose column. ACLK was eluted with a KCl gradient; the active pool was diluted 4-fold with buffer B and loaded on to a Blue Sepharose column. After washing with buffer B containing 0.03 M-KCl, ACLK was eluted with a 0.03–0.4 M-KCl gradient in buffer B. After concentration, the activity was subjected to gel filtration on Sepharose S-200 in buffer B containing 0.15 M-NaCl and 0.1% Nonidet P40. The active fractions were diluted 3-fold with buffer B and re-applied to the Blue Sepharose column and eluted with a KCl gradient. After

Abbreviations used: ACLK, ATP-citrate lyase kinase; GSK-3, glycogen synthase kinase-3; MFPK, multifunctional protein kinase; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol.

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Table 1. Purification of ACLK from rat liver (*a*) and bovine brain (*b*)

Species and organ	Step	Total activity (nmol/min)	Protein (mg)	Activity (nmol/min per mg)	Yield (%)	Purification (fold)
(*a*) Rat liver	1. Homogenate	6341	576450	0.011	100	1
	2. DEAE-Sepharose	6024	120480	0.06	95	5
	3. P-11	4704	2345	2.0	74	182
	4. Fast S-Sepharose	2346	480	4.9	37	444
	5. Phosvitin–Sepharose	1884	200	9.4	30	856
	6. Blue Sepharose	713	5832	12.2	11	1111
	7. S-200	452	10.25	44.1	7	4010
	8. Blue Sepharose	266	1.8	148.5	4	13500
	9. CM-300	218	0.95	229	3	20800
	10. Mono S	119	0.36	330	2	29900
(*b*) Bovine brain	1. DEAE-Sepharose	10800	360000	0.03	100	1
	2. P-11	8700	6000	1.45	81	48
	4. Fast S-Sepharose	6920	1250	5.54	64	184
	5. Heparin–Sepharose	2820	105	26.9	26	895
	6. Phenyl-Sepharose	1176	15.5	76	11	2529
	7. Mono Q	1008	9.5	106	9	3530
	8. CM-300	798	3.25	245	7	8185
	9. Superose 12	414	1.05	394	4	13130
	10. Mono S	244	0.42	574	2	19130

concentration the ACLK activity was applied to a CM-300 Synchropak column connected to a Beckman Gold h.p.l.c. system. Protein kinase activity was eluted with a 0.05–0.4 M-NaCl gradient in buffer B containing 0.1 % Nonidet P40. Portions of this material were further chromatographed on a Mono S f.p.l.c. column (HR5/5) and eluted with a 0.03–0.3 M-NaCl gradient. Aliquots of the eluted fractions were subjected to SDS/PAGE and stained with silver. Prominent proteins migrating between 45 and 55 kDa correlated with ACLK activity (results not shown). A summary of the purification is presented in Table 1(*a*). ACLK activity was unstable after Mono S chromatography.

ACLK was also purified from bovine brain (4 kg). The purification procedure was similar to that described for the liver preparation through to chromatography on S200-Sepharose. The eluent from this column was diluted 4-fold with buffer B and mixed with heparin–Sepharose. After packing the material into a column and washing with buffer B, ACLK was eluted with a 0.05–0.4 M-KCl gradient in buffer B. KCl (1.5 M) was added to yield a final concentration of 1 M. The protein was applied to a phenyl-Sepharose column and eluted with a 1.0–0 M-KCl gradient. After concentration, the pool of ACLK activity was concentrated, dialysed against buffer B and applied to a Mono Q column. The unbound fraction was loaded on to a CM-Synchropak 300 column. From this step, 0.1 % Nonidet P40 was added to all buffers. The ACLK was eluted from the column with a 0.05–0.4 M-KCl gradient. After concentration, the kinase active fractions were applied to a Superose 12 HR10/30 column equilibrated in buffer B containing 0.2 M-KCl. Active fractions were diluted 4-fold with buffer B and applied to a Mono Q HR 5/5 column in buffer B containing 0.03 M-NaCl. The kinase was eluted by a linear increase in KCl to 0.3 M. The final preparation represented a purification of ~ 20000-fold (Table 1*b*), and exhibited two prominent bands of 52 and 46 kDa after SDS/PAGE (Fig. 1). The preparation became unstable after the final step, and most of the experiments performed here used material purified up to and including the Superose chromatography.

Purification of GSK-3 isoforms

GSK-3 was purified from bovine brain or rabbit skeletal muscle as described in [21]. The former source yields 1:1 proportions of the α- and β-forms and this is indicated in the text below as 'GSK-3$\alpha+\beta$'. For analysis of the isolated isoforms, each was expressed in insect cells using recombinant baculovirus and purified to homogeneity essentially as described for GSK-3β [25].

Assay of protein kinase activities

All kinase reactions were performed in 20 mM-sodium glycerophosphate (pH 7.0)/1 mM-DTT/0.2 mM-EGTA/heparin (10 μg/ml)/WIPtide (10 μg/ml) (Sigma) to inhibit any trace Ca^{2+}-dependent protein kinase, casein kinase-II and cyclic AMP-dependent protein kinase. Reactions were carried out at 30 °C on the basis of those described for glycogen synthase phosphorylation by Woodgett & Cohen [20]. Reactions were performed with excess substrate, giving initial-rate conditions, and terminated with an equal volume of protein sample buffer. Proteins were resolved by SDS/PAGE, and phosphorylation of proteins was detected by autoradiography.

Immunological methods

Polyclonal rabbit antisera were raised against bacterially expressed TrpE fusion proteins containing the *C*-terminal region of rat GSK-3α (181 amino acids) and GSK-3β (231 amino acids) as described in [21]. Rabbit antibodies were also generated against a 12-residue synthetic peptide based on the *C*-terminal sequence of rat GSK-3α:

Ala-Pro-Asp-Ala-Thr-Pro-Thr-Leu-Thr-Asn-Ser-Ser

Immunoglobulins were purified using a DEAE-Affigel Blue column (Pharmacia) before use. Protein samples were resolved by SDS/PAGE and electroblotted on to PVDF membrane using standard procedures [26]. After incubation with 200-fold diluted antiserum, the immunoreactive proteins were revealed by using enhanced chemiluminescence (ECL; Amersham International).

Partial phosphopeptide analysis of phosphorylated ATP citrate lyase

Partial proteolytic mapping using *Staphylococcus aureus* V8 proteinase was performed using the method of Cleveland *et al.* [27]. Briefly, 20 μl-volume kinase reactions were performed with

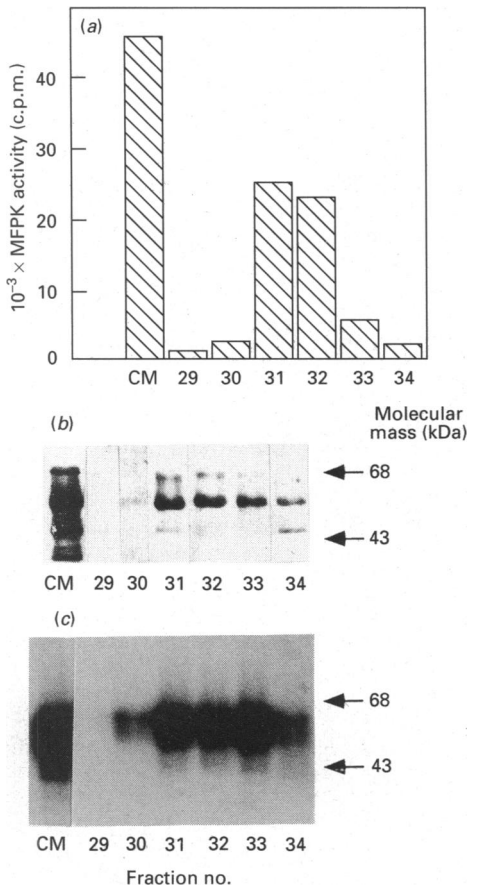

Fig. 1. Mono S chromatography of bovine brain ACLK

(*a*) The activity eluted from the Synchropak CM-300 column was applied to a Mono S HR5/5 f.p.l.c. column as described in the Methods section. Activity towards ATP-citrate lyase is shown in the eluted fractions. (*b*) Fractions corresponding to those in (*a*) were subjected to SDS/PAGE and the gel stained with silver to reveal proteins. (*c*) Autophosphorylation of ACLK.

purified GSK-3α, GSK-3β, liver ACLK or brain ACLK in the presence of ATP-citrate lyase (100 μg/ml). Reactions were allowed to proceed for 10 min at 30 °C before termination by addition of an equal volume of SDS sample buffer. After SDS/PAGE the gel was dried on to filter paper without fixation. The ATP-citrate lyase bands were excised after autoradiography and rehydrated for 2 min in 100 μl of 125 mM-Tris (pH 6.8)/1 mM-EDTA/0.1 % SDS/1 mM-2-mercaptoethanol/30 % (v/v) glycerol/0.001 % Bromophenol Blue. The gel pieces were then inserted into sample wells of a second SDS/polyacrylamide gel and treated with 50 ng of V8 proteinase in the rehydration buffer containing 10 % (v/v) glycerol. The gel was electrophoresed at 20 mA until the dye front was 5 mm above the resolving gel. The power was interrupted for 30 min to allow proteolytic digestion before resumption of electrophoresis and processing for autoradiography.

RESULTS

The substrate specificity of ACLK from bovine brain and rat liver were compared with GSK-3α and GSK-3β purified from insect cells infected with recombinant baculovirus harbouring a rat GSK-3α or GSK-3β cDNA respectively [25]. As expected, the ACLK preparations phosphorylated acetyl-CoA carboxylase and ATP-citrate lyase (Table 2). These substrates were also efficiently phosphorylated by both forms of GSK-3. ACLK also phosphorylated several proteins previously characterized as GSK-3 substrates, including glycogen synthase, inhibitor-2, myelin basic protein and microtubule-associated protein-2 (Table 2) [28,29]. Bacterially expressed proto-oncoprotein c-Jun was also a substrate for both forms of ACLK. The sites targeted on this transcription factor were identical with those phosphorylated by GSK-3α and β (Fig. 2). Phosphorylation occurred at *C*-terminal sites Thr-239, Ser-243 and Ser-249, an event which inhibits the binding of the c-Jun protein to the AP1 target promoter in DNA [28]. ACLK also phosphorylates the c-Myc and c-Myb proto-oncoproteins, a specificity characteristic of GSK-3 (results not shown).

Relative rates of phosphorylation of substrate proteins were similar among the different kinases, although both liver and brain ACLK preparations exhibited kinetic parameters most similar to those of GSK-3α purified from rabbit skeletal muscle. ACLK phosphorylated glycogen synthase with a K_m of 0.16 mg/ml (using 50 μM-ATP) (Table 3). This is similar to that of purified GSK-3α (0.3 mg/ml), but somewhat lower than for GSK-3β (0.6 ml/ml). The K_m for ATP (using 1.0 mg of glycogen synthase/ml) was 25 μM, identical with that measured for GSK-3α. The GSK-3 enzymes display the unusual property of using both ATP and GTP as phospho donors [19]. This property was shared by both ACLK preparations. The ACLK preparations yielded a K_m for GTP (using 1.0 mg of glycogen synthase/ml) of 400 μM, with $V_{max.}$ being half that of ATP, again identical with

Table 2. Substrate specificity of brain (B-) and liver (L-) ACLK

ACLK displayed kinase activity against all tested substrates of GSK-3. Initial rates were determined, due to substrate availability and suitability for kinetic comparison. Assays were performed simultaneously with identical substrate concentrations. All data are normalized for activity against glycogen synthase. Abbreviation: MAP2, microtubule-associated protein 2.

Substrate	Final concn. (μg/ml)	Relative rate of phosphorylation by: GSK-3α	GSK-3β	B-ACLK	L-ACLK
Glycogen synthase	1000	100	100	100	100
ATP-citrate lyase	100	21	25	24	27
Myelin basic protein	200	25	88	33	42
Acetyl-CoA carboxylase	250	5	12	6	6
Casein	1000	< 1	8	< 1	2
Histone H1	1000	2	6	3	4
Inhibitor-2	120	6	22	6	7
MAP2	1000	50	65	44	57

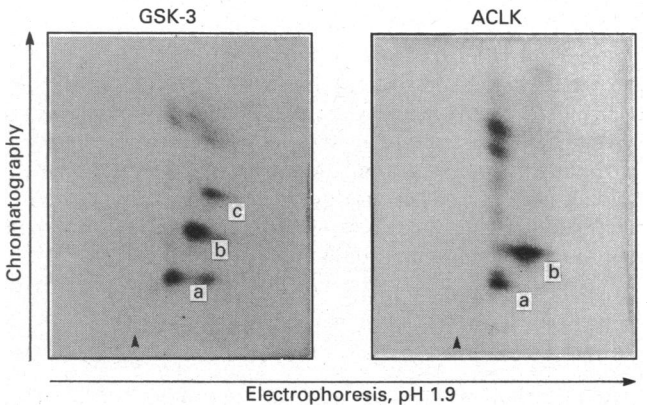

Fig. 2. Phosphopeptide mapping of cellular proto-oncoprotein c-Jun after phosphorylation by GSK-3 and ACLK

Bacterially expressed c-Jun (40 kDa) was a substrate for all forms of GSK-3 and ACLK. Radiolabelled spots a, b and c are identified as tri-, di- and mono-phosphorylated forms respectively of the same tryptic phosphopeptide. The absence of spot c in the ACLK map reflects a slightly higher stoichiometry of phosphorylation than by GSK-3 in the experiment shown. Spot c is often absent in corresponding '*in vitro*' GSK-3 maps [25]. Tryptic peptide abc contains the *C*-terminal phosphorylation sites Thr-239, Ser-243 and Ser-249, phosphorylation of which inhibits the binding of c-Jun to its DNA promoter. Co-electrophoresis and mapping experiments, where equal amounts of radioactivity from GSK-3- and ACLK-phosphorylated c-Jun were pooled, gave no additional phosphopeptides, showing that phosphorylation sites a, b and c are common to both ACLK and GSK-3 (results not shown).

Table 3. Enzyme kinetics of ACLK/GSK-3

Phosphorylation reactions were performed as described in the Methods section. Michaelis–Menton substrate-affinity constants (K_m) were calculated by Lineweaver–Burk analysis. K_m values for glycogen synthase and ATP-citrate lyase were determined in the presence of 50 μM-ATP. ATP and GTP K_m values were calculated in the presence of 1.0 mg of glycogen synthase/ml.

Substrate	GSK-3α	GSK-3β	Brain ACLK	Liver ACLK
Glycogen synthase*	0.3	0.6	0.16	0.16
ATP-citrate lyase*	0.4	0.35	0.35	0.38
ATP†	25	55	25	25
GTP†	400	95	400	400

* K_m values expressed in mg/ml.
† K_m values expressed as μM.

the behaviour of GSK-3α. GSK-3β displays a higher affinity for GTP (K_m = 95 μM [25]).

GSK-3α and GSK-3β phosphorylated ATP-citrate lyase with a similar relative rate to that of the ACLKs. The K_m for ATP-citrate lyase was 350–400 μg/ml for all GSK-3 and ACLK preparations (50 μM-ATP). Partial V8-proteinase phosphopeptide mapping of ATP-citrate lyase phosphorylated by ACLK or either of the GSK-3s revealed qualitatively indistinguishable patterns, indicating an identical site-specificity for these kinases against this substrate (Fig. 3).

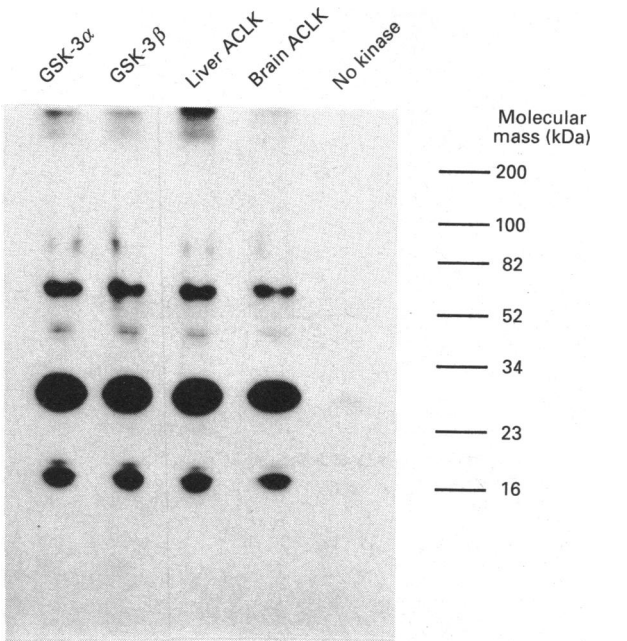

Fig. 3. One-dimensional phosphopeptide mapping of ATP-citrate lyase

ATP-citrate lyase was phosphorylated by the protein kinases as described in the text, excised from a gel, subjected to partial proteolysis with V8 proteinase and the fragments resolved on a second polyacrylamide gel.

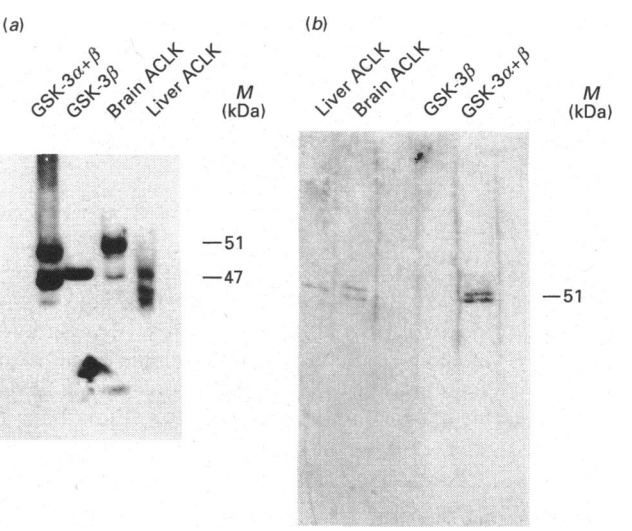

Fig. 4. Immunoblotting of ACLK with polyclonal antisera directed against GSK-3α and GSK-3β

Purified preparations of ATP-citrate lyase kinase from brain or liver, purified GSK-3$\alpha+\beta$ from bovine brain and baculovirus-expressed GSK-3β were immunoblotted in (*a*) with a mixture of polyclonal antibodies raised against TrpE fusion proteins containing either rat GSK-3α or -β sequences as described in [21] or, in (*b*), with anti-peptide antibodies raised against a peptide derived from the *C*-terminal 12 residues of rat GSK-3α. Detection was by enhanced chemiluminescence. Abbreviation: *M*, molecular mass.

Immunoblotting of ACLK

To investigate the relatedness of the protein kinase preparations at the protein level, the GSK-3 and ACLK enzymes were subjected to immunoblotting with antisera raised to bacterial fusion proteins containing the *C*-terminal 180–240 amino acids of rat GSK-3α or GSK-3β [21]. Both liver and brain ACLKs were detected by these antisera (Fig. 4). It was also noted that antisera directed against the GSK-3α fusion protein were

more avid in their recognition of ACLK preparations (results not shown). The cross-reacting polypeptides in the ACLK preparations exhibit distinct electrophoretic mobilities during electrophoresis. Bovine brain ACLK contains a major immunoreactive band of similar molecular mass to that observed for '*bona fide*' GSK-3α (51 kDa), whereas liver ACLK exhibits several polypeptides between 40 and 45 kDa (Fig. 4*a*), which are probably derived by proteolytic degradation during purification. Rat GSK-3β has a molecular mass of 47 kDa [21,25]. Immunoblotting of the preparations with sera raised to a synthetic peptide modelled on the *C*-terminal 12 amino acids of rat GSK-3α specifically detected proteins of ~ 51 kDa in the GSK-3α+β preparation. GSK-3β was not detected, consistent with the considerable divergence between the α- and β-proteins in the region used for the antigen. However, 50 kDa proteins were detected by this antiserum in both the liver and brain ACLK preparations (Fig. 4*b*), indicating the presence of GSK-3α polypeptides. Antibodies raised against the *C*-terminal 12 amino acids of GSK-3β failed to cross-react with the ACLK preparations (results not shown). However, the titre of these antibodies was severalfold lower than those raised against the GSK-3α peptide, as determined by immunoblotting of the baculovirus-expressed GSK-3β protein.

DISCUSSION

In comparing the enzymic and immunoreactive properties of ACLK from two tissue sources with those of GSK-3α and GSK-3β, we have demonstrated a high degree of functional and structural similarity between these proteins. Although absolute proof of identity requires isolation and sequencing of peptides from purified ACLK, the data demonstrate ACLK to be functionally equivalent to the GSK-3 subclass of protein-serine kinases.

Molecular cloning revealed the presence of two related forms of GSK-3, α and β [21]. Despite several cDNA library screenings and PCR-based strategies, no further sequences related to these two genes have been detected in mammalian species (E. Nikolakaki and J. R. Woodgett, unpublished work). Immunoblotting of tissue extracts with the antisera used here detects only proteins of ~ 51 and 47 kDa [21]. (The higher molecular mass of the brain ACLK preparation may reflect post-translational modification, since *in vitro* the electrophoretic mobility of GSK-3α is affected by autophosphorylation.) Given the level of structural conservation revealed by the immunoblotting, it is unlikely that the ACLK cDNA(s) would have avoided detection by the above-mentioned approaches, supporting our contention that ACLK and GSK-3 polypeptides are likely to be identical. As the two forms of GSK-3 are very difficult to completely separate by chromatography or catalytic properties, it is possible that ACLK preparations used here contain both forms, although we have only directly demonstrated the presence of the α-isoform.

This is the first report that '*bona fide*' GSK-3 can efficiently phosphorylate ATP-citrate lyase and acetyl-CoA carboxylase. The failure of other groups to observe this effect is likely related to the method of preparation of the substrate proteins [19]. Phosphorylation of ATP-citrate lyase by ACLK requires prior phosphorylation of a serine residue in an adjacent peptide (peptide A) [15]. Dephosphorylated ATP-citrate lyase is not a substrate for ACLK. A similar dependency upon prior phosphorylation of a neighbouring residue by a distinct protein kinase has been observed for several GSK-3 substrates, including glycogen synthase, inhibitor-2 and the type II regulatory subunit of cyclic AMP-dependent protein kinase [30–32]. We suggest that the amount of phosphate in peptide A can vary depending on the methods of isolation. Preparations containing low levels of phosphate in this site will not be phosphorylated by GSK-3/ACLK.

The effect of insulin on ATP-citrate lyase is complex, involving stimulation at one site (in peptide A) and dephosphorylation of another (peptide B) [9]. The rate of phosphorylation of these sites is interdependent; as well as phosphorylation of peptide A being required for phosphorylation of peptide B, dephosphorylation of peptide B stimulates phosphorylation of peptide A. Insulin treatment causes a decrease in assayable ACLK activity [14]. It has thus been proposed that insulin inactivation of ACLK results in a decrease in phosphorylation of peptide B, causing a stimulation of phosphopeptide A. In skeletal muscle, administration of insulin causes a specific decrease in the phosphorylation of residues targeted by GSK-3 [3,33]. One proposed mechanism to account for this observation is via insulin-stimulated phosphorylation of the glycogen-binding subunit of protein phosphatase-1, which increases the activity of this phosphatase towards glycogen synthase [34]. Given the reported effects of insulin on ACLK in adipose tissue and the similarity between this protein kinase and GSK-3α, it is tempting to speculate that inhibition of GSK-3 activity also contributes to the effect of insulin on glycogen synthase.

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