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Regulation of multisite phosphorylation and 14-3-3 binding of AS160 in response to insulin-like growth factor 1, EGF, PMA and AICAR

¹*Kathryn Geraghty, ¹*Shuai Chen, *Jean E. Harthill, *Adel F. Ibrahim, *Rachel Toth, *Nick A. Morrice, *Franck Vandermoere, †Greg B. Moorhead, ‡D. Grahame Hardie and *Carol MacKintosh

*MRC Protein Phosphorylation Unit, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

†Department of Biological Sciences, University of Calgary, 2500 University Dr. N.W., Calgary, Alberta, Canada T2N 4N1.

‡Division of Molecular Physiology, College of Life Sciences, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

¹These two authors contributed equally to this work.

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Correspondence to:

Carol MacKintosh,
MRC Protein Phosphorylation Unit,
College of Life Sciences,
University of Dundee,
Dundee DD1 5EH,
Scotland, U.K.

Tel: 44-1382-344241

FAX: 44-1382-223778

E-mail: c.mackintosh@dundee.ac.uk

Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside; AS160, Akt substrate of 160 kDa; DSP, dithiobis[succinimidyl propionate]; EGF, epidermal growth factor; ERK, extracellular-signal-regulated protein kinase; GAP, GTPase-activating protein; GST, glutathione S-transferase; GSV, GLUT4 storage vesicle; HA, haemagglutinin; IGF1, insulin-like growth factor-1; IRAP, insulin-responsive aminopeptidase; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PI 3-kinase, phosphatidylinositol 3-kinase; PI(3,4,5)P₃, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B (also known as Akt); PTB, phosphotyrosine binding domain; Rheb, Ras enriched in brain; TORC1, protein complex that contains mTOR and raptor; TOS motifs, mTOR signalling motifs; TSC1/TSC2, the Tuberous Sclerosis heterodimer.

ABSTRACT

AS160 mediates insulin-stimulated GLUT4 translocation, but is widely expressed in insulin-insensitive tissues lacking GLUT4. Having isolated AS160 by 14-3-3-affinity chromatography we found that binding of AS160 to 14-3-3 isoforms in HEK293 cells was induced by insulin-like growth factor 1 (IGF1), epidermal growth factor (EGF), phorbol ester (PMA), and to a lesser extent by AICAR. AS160/14-3-3 interactions were stabilised by chemical cross-linking, and abolished by dephosphorylation. Eight residues on AS160 (Ser318, Ser341, Thr568, Ser570, Ser588, Thr642, Ser666, and Ser751) were phosphorylated differentially in response to IGF1, EGF, PMA and AICAR. The binding of 14-3-3s to HA-AS160 was markedly decreased by mutation of Thr642 and abolished in a Thr642Ala/Ser341Ala double mutant. The AGC kinases RSK1, SGK1 and PKB displayed distinct signatures of AS160 phosphorylation in vitro: All three kinases phosphorylated Ser318, Ser588 and Thr642; RSK1 also phosphorylated Ser341, Ser751 and to a lesser extent Thr568; and SGK1 phosphorylated Thr568 and Ser751. AMPK preferentially phosphorylated Ser588, with lesser phosphorylation of other sites. In cells, the IGF1-stimulated phosphorylations, and certain EGF-stimulated phosphorylations, were inhibited by PI 3-kinase inhibitors, while the RSK inhibitor BI-D1870 inhibited the PMA-induced phosphorylations. The expression of LKB1 in HeLa cells and use of AICAR in HEK293 cells promoted phosphorylation of Ser588, but only weak Ser341 and Thr642 phosphorylations and binding to 14-3-3s. Paradoxically however, phenformin activated AMPK without promoting AS160 phosphorylation. The IGF1-induced phosphorylation of the novel phosphoSer666-Pro site was suppressed by AICAR, and by combined mutation of a TOS-like sequence (FEMDI) and rapamycin. Thus, while AS160 is a common target of insulin, IGF1, EGF, PMA and AICAR, these stimuli induce distinctive patterns of phosphorylation and 14-3-3 binding, mediated by at least four protein kinases.

