

Identification of filamin C as a new physiological substrate of PKB α using KESTREL

James T. MURRAY^{*1}, David G. CAMPBELL^{*}, Mark PEGGIE^{*}, Alfonso MORA^{*} and Philip COHEN^{*†}

^{*}MRC Protein Phosphorylation Unit, School of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, U.K., and [†]Division of Signal Transduction Therapy, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

We detected a protein in rabbit skeletal muscle extracts that was phosphorylated rapidly by PKB α (protein kinase B α), but not by SGK1 (serum- and glucocorticoid-induced kinase 1), and identified it as the cytoskeletal protein FLNc (filamin C). PKB α phosphorylated FLNc at Ser²²¹³ *in vitro*, which lies in an insert not present in the FLNa and FLNb isoforms. Ser²²¹³ became phosphorylated when C2C12 myoblasts were stimulated with insulin or epidermal growth factor, and phosphorylation was prevented by low concentrations of wortmannin, at which it is a relatively specific inhibitor of phosphoinositide 3-kinase. PD 184352 [an inhibitor of the classical MAPK (mitogen-activated protein kinase)

cascade] and/or rapamycin [an inhibitor of mTOR (mammalian target of rapamycin)] had no effect. Insulin also induced the phosphorylation of FLNc at Ser²²¹³ in cardiac muscle *in vivo*, but not in cardiac muscle that does not express PDK1 (3-phosphoinositide-dependent kinase 1), the upstream activator of PKB. These results identify the muscle-specific isoform FLNc as a new physiological substrate for PKB.

Key words: cytoskeleton, filamin, kinase substrate tracking and elucidation (KESTREL), phosphorylation, protein kinase B (PKB).

INTRODUCTION

Signals that activate PI 3-kinase (phosphoinositide 3-kinase) and elevate the intracellular concentration of PtdIns(3,4,5)P₃ trigger the activation of several protein kinases, including PKB (protein kinase B; also called Akt). PtdIns(3,4,5)P₃ binds to the PH (pleckstrin homology) domains of PKB and PDK1 (3-phosphoinositide-dependent protein kinase 1), co-localizing them at the plasma membrane. This allows PDK1 to activate PKB by phosphorylation of Thr³⁰⁸ (reviewed in [1]).

PKB appears to play a central role in mediating many of the actions of signals that elevate PtdIns(3,4,5)P₃, including many of the metabolic actions of insulin and the anti-apoptotic effects of survival factors (reviewed in [2]). However, although a number of physiological substrates for PKB have been identified, many more remain to be discovered. For example, mice that do not express the β -isoform of PKB are unable to recruit the glucose transporter GLUT4 to the plasma membrane or to stimulate glucose uptake in response to insulin [3], but the substrate(s) for PKB β that might mediate this effect are unknown.

PKB isoforms belong to the AGC subfamily of protein kinases, and are most similar to SGK (serum- and glucocorticoid-induced protein kinase) isoforms [4–6], with which they share 55 % identity in the catalytic domain. Based on studies with synthetic peptide substrates, PKB was found to phosphorylate serine and threonine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr- motifs [7]. However, several other protein kinases, including SGK [4,6], RSK (p90 ribosomal S6 kinase) and S6K (p70 S6 ribosomal kinase) isoforms [8], have similar substrate specificities *in vitro*, making it difficult to predict substrates for PKB simply by searching protein sequence databases with this motif.

In order to try and identify new physiological substrates for PKB, we therefore decided to adopt the KESTREL (kinase substrate tracking and elucidation) approach [9]. In this method, cell extracts are subjected to ion exchange chromatography, and aliquots of the fractions collected are incubated with MgATP in the absence or presence of closely related protein kinases with similar specificity requirements. The aim is to detect proteins that are phosphorylated rather selectively by just one of these kinases and then investigate whether such proteins are *bona fide* physiological substrates in appropriate follow-up studies. Using this approach, we have identified elongation factor 2-kinase as a protein that is phosphorylated at Ser³⁵⁹ and inactivated by SAPK4 (stress-activated protein kinase 4; also called p38 δ), but not by the closely related isoforms SAPK2a/p38 α or SAPK3/p38 γ [9].

In the preceding paper [10], we used KESTREL to identify NDRG2 (n-myc downstream-regulated gene 2) as a protein that is phosphorylated much more rapidly by SGK1 than by PKB α . Here we identify the muscle-specific filamin isoform FLNc (filamin C) as a protein that is phosphorylated efficiently by PKB α , but not by SGK1. We go on to show that FLNc is indeed a new physiological substrate for PKB.

MATERIALS AND METHODS

Materials

Human insulin was purchased from Roche Molecular Biochemicals (Lewes, E. Sussex, U.K.), EGF (epidermal growth factor) and precast Tris/acetate SDS/3–8%-polyacrylamide gels from Invitrogen (Paisley, Scotland, U.K.), and wortmannin and rapamycin from Merck Biosciences (Nottingham, U.K.). PD 184352

Abbreviations used: EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FLNa, FLNb and FLNc, filamin isoforms A, B and C respectively; GST, glutathione S-transferase; KESTREL, kinase substrate tracking and elucidation; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; mTOR, mammalian target of rapamycin; NDRG, n-myc downstream-regulated gene; PI 3-kinase, phosphoinositide 3-kinase; PDK1, 3-phosphoinositide-dependent kinase 1; PH, pleckstrin homology; PKB, protein kinase B; RSK, p90 ribosomal S6 kinase; S6K, p70 ribosomal S6 kinase; SAPK, stress-activated protein kinase; SGK1, serum and glucocorticoid-induced kinase 1; SHIP, Src homology 2-interacting protein.

¹ To whom correspondence should be addressed (email j.t.c.murray@dundee.ac.uk).

[11] was obtained by chemical synthesis, while peptides were synthesized by Dr Graham Bloomberg (University of Bristol, Bristol, U.K.).

Antibodies

Antibodies that recognize PKB phosphorylated on Ser⁴⁷³ were raised in sheep. Antibodies that recognize PKB phosphorylated on Thr³⁰⁸ and those that recognize ERK1 (extracellular-signal-regulated kinase 1) and ERK2 phosphorylated at their pThr-Glu-pTyr motifs (where pThr and pTyr are phosphothreonine and phosphotyrosine respectively) were purchased from Cell Signalling (Hitchin, Herts., U.K.). Antibodies that recognize ribosomal protein S6 phosphorylated at Ser²³⁵ were obtained from Upstate (Milton Keynes, U.K.). Antibodies against human FLNc were raised in a rabbit against the peptide SKTRGGETKREVRVEEST, corresponding to residues 2160–2177. A phospho-specific antibody that recognizes FLNc phosphorylated at Ser²²¹³ was raised in sheep against the phosphopeptide GRERLGpSFGSITR (where pS is phosphoserine), corresponding to residues 2207–2219 of human FLNc. Each peptide was coupled separately to keyhole limpet haemocyanin and BSA, and the conjugates were mixed before injection at Diagnosticon Scotland (Edinburgh, U.K.). Both antisera were affinity purified on CH-Sepharose to which the relevant peptide antigen had been coupled covalently. The phospho-specific antibody that recognizes Ser²²¹³ was used for immunoblotting in the presence of the unphosphorylated peptide antigen (10 µg/ml) to neutralize any antibodies that recognized unphosphorylated FLNc.

Cloning and expression of GST (glutathione S-transferase)–FLNc-(1915–2446)

The DNA coding for amino acids 1915–2446 of human FLNc (XP_045856) was amplified with the Expand HiFidelity PCR System (Roche) from IMAGE EST 4121595 using oligonucleotides MP339 (GAATTCAAGCATCCCGGGAGCCC) and MP340 (GCGGCCGCTTACGGATGGTGTGCTTGCTACTGTCC). This produced a fragment containing 5' *Eco*RI and 3' *Not*I restriction enzyme sites. The PCR product was cloned into pCR2.1 (Invitrogen), sequenced and subcloned into pGEX6P-1.

GST–FLNc-(1915–2446) was expressed in *Escherichia coli* BL21 pLysS (Merck Biosciences) and purified on glutathione-Sepharose. The purified protein was dialysed against 50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 0.1 % (v/v) 2-mercaptoethanol, 0.2 mM PMSF and 1 mM benzamidine, snap frozen in liquid nitrogen and stored at –80 °C.

Preparation and chromatography of human skeletal muscle tissue extracts

Human soleus muscle was minced and homogenized with 5 vol. of 4 mM EDTA, 4 mM EGTA, 1 mM benzamidine, 0.2 mM PMSF and 0.1 % (v/v) 2-mercaptoethanol. The suspension was centrifuged at 2500 g for 45 min at 4 °C, the floating fat pellet was removed and the supernatant was decanted through glass wool. The extract was centrifuged at 25 000 g for 20 min at 4 °C, then the supernatant was passed through Sephadex G-25 equilibrated in 30 mM Mops, pH 7.0, 5 % (v/v) glycerol, 0.1 % (v/v) 2-mercaptoethanol and 0.03 % (w/v) Brij 35 (buffer A), and this material (750 mg of protein) was chromatographed on a 25 ml column of heparin–HP-Sepharose. The column was washed with 125 ml of buffer A and the column developed with a 500 ml non-linear gradient from 0 to 1 M NaCl in buffer A. Fractions of 12.5 ml were collected at a flow rate of 2 ml/min. Aliquots of each fraction were diluted 5-fold into 30 mM Tris/HCl, pH 7.5, 2 mM

MgCl₂, 10 mM 2-mercaptoethanol, 0.1 mM EGTA, 1.0 µg/ml aprotinin and 1.0 µg/ml leupeptin. A 25 µl aliquot was then incubated for 4 min at 30 °C with 5 µl of 20 nM [γ -³²P]ATP (2.5 × 10⁶ c.p.m.) with or without PKB α or SGK1 (0.3 unit/ml). The reactions were stopped by the addition of 10 µl of 320 mM Tris/HCl, pH 6.8, 8 % (w/v) SDS, 20 mM EDTA, 32 % (v/v) glycerol, 1.14 M 2-mercaptoethanol and 0.02 % (w/v) Bromophenol Blue (SDS sample buffer), heated for 3 min at 100 °C, subjected to SDS/PAGE, electroblotted on to ImmobilonP membranes and autoradiographed to reveal phosphorylated proteins.

Assay of protein kinases

These were assayed at 30 °C as described previously [12,13]. One unit of PKB α or SGK1 activity was that amount which catalysed the phosphorylation of 1 nmol of the standard substrate peptide CROSSide (GRPRTSSFAEG) in 1 min [14].

Cell culture, and preparation of cell and mouse tissue extracts

C2C12 myoblasts were cultured on 15 cm-diameter dishes in Dulbecco's modified Eagle's medium supplemented with 2 mM glutamine, 10 % (v/v) foetal bovine serum, penicillin and streptomycin. Cells were lysed in 0.5 ml of lysis buffer, comprising 50 mM Tris/HCl (pH 7.5), 1 mM EGTA, 1 mM EDTA, 1 % (w/v) Triton-X 100, 1 mM sodium orthovanadate, 50 mM NaF, 5 mM sodium pyrophosphate, 0.27 M sucrose, 1 µM microcystin-LR, 0.1 % (v/v) 2-mercaptoethanol and 'complete' proteinase inhibitor cocktail. Mice were killed and cardiac muscle was removed, snap frozen in liquid nitrogen, powdered and extracted with the lysis buffer described above, then centrifuged at 16 000 g for 15 min at 4 °C to pellet insoluble material. Protein concentrations were determined by the Bradford method using BSA as standard. Lysates were denatured in lithium dodecyl sulphate and heated for 10 min at 70 °C before being subjected to SDS/PAGE.

RESULTS

Identification of a high-molecular-mass protein phosphorylated by PKB α in skeletal muscle extracts

Desalted human skeletal muscle extracts were chromatographed on heparin-Sepharose and the fractions collected were phosphorylated with PKB α or SGK1 as described in the Materials and methods section. A protein was identified of apparent molecular mass > 250 kDa, eluting at 0.15 M NaCl, that was phosphorylated by PKB α , but more weakly by SGK1 (Figure 1), and therefore merited further investigation.

The peak fraction from heparin-Sepharose contained many proteins, but only one co-migrated on a 3–8 % gradient gel with the unusually high molecular mass of the ³²P-labelled band (Figure 2A). This band was excised from the gel, and tryptic mass fingerprinting revealed that it was FLNc, the muscle-specific isoform of filamin (Table 1), suggesting that this protein might be the PKB substrate.

Identification of the residue in FLNc phosphorylated by PKB

To investigate whether the PKB substrate and FLNc were the same protein, the partially purified material was phosphorylated as in Figure 2(A), and the ³²P-labelled band was digested with trypsin and chromatographed on a C₁₈ column. One major peptide was detected (Figure 2B, left panel), whose molecular mass corresponded to that of a peptide comprising residues 2205–2217 of

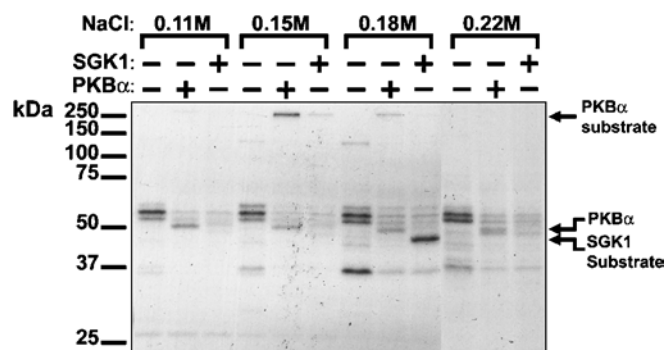


Figure 1 Detection of a >250 kDa PKB α substrate in human skeletal muscle

A skeletal muscle extract (750 mg of protein) was chromatographed on a heparin–Sephacrose column, and aliquots of each fraction were incubated with 2 mM MgCl₂ and 20 nM [γ -³²P]ATP in the absence or presence of 0.3 unit/ml PKB α or SGK1. After SDS/PAGE and autoradiography, a >250 kDa protein, eluting at 0.15 M NaCl, was detected that was phosphorylated by PKB α , but only weakly by SGK1. The position of PKB α , which phosphorylates itself, is indicated. Autophosphorylation of SGK1 is negligible under the conditions used, but the fraction eluting at 0.18 M NaCl contains a protein that is phosphorylated by SGK1, but not by PKB.

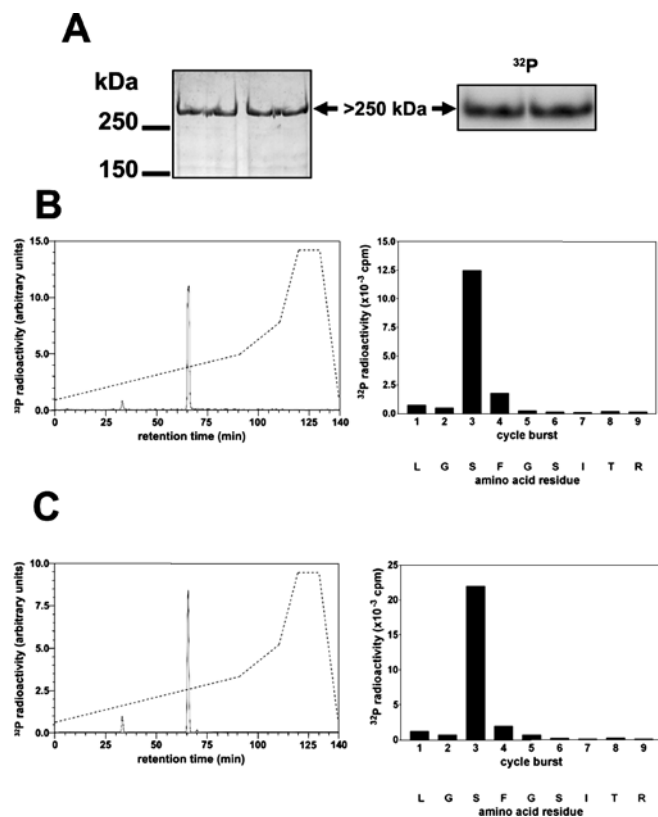


Figure 2 Identification of the residue in FLNc phosphorylated by SGK1 *in vitro*

The partially purified PKB α substrate from Figure 1 was phosphorylated by incubation for 30 min with 10 mM MgCl₂/0.1 mM [γ -³²P]ATP (10⁶ c.p.m./nmol) and 1.0 unit/ml PKB α , denatured in lithium dodecyl sulphate and subjected to SDS/PAGE. (A) The gel was stained with colloidal Coomassie Blue (left panel) or autoradiographed (right panel). (B) The >250 kDa ³²P-labelled band from (A) was excised, digested with trypsin and the digest chromatographed on a Vydac C₁₈ column (Separations Group) equilibrated in 0.1% (v/v) trifluoroacetic acid. The column was developed with an acetonitrile gradient (broken line) at a flow rate of 0.8 ml/min, and fractions of 0.4 ml were collected. The major ³²P-labelled peptide P1 (solid line in left panel) was subjected to MS and the sites of phosphorylation were identified by solid-phase sequencing (right panel) after coupling the peptide to a Sequelon-AA membrane [33]. (C) Same as (B), except that GST–FLNc–(1915–2446) was used instead of partially purified human FLNc.

Table 1 Identification of the >250 kDa substrate of PKB α as the muscle-specific isoform of filamin

The ³²P-labelled 250 kDa substrate (see Figure 2A) was excised from the gel, digested with trypsin and analysed on a Perseptive Biosystems Elite STR MALDI-TOF (matrix-assisted laser-desorption–time-of-flight) mass spectrometer with saturated α -cyanocinnamic acid as the matrix, as described in [28]. The mass spectrum was acquired in the reflector mode and was internally mass calibrated. The tryptic peptide ions obtained were scanned against the Swiss-Prot and Genpep databases using the MS-FIT program of Protein Prospector.

Mass		Residue no.		
Submitted	Matched	Start	End	Peptide sequence
977.5	977.5	1519	1526	YADQEVPR
1057.6	1057.5	992	1002	GAGGQGLDVR
1180.6	1180.6	756	766	VNVGEGSHPER
1188.6	1188.6	2609	2620	GPGLSQAFVGQK
1226.8	1226.8	70	80	LIALLEVLSQL
1230.7	1230.7	1147	1157	ATIRPVFDPK
1267.6	1267.6	1268	1278	EVTFETVDAR
1298.7	1298.7	173	183	VPQLPITNFR
1303.7	1303.7	1255	1267	VSGPGVEPHGLR
1346.8	1346.7	350	361	VTVLVAGQNIK
1386.7	1386.7	2544	2556	YGGPQHIVGSPFK
1400.7	1400.6	1616	1627	YGGDEIPYSPFR
1489.8	1489.8	2294	2307	GVAGVPAEFSIWR
1505.8	1505.8	978	991	VAVGQEQAFSVNTR
1518.8	1518.8	97	109	LENVSVALEFLER
1533.8	1533.8	1936	1949	ITESDLSQLTASIR
1565.7	1565.7	1461	1474	VPQTFVTDCSQAGR
1571.8	1571.9	114	128	LVSDSKAIVDGNLK
1571.8	1571.8	2390	2404	VNQPASFAVLNGAR
1595.9	1595.9	545	558	YVVTITWGGYAIK
1601.8	1601.8	559	573	SPFEVQVSEAGVQK
1823.9	1823.9	2228	2244	VEAAEIVEGEDSAYSVR
1923.9	1924.0	1639	1657	CLVTVISIGGHGLGACLGPR
1941.8	1941.9	462	478	SPFPVHVSEACNPACR
2002.1	2002.1	1475	1494	APLQAVALLGPTGVAEPVEVR
2006.0	2006.1	1009	1027	RPIPCCKLEPGGGAQAQR
2006.0	2006.0	2017	2034	GLSEGHFTQVAEIVDTR
2324.1	2324.1	2174	2195	VEESTQVGGDPFPAVFGDGLGR
2363.1	2363.1	1807	1827	YDGNHIPSPLQFYVDAINSR
2527.2	2527.2	2354	2376	FNDEHIDPSFVVPVSLSDAR
2729.2	2729.3	2411	2434	VHTPSGAVEECYVSELDSDKHTIR

FLNc plus one phosphate group. Solid-phase sequencing (Figure 2B, right panel) identified the site of phosphorylation as Ser²²¹³, establishing that the ³²P-labelled protein was indeed FLNc. This residue lies in an optimal consensus sequence for PKB (RERLGSF). We also phosphorylated a fragment of FLNc, expressed as a GST fusion protein, and showed that it was again phosphorylated specifically at Ser²²¹³ (Figure 2C). This fragment was phosphorylated equally well by PKB α and PKB β when the two protein kinases were matched for activity towards CROSStide (results not shown).

Generation of antibodies that recognize unphosphorylated and phosphorylated FLNc

To examine whether Ser²²¹³ of FLNc becomes phosphorylated in cells, we raised antibodies that recognize FLNc only when it is phosphorylated at Ser²²¹³ (anti-pSer²²¹³), as well as antibodies that recognize phosphorylated and unphosphorylated FLNc equally well (anti-FLNc) (Figure 3). The latter antibody was raised against a peptide sequence, conserved in mouse and human FLNc, that is present within the unique insert region of FLNc, but not in the closely related FLNa and FLNb isoforms. The peptide sequence

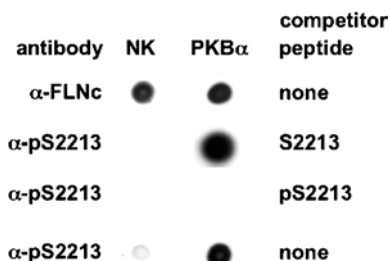


Figure 3 Characterization of antibodies that recognize FLNc

Bacterially expressed FLNc (residues 1915–2446) was left unphosphorylated (no kinase; NK) or maximally phosphorylated with PKBα. Aliquots (50 ng) were spotted on to a nitrocellulose membrane (Schleicher and Schuell, London, U.K.) and immunoblotted using the anti-pSer²²¹³ antibody (α-pS2213) or an antibody that recognizes phosphorylated and unphosphorylated FLNc equally well (α-FLNc) in the presence or absence ('none') of the competitor peptides shown. The sequences of these peptides are given in the Materials and methods section. The prefix p denotes a phosphorylated peptide.

to which the anti-pSer²²¹³ antibody was raised was also conserved in mouse and human FLNc. This antibody recognized GST–FLNc only after phosphorylation by PKBα (Figure 3), and recognition was blocked by incubation with the phosphopeptide immunogen, but not with the unphosphorylated form of this peptide. These antibodies were then used to study the phosphorylation of FLNc by PKBα in cells.

Insulin and EGF stimulate phosphorylation of FLNc on Ser²²¹³ in C2C12 myoblasts

Insulin induced rapid phosphorylation of FLNc at Ser²²¹³ that was maximal after a few minutes and was sustained for at least 2 h. The phosphorylation of FLNc paralleled the activation of PKBα, as assessed by the phosphorylation of Ser⁴⁷³ of PKBα (Figure 4A). The same result was obtained if the extracts were immunoblotted directly (Figure 4A) or if FLNc was immunoprecipitated first (results not shown).

EGF also induced a rapid, but transient, phosphorylation of FLNc at Ser²²¹³, with maximal phosphorylation at 3–10 min. The phosphorylation and subsequent dephosphorylation of FLNc lagged slightly behind the activation and inactivation of PKBα, consistent with FLNc being a substrate of PKB (Figure 4A).

Phosphorylation of FLNc at Ser²²¹³ is severely impaired in cardiac muscle from PDK1^{-/-} mice

Since FLNc is highly expressed in striated muscles, we analysed the phosphorylation of this protein in the cardiac muscle of both wild-type mice (mPDK1^{+/+}) and 'conditional knockout' mice (mPDK1^{-/-}) that no longer express PDK1 (3-phosphoinositide-dependent kinase 1), which activates PKB by phosphorylating it on Thr³⁰⁸ [15]. Mice were starved for 12 h before injection with insulin, and cardiac muscle was removed immediately or after 20 min of insulin stimulation. Immunoblot analysis showed that the expression of FLNc was similar in cardiac muscle from wild-type and conditional knockout mice (Figure 4B). In wild-type tissues, FLNc had low basal phosphorylation on Ser²²¹³, which was increased after 20 min of insulin stimulation. In contrast, the basal phosphorylation of Ser²²¹³ in cardiac muscle of the mPDK1^{-/-} mice was not increased by insulin (Figure 4B). The insulin-stimulated phosphorylation of Ser²²¹³ in wild-type cardiac muscle was correlated with activation of PKB, as determined by phosphorylation of Thr³⁰⁸ (Figure 4B).

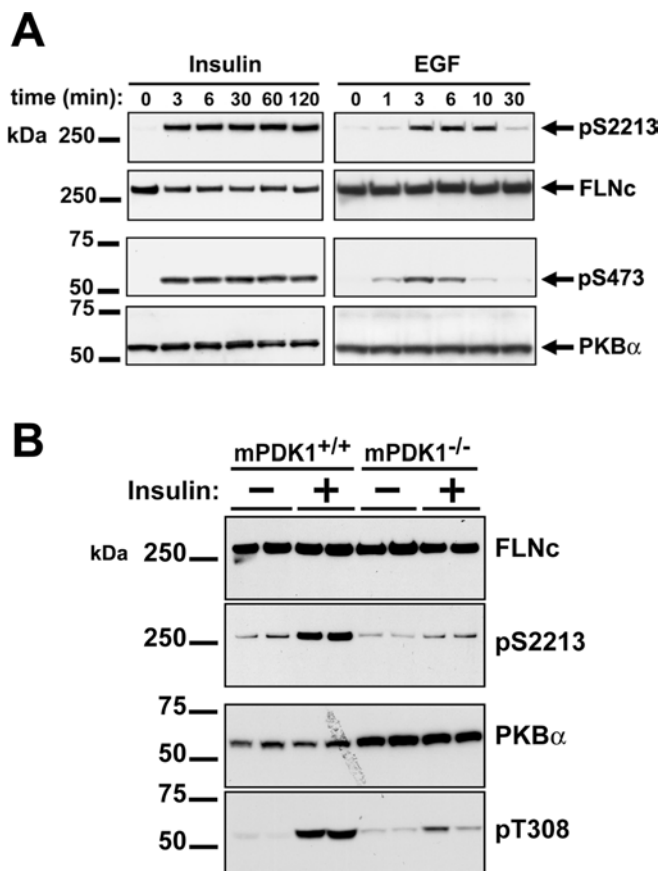


Figure 4 Insulin and EGF induce the phosphorylation of FLNc at Ser²²¹³ in C2C12 cells

(A) Murine C2C12 myoblasts were deprived of serum for 12 h, then stimulated with 250 ng/ml insulin or 20 ng/ml EGF for the times indicated. The cells were lysed and the lysates subjected to SDS/PAGE followed by transfer to an ImmobilonP membrane. The membranes were then probed with the anti-pSer²²¹³ and anti-FLNc antibodies, and with further antibodies that recognize PKBα phosphorylated at Ser⁴⁷³ (p473) and the phosphorylated and unphosphorylated forms of PKBα equally well (PKBα). (B) Cardiac muscle extracts (10 µg of protein) were prepared from either wild-type or PDK1 conditional knockout duplicate individual mice that had been fasted for 12 h before injection with or without insulin (10 m-units/g) for 20 min. Extracts were subjected to SDS/PAGE, transferred to ImmobilonP membranes and immunoblotted with antibodies as in (A), except that phosphorylation of PKBα was detected with an antibody that recognized PKBα phosphorylated at Thr³⁰⁸ (pT308).

The phosphorylation of FLNc at Ser²²¹³ is prevented by inhibition of PI 3-kinase

To investigate the signalling pathway involved in the insulin- and EGF-induced phosphorylation of FLNc at Ser²²¹³, we first incubated C2C12 myoblasts in the absence or presence of inhibitors of different signalling pathways, prior to stimulation with these agonists. These experiments showed that the insulin- or EGF-induced phosphorylation of FLNc at Ser²²¹³ and the phosphorylation of PKB at Ser⁴⁷³ were completely prevented by wortmannin, an inhibitor of PI 3-kinase (Figure 5). In contrast, the phosphorylation of FLNc at Ser²²¹³ was unaffected by PD 184352, an inhibitor of the classical MAPK (mitogen-activated protein kinase) cascade and/or by rapamycin, an inhibitor of the protein kinase mTOR (mammalian target of rapamycin). However, as expected, PD 184352 prevented the activation of ERK1 and ERK2, the MAPKs of the classical MAPK cascade, while rapamycin prevented the phosphorylation of ribosomal protein S6, a substrate of S6K which is a downstream component of the mTOR pathway (Figure 5).

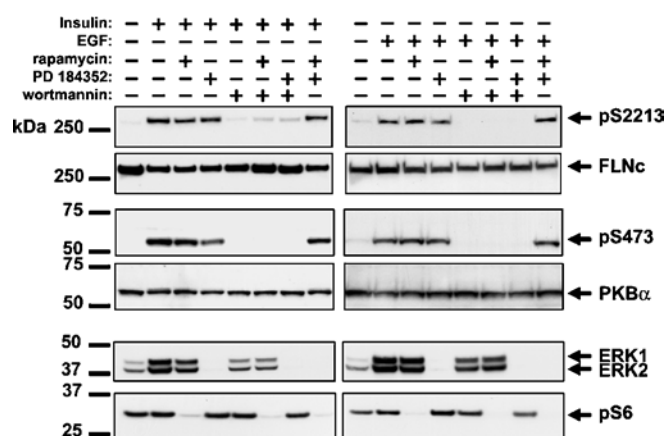


Figure 5 Effects of inhibitors of signalling pathways on the insulin- and EGF-stimulated phosphorylation of FLNc on Ser²²¹³ in C2C12 cells

The experiments were carried out as in Figure 4(A), except that, prior to stimulation with insulin for 3 min or EGF for 6 min, the cells were incubated for 1 h without (–) or with (+) 50 nM rapamycin, 2 μ M PD 184352 or 100 nM wortmannin. The membranes were immunoblotted with the antibodies described in the legend to Figure 4, as well as with antibodies that recognize the active, phosphorylated forms of ERK1 and ERK2 and ribosomal protein S6 phosphorylated at Ser²³⁵ (pS6).

DISCUSSION

In this paper, we have used the KESTREL method to identify FLNc as a new physiological substrate for PKB that is specific to striated muscle. PKB phosphorylated FLNc at Ser²²¹³ *in vitro*, and at a much higher rate than did SGK. Moreover, Ser²²¹³ became phosphorylated in C2C12 myoblasts in response to insulin or EGF, with phosphorylation and dephosphorylation correlating with the activation and inactivation respectively of PKB. The phosphorylation of FLNc, like that of PKB, was prevented by the PI 3-kinase inhibitor wortmannin. The phosphorylation of Ser²²¹³ was not suppressed by rapamycin or PD 184352, which prevent activation of S6K1/S6K2 and RSK isoforms respectively, protein kinases that have similar substrate preferences to PKB, and are also activated in response to insulin or EGF. Consistent with this finding, PMA, a potent activator of the classical MAPK cascade and hence the activation of RSK isoforms, did not induce the phosphorylation of FLNc at Ser²²¹³ (results not shown). The present study, in conjunction with the results described in the preceding paper [10], in which NDRG1 and NDRG2 were identified as physiological substrates for SGK, again illustrates the power of the KESTREL method for identifying physiological substrates of closely related protein kinases with similar substrate preferences.

Filamin, which was first identified over 25 years ago [16], is expressed in cells and tissues as three highly related isoforms, termed FLNa, FLNb and FLNc. It is an actin-binding protein that is thought to stabilize three-dimensional networks of actin filaments, linking them to cell membranes. However, it may also act as a scaffolding protein, tethering components of signalling pathways to enhance their activation by particular agonists. For example, FLNa has been reported to bind to MKK4 [MAPK kinase 4, also called SEK1 (SAPK/ERK kinase 1)], one of the activators of JNK (c-Jun N-terminal kinase), and to be required for the tumour necrosis factor α -induced activation of MKK4 in melanoma cells [17]. FLNa has also been reported to bind to SHIP2 (Src homology 2-interacting protein 2) [18], a lipid phosphatase that converts PtdIns(3,4,5) P_3 into PtdIns(3,4) P_2 and is involved in modulating signalling through PI 3-kinase.

FLNc, the muscle-specific isoform of filamin, contains a 78-amino-acid residue insert that is not present in either FLNa or FLNb, and which is located between the 19th and 20th immunoglobulin-like repeats, of which 24 are present in all three isoforms [19]. Strikingly, it is within this region unique to FLNc that the PKB-phosphorylation site Ser²²¹³ is situated. In contrast, FLNa is not phosphorylated by PKB [20]. In skeletal muscle, FLNc is associated with the Z-disc of the myofibrillar apparatus and binds directly to the Z-disc proteins FATZ [21] and myotilin [22,23]. It also interacts with sarcoglycans γ and δ , which are components of the dystrophin–dystroglycan complex at the sarcolemma [24]. Such a localization at regions where the sarcolemma interacts with myofibrils suggests that FLNc, like other filamin isoforms, may be involved in assembling signalling complexes near the cell membrane. It is therefore of considerable interest that LL5 β , a large 160 kDa protein containing a PtdIns(3,4,5) P_3 -binding PH domain at its C-terminus, has been found to interact with FLNc [25].

Although FLNc is a muscle-specific protein, it is also expressed in some cell lines and, in such cells, agonists that activate PI 3-kinase cause LL5 β to translocate to the cell periphery. This is presumably a consequence of the interaction of its PH domain with membrane-associated PtdIns(3,4,5) P_3 , because translocation did not occur in mutants of LL5 β in which the PH domain was deleted. However, incubation of the cells with inhibitors of PI 3-kinase causes LL5 β to relocate to an unidentified intracellular vesicular compartment. In COS7 cells co-transfected with LL5 β and FLNc, treatment with wortmannin caused FLNc to partially redistribute to this compartment. In cells not treated with PI 3-kinase inhibitors, or in cells transfected with FLNc alone, FLNc adopted a punctate distribution that was insensitive to wortmannin. Interestingly, a similar relocation to the intracellular vesicular compartment occurred in cells co-transfected with FLNc and a mutant of LL5 β that lacks the C-terminal PH domain [25]. This indicates that wortmannin does not induce relocation by preventing the interaction of PtdIns(3,4,5) P_3 with the PH domain of LL5 β , but by inhibiting a distinct PtdIns(3,4,5) P_3 -dependent mechanism. It will clearly be of great interest to investigate whether inhibition of the PKB-catalysed phosphorylation of FLNc at Ser²²¹³ underlies this effect.

We have carried out several experiments to address the function of phosphorylation of FLNc on Ser²²¹³. First, we examined the localization of FLNc in MRC5 fibroblasts, which express this protein, but could not detect any change in FLNc localization or its co-localization with actin stress fibres after stimulation with insulin in the presence or absence of wortmannin (results not shown). Since SHIP2 is reported to associate with FLNa, we also analysed whether it interacted with FLNc, but were unable to detect any SHIP2 activity associated with FLNc immunoprecipitates from control or insulin-stimulated C2C12 cells (results not shown). The muscle-specific proteinase calpain III regulates the ability of FLNc to interact with sarcoglycans γ and δ by cleaving FLNc during calpain-mediated remodelling of cytoskeleton–membrane interactions, including myoblast fusion and muscle repair [26]. FLNa becomes resistant to cleavage by calpain upon phosphorylation by cAMP-dependent protein kinase [27]. We immunoprecipitated FLNc from control or insulin-stimulated C2C12 cells, but the rate and extent of proteolytic cleavage that occurred upon incubation with recombinant calpain II *in vitro* were unaltered (results not shown). In summary, our current data provided no evidence that the phosphorylation of Ser²²¹³ affects the localization of FLNc, its interaction with SHIP2 or its susceptibility to cleavage of calpain.

Finally, it should be mentioned that FLNc is likely to be phosphorylated at sites additional to Ser²²¹³. The C-terminal region in

FLNa, which is conserved in FLNc, is reported to be phosphorylated *in vitro* by several protein kinases, including cAMP-dependent protein kinase [27,28], calcium/calmodulin-dependent protein kinase II [29], protein kinase C [30,31] and PAK1 (p21-activated kinase 1) [32]. Although RSK isoforms do not phosphorylate FLNc at Ser²²¹³, RSK2 has been reported to phosphorylate FLNa at Ser²¹⁵², and mutation of this site to alanine blocked the EGF-stimulated phosphorylation of FLNa [20]. The multisite phosphorylation of FLNc and other filamin isoforms may therefore influence their interaction with other proteins and their ability to modulate signal transduction in a complex manner.

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