INTRODUCTION

14-3-3s are C-shaped proteins that dock onto specific phosphorylated sites, forcing changes in the conformations of their targets and/or their interactions with other molecules [1]. Hundreds of proteins that bind to 14-3-3s directly, or as components of multi-subunit complexes, have been purified from mammalian cells using their affinity for 14-3-3s [2-5]. Thus, binding of 14-3-3s to phosphoproteins is a common mechanism for linking signalling pathways to the regulation of cellular processes.

A sense of the global regulation of 14-3-3/target interactions can be gleaned by using Far Western (14-3-3 overlay) assays to observe how the patterns of phosphorylated 14-3-3 binding proteins change in extracts of cells stimulated in different ways [5, 6]. Such experiments show that the phosphorylation of a substantial subset of 14-3-3 targets is promoted by the phosphatidylinositol 3-kinase (PI 3-kinase) pathway, including cardiac PFK-2 [7], TSC2 [8, 9], p27(Kip1) [10], FOXO3a [11], and AS160/TBC1D4 [5, 12, 13]

AS160/TBC1D4, protein kinase B (PKB/Akt) substrate of 160 kDa [14], has been implicated in mediating the translocation of the GLUT4 glucose transporter to the plasma membrane in response to insulin, a process whose deregulation occurs early in the pathophysiology of insulin resistance and Type II diabetes [15]. The emerging role of AS160 in insulin-stimulated GLUT4 trafficking is:-

- 1) *In the basal state*, most GLUT4 is in intracellular GLUT4 storage vesicles (GSVs). AS160 is a Rab GTPase activating protein (RabGAP) that promotes hydrolysis of GTP to GDP by Rabs 8A, 10 and 14 on the GSV [16-19]. It is proposed [19-21] that in their inactive GDP-bound state the GSV-bound Rabs inhibit GLUT4 exocytosis.
- 2) *Upon insulin stimulation*, AS160 is phosphorylated [16, 22], which leads to its binding to 14-3-3s [13] and the inactivation of the RabGAP activity of AS160 and/or its dissociation from GSVs [17, 23]. Thus, GSV-associated Rabs are thought to become loaded with GTP and promote events that lead to expression of GLUT4 at the cell surface, thereby mediating the influx of glucose.

The anti-diabetic drug metformin activates the LKB1/AMPK signalling pathway [24-26], which has an insulin-sensitizing effect on the liver leading to decreased hepatic glucose production [25], and also enhances glucose uptake into tissues, possibly by altering the kinetics of GLUT4 trafficking to and from the plasma membrane. AICAR (5-

aminoimidazole-4-carboxamide-1- β -D-ribofuranoside), which also activates AMPK, has been reported to promote phosphorylation of AS160 [27, 28], raising the question of whether metformin also regulates AS160. Other PI 3-kinase-independent pathways have also been reported to regulate AS160 [28].

Together, these findings suggest that AS160 may be a convergence point for signalling pathways that promote GLUT4 translocation. The tissue distribution of AS160 is wider than GLUT4 however, suggesting that AS160 may also regulate trafficking of vesicles carrying other cargoes. Moreover, while AS160 is subject to multisite phosphorylation [22], most regulatory studies have focussed on Thr642, whose phosphorylation is linked to insulin-mediated 14-3-3 binding [13, 17] and GLUT4 trafficking [22], and/or monitored phosphorylation of AS160 using the PAS antibody, which recognises sequences phosphorylated on a generic Akt/PKB phosphomotif [27-30]. Here, our initial aim was to define further mechanistic details of 14-3-3 binding to AS160. Our findings led to a wider study in which we showed that IGF1, EGF, PMA and AICAR induce distinctive patterns of multisite phosphorylation and 14-3-3 binding of AS160, involving at least four protein kinases.

EXPERIMENTAL

Materials

Synthetic peptides were from Graham Bloomberg (University of Bristol), oligonucleotides from MWG-Biotech, IGF1 (insulin-like growth factor 1) was from Biosource, microcystin-LR from Linda Lawton (Robert Gordon's University, Aberdeen), frozen human HeLa cell pellets from 4c Biotech (Ghent, Belgium), Vivaspin concentrators from Vivascience, tissue culture reagents from Life Technologies, protease-inhibitor cocktail tablets (no. 1697498) and sequencing grade trypsin from Roche Molecular Biochemicals, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICAR) from Toronto Research Chemicals (North York, ON, Canada), precast SDS-polyacrylamide gels from Invitrogen, and dithiobis[succinimidyl propionate] (DSP) from Perbio. Protein G-Sepharose and other chromatographic matrices were from GE-Healthcare. All other chemicals were from BDH Chemicals or Sigma-Aldrich.

Antibodies and protein kinases

Sheep anti-HA was raised against the peptide YPYDVPDYA, and sheep anti-AS160 against KAKIGNKP (near the C-terminus of human AS160; Supplementary Fig 1). Phosphospecific antibodies were raised against the following phosphopeptides:- FRSRCSpSVTGVQR (residues 311 to 324, where pS represents phosphorylated Ser318); CPRRRHApSAPSHVQ (Cys + 335 to 347, pSer341); CKAKRSLpTSSLENI (Cys + 562 to 574, where pT is pThr568); CKRSLTSpSLENIFS (Cys + 564 to 576, pSer570); CMRGRLGpSVDSFER (Cys + 582 to 594, pSer588); CFRRRHApTFSHPPS (Cys + 636 to 648, pThr642); CAQGVRpSPLLQRS (Cys + 661 to 672, pSer666); and GRKRTSpSTCSNES (745 to 757, pSer751). The pSer318 and pSer751 peptides were conjugated via their N-termini, whereas the others were coupled via the added cysteines. Peptides were coupled separately to BSA and keyhole-limpet haemocyanin, mixed and injected into sheep at Diagnostics Scotland (Penicuik, UK). The antibodies were affinity-purified on phosphopeptide-Sepharose columns, and in some cases were also passed through columns coupled to the cognate unphosphorylated peptides and the flow-throughs collected. In each case, the antibodies recognised the synthetic phosphopeptide immunogen, but not the corresponding unphosphorylated peptide in dot blot assays (not shown). Anti-phosphoErk1/2 (pThr202/pTyr204), anti-phosphoThr308-PKB and anti-PKB/Akt were from Cell Signalling Technology. Western blots used the indicated antibodies at 1 μ g/ml. Fig 1, all K19 Western blots, and blots of cell lysates were visualised by ECL (enhanced chemiluminescence reagent), whereas other Western blots used secondary antibodies that were detected using the Odyssey Infrared Imaging System (LI-COR, Inc.). 14-3-3 overlays (using DIG-labelled 14-3-3 in place of primary antibody) used ECL detection [5].

Purified recombinant protein kinases, generated in the Division of Signal Transduction Therapy (DSTT), were His-PKBalph-S473D (residues 118 to 480 of human protein) expressed in insect cells and activated by PDK1; His-RSK1 (1 to 735 of rat) expressed in insect cells and activated by p42MAPK and PDK1; SGK1-S422D (60 to 431 of human) expressed in insect cells and activated by PDK1; and bacterially-expressed GST-AMPK-T172D (3 to 308 of rat protein). AMPK was purified from rat liver by Kevin Green in Grahame Hardie's laboratory. Unless indicated, the protein kinases were used at 1 Unit/ml, where 1 Unit is nmole phosphate per min at 30°C incorporated into the substrate peptides

Crosstide (GRPRTSSFAEG) for PKB and SGK1, Long-S6 (KEAKEKRQEQIAKRRRLSSLRASTSKSGGSQK) for RSK1, and AMARA peptide (AMARAASAAALARRR) for AMPK.

Molecular biology: splice variants of AS160

Two forms of AS160 appear to be products of one locus (NM_014832; gene id: ENSG00000136111). One form (termed AS160B) is identical to Z25171 from a skeletal muscle cDNA library. A shorter form (AS160A), more commonly represented in the sequence databases, is identical to AS160B except for deletion of residues 679 to 733 encoded by exon 11. Constructs to generate HA-AS160A (pCMV5.HA AS160A) and HA-AS160B (pCMV5.HA AS160B) were generated by RT-PCR amplification from RNA extracted from HeLa cells and human skeletal muscle, respectively. Bacterial expression plasmids for GST-AS160 fusions (pGEX6P AS160A and pGEX6P AS160B) were generated by standard procedures. Site-directed mutagenesis used the Quickchange protocol (Stratagene) and KOD HotStart DNA polymerase (Novagene). Sequences of DNA constructs were checked (www.dnaseq.co.uk).

Results are shown for the HA-AS160A and GST-AS160A form, though experiments in Fig 7 were repeated with AS160B and gave similar results (not shown). Features of AS160 and the related TBC1D1 are in Supplementary Fig 1. Unless stated, residue numbers given correspond to AS160B.

Cell stimulations, cross-linking, lysis and immunoprecipitations

Human HEK293 cells cultured on 10-cm diameter dishes in medium containing 10% (v/v) serum were used untransfected, or 16 to 30 h after transfection with the indicated plasmids. Cells were serum-starved for a further 4 to 12 h (unstimulated), then stimulated for 20 min with 50 ng/ml IGF1. Where indicated, calyculin A was used at 100 nM for ~3 min, and AICAR at 2 mM for 60 min, phenformin at 2 mM for 60 min, EGF at 50 ng/ml for 15 min, and PMA at 100 ng/ml for 30 min. Where indicated, cells were incubated with LY294002 (LY; 100 μ M for 1 h), wortmannin (100 nM for 1 h), U0126 (U; 10 μ M for 1 h), rapamycin (Ra; 50 nM for 30 min), Go6983 (1 μ M for 30 min) and BI-D1870 (BI; 10 μ M for 30 min) prior to stimulation with IGF1 and other stimuli. After stimulation, cells were lysed in 0.2 ml ice-cold lysis buffer [5]. For experiments using chemical crosslinker, cells were rinsed with cold PBS, lysed in 0.3 ml lysis buffer containing dithiobis[succinimidyl

propionate] (DSP, 2.5 mg/ml from 250 mg/ml in DMSO) for 30 min on ice, and unreacted cross-linker was quenched with 75 μ l of 1 M Tris-HCl pH7.4 with a further 30 min incubation [31].

For immunoprecipitations with anti-AS160 and anti-HA, 4 μ g antibody/mg lysate was mixed at 4°C for 1 h, then protein G-Sepharose (30 μ l of a 50% suspension in lysis buffer) added and mixed for a further hour. The suspension was centrifuged at 12,000 *g* for 1 min between washes.

The human HeLa cell-lines stably transfected with wild-type LKB1 and kinase-dead LKB1 were those derived by Sapkota et al [32].

RESULTS

IGF1 promotes the phosphorylation and binding of AS160 to 14-3-3 proteins

Previously, AS160 was isolated from HeLa cell extracts by 14-3-3-affinity chromatography [5]. Western blotting confirmed that AS160 bound to 14-3-3-Sepharose and was specifically eluted by a canonical 14-3-3 binding phosphopeptide, ARAApSAPA, but not a control peptide (Fig 1A).

When endogenous AS160 was immunoprecipitated from extracts of HEK293 cells that had been stimulated with serum, IGF1 or the protein phosphatase inhibitor calyculin A, the isolated AS160 bound directly to 14-3-3 proteins in an overlay assay (Fig 1B, middle panel). There was only a trace 14-3-3-binding signal for AS160 from unstimulated cells, or cells stimulated with IGF1 in the presence of the PI 3-kinase inhibitor LY294002 (Fig 1B, middle panel). Similarly, AS160 bound to 14-3-3-Sepharose in extracts of cells that had been stimulated with serum, IGF1 and calyculin A (Fig 1C, lower panel). The IGF1-induced 14-3-3-binding signal was unaffected by the mTOR inhibitor rapamycin, or U0126, which inhibits the activation of the classical MAPK pathway (data not shown).

In contrast to the 14-3-3 overlays, only small sub-stoichiometric amounts of 14-3-3 proteins were detected in anti-AS160 immunoprecipitates from IGF1-stimulated cells (not shown). However, 14-3-3s were co-immunoprecipitated with AS160 from IGF1- or calyculin A-stimulated cells when protein-protein interactions were stabilised by DSP, which reversibly links primary amines (Fig 1B, bottom panel). The co-precipitation of 14-3-3s

with AS160 was specifically induced by IGF1 and calyculin A, and blocked by LY294002 (Fig 1B, bottom panel).

The ability of AS160 from IGF1-induced cells to bind to 14-3-3s was abolished by dephosphorylation of the AS160 with protein phosphatase 2A (PP2A) *in vitro*, and this was prevented by the PP2A inhibitor microcystin-LR (Fig 1D).

IGF1 also induced the binding of 14-3-3s to HA-AS160 in transfected cells, as determined by binding of the extracted HA-AS160 to 14-3-3s in the overlay assay (Fig 1E, middle), and co-precipitation of 14-3-3s (Fig 1E bottom panel and 1F). MS analysis of tryptic digests showed that at least six of the seven human 14-3-3 isoforms were bound to the HA-AS160 extracted from IGF1-stimulated cells in the presence of DSP (Supplementary Table 1): The upper Coomassie-stained band (Fig 1F) contained peptides that are unique to the 14-3-3epsilon. The lower band (Fig 1F) contained peptides specific to 14-3-3theta, gamma, alpha/beta, eta, and delta/zeta. No peptides specifying 14-3-3sigma were identified.

Identification of phosphorylated sites on AS160, including known sites and the novel pSer666-Pro site

Digests of endogenous AS160 were analysed by liquid chromatography mass spectrometry [33] to identify IGF1-induced phosphorylations. Initially, precursor ion scans were run to detect phosphopeptides (Fig 2 and not shown). A Multiple Reaction Monitoring (MRM) analysis was then performed to look for the known phosphorylated sites on AS160 (Supplementary Table 2) [22]. In addition, phosphoSer318 and phosphoThr642 (also identified in [22]) were detected in endogenous AS160 using phosphospecific antibodies (later results), and by mass spectral analysis of recombinant AS160 isolated from human cells (not shown).

In addition to known sites, a new phosphopeptide from endogenous AS160 was identified by tandem mass spectrometry as AQGVRpSPLL^R (Fig 2). The phosphorylated Ser666 within this phosphopeptide lies within a pSer-Pro motif that is not a consensus site for PKB. The MS data indicated a marked increase in phosphorylation at Ser666 in response to IGF1, and the increased phosphorylation was blocked by LY294002 (Supplementary Table 3).

Mutation analysis indicates that 14-3-3 binds to phosphorylated Thr642 and Ser341

HA-AS160 with each of these seven phosphorylation sites individually mutated from Ser/Thr to Ala was tested for 14-3-3 binding using overlay assays and by co-precipitation of 14-3-3s with HA-AS160 from cell extracts. Only the Thr642Ala mutation had a marked effect in preventing the IGF1-induced binding of 14-3-3s to HA-AS160 (Fig 3A).

The 14-3-3 binding of Thr642Ala-AS160 was not completely abolished however (Fig 3A), indicating the existence of a second lower affinity 14-3-3-binding site. Therefore, double mutants of Thr642Ala combined with Ser to Ala mutations of each of the other phosphorylated sites were generated. Of the double mutants, the combination of Thr642Ala and Ser341Ala completely prevented the IGF1-induced binding of 14-3-3s to AS160 (boxed lanes in Fig 3B). Consistent with phosphorylation of Ser341 being responsible for the basal binding of 14-3-3s to AS160, the Ser341Ala mutation abolished the binding of 14-3-3s to AS160 in unstimulated cells (Fig 3B and data not shown).

In combination with Thr642Ala, the mutation of Ser318 to Ala also decreased the binding of 14-3-3s to AS160 (Fig 3B), consistent with the Ser318Ala mutation causing a decrease in the phosphorylation of Ser341 (Fig 3B and Supplementary Fig 2A).

The generation of phosphospecific-AS160 antibodies to monitor the phosphorylation of seven sites on AS160

To examine the cellular regulation of these phosphorylations further, we raised phospho-specific antibodies against each of the seven phosphorylated residues on AS160 (pSer318-AS160, pSer341-AS160, pSer570-AS160, pSer588-AS160, pThr642-AS160, pSer666-AS160, and pSer751-AS160) (Supplementary Fig 2A).

The recognition of AS160 by anti-PAS was largely abolished by the Thr642Ala mutation indicating that phosphoThr642 is the prime anti-PAS recognition site on AS160 from cells stimulated with serum, IGF1 and insulin (Supplementary Fig 2B and data not shown).

Phosphorylation of AS160 in response to IGF1 and AICAR, but not phenformin, in HEK293 cells

The phosphospecific antibody data for endogenous AS160, and HA-AS160 from transfected cells, showed that the phosphorylation of each of the seven sites was enhanced when cells were stimulated with serum and IGF1. These phosphorylations were prevented

by LY294002 (Fig 4A and data not shown), except for phosphoSer588 whose phosphorylation was partly inhibited by LY294002 (Fig 4A and not shown). There was a high basal phosphorylation of Ser341 and Ser666 in unstimulated cells, which was also abolished by LY294002 (Fig 4A).

The AMPK activator AICAR promoted phosphorylation of Ser588 of AS160 (Fig 4A), with much weaker phosphorylations of pSer341, Thr642 and Ser751 (Fig 4A and later results). Consistent with the much lesser phosphorylation of Ser341 and Thr642 compared to that seen with IGF1, AICAR promoted only weak binding of 14-3-3s to AS160 that was more easily detected in the overlay assay (Fig 4A) than by co-immunoprecipitation of AS160 with 14-3-3s from cell extracts (not shown).

Phenformin promoted a much stronger phosphorylation of Thr172 in the activation loop of AMPK, and of the AMPK target acetyl CoA carboxylase (ACC), compared to AICAR and metformin (Fig 4A). Despite its strong activation of AMPK however, phenformin did not stimulate phosphorylation of AS160 (Fig 4A). Metformin promoted only weak phosphorylation of several sites on AS160 (Fig 4A), and, in particular, metformin did not stimulate phosphorylation of Ser588 to the same extent as AICAR (Fig 4A). We also noticed that phosphorylation of both AMPK and ACC was strongly promoted by LY294002 in HEK293 cells (Fig 4A), but not in other cells tested (not shown). Neither phenformin nor metformin inhibited any of the IGF1- and AICAR-stimulated phosphorylations of AS160 in HEK293 cells (not shown).

In view of the paradoxical effects of AICAR and phenformin, we examined whether LKB1, an upstream activator of AMPK has any role in regulating phosphorylation of AS160. LKB1 is absent from HeLa cells [32] and AICAR did not promote phosphorylation of AS160 in these cells (data not shown). In HeLa cells that were stably transfected with wild-type LKB1, the presence of the active LKB1 was itself sufficient to induce phosphorylation of Ser588 of endogenous AS160, even in the absence of any AMPK activators (Fig 4B, left side). Kinase-dead LKB1 did not induce phosphorylation of AS160 at Ser588 (Fig 4B, right side).

In vitro phosphorylation of GST-AS160 by PKB, AMPK, SGK1 and RSK1; identification of an eighth phosphorylation site at Thr568

Consistent with its proposed role in phosphorylation of AS160 [14], PKB preferentially phosphorylated Ser318, Ser588 and Thr642, and also catalysed trace phosphorylation of Ser570 and Ser751 in vitro (Fig 5, right and left hand panels). In contrast, AMPK preferentially phosphorylated Ser588 of AS160, phosphorylated Ser570 to a similar degree as did PKB, and phosphorylated Thr642 to a lesser extent (Fig 5, right side). Compared with PKB, other AGC kinases displayed distinct signatures of AS160 phosphorylation in vitro (Fig 5, left side; and data not shown). In common with PKB, SGK1 and RSK1 phosphorylated Ser318, Ser588 and Thr642 (Fig 5, left side). In addition, RSK1 also phosphorylated Ser341 and Ser751; and SGK phosphorylated Ser751. MS analysis of tryptic digests of in vitro-phosphorylated AS160 also revealed a new phosphorylated site at Thr568, within the sequence KAKRSLpT₅₆₈SSLENI, two residues N-terminal to the phosphoSer570 site (data not shown). A phospho-specific pThr568-AS160 antibody was raised and tested for phospho- and sequence specificity (Supplementary Fig 2C), and using this antibody it was found that SGK1 was the best Thr568 kinase of those tested, though this site was also phosphorylated by RSK1 (Fig 5, left side panel). These kinases displayed no or barely detectable phosphorylation of Ser666 (Fig 5).

Phosphorylation and 14-3-3 binding of AS160 in response to EGF and PMA in HEK293 cells: a role for RSK in mediating the response to PMA

We next assessed whether Thr568 of AS160 is phosphorylated in cells, and whether RSK is a physiological AS160 kinase. Alongside IGF1, AICAR and phenformin, cells were treated with EGF and the phorbol ester PMA, which stimulate RSK isoforms via the mitogen-activated protein kinase family members ERK1 and ERK2 ([34] and references within) (Fig 6 and Supplementary Fig 3).

HA-AS160 and 14-3-3s were co-immunoprecipitated, and AS160 was phosphorylated on several sites and could bind to 14-3-3s in overlay assays, when extracted from cells stimulated with serum, IGF1, EGF and PMA (Fig 6 and Supplementary Fig 3). Of these stimuli, PMA gave the weakest 14-3-3-binding signals for AS160, consistent with its lesser stimulation of Thr642 phosphorylation (Fig 6 and Supplementary Fig 3). In

contrast, PMA induced the strongest phosphorylation of Thr568, showing that this site is phosphorylated in cells (Fig 6) as well as in vitro (Fig 5).

When PMA-induced and PKC-mediated phosphorylation of Erk1 and Erk2 was blocked by Go6983 (Fig 6, [35], this inhibitor largely abolished phosphorylation of AS160 in response to PMA (Fig 6). In addition, the RSK inhibitor BI-D1870 [34] markedly suppressed the responses to PMA, except the weak Thr642 phosphorylation (Fig 6). Go6983 and BI-D1870 also inhibited PMA-induced binding of AS160 to 14-3-3s (data not shown). Wortmannin had no effect on PMA-induced phosphorylations of AS160 (Fig 6).

In contrast, wortmannin inhibited EGF-induced phosphorylation of Ser318 and Thr642, with little or no effect on the other EGF-regulated sites (Fig 6), though the RSK inhibitor BI-D1870 inhibited all the EGF-stimulated phosphorylations to different extents (Fig 6). In contrast to PMA, Go6983 inhibited phosphorylation of neither Erk1/2 nor AS160 in response to EGF.

As before, the IGF1-stimulated phosphorylations were blocked by LY294002, and largely inhibited by wortmannin (Fig 6).

IGF1-induced phosphorylation of Ser666 is partially inhibited by combined mutation of a TOS-like motif in AS160 and rapamycin, and blocked by AICAR

The IGF1-induced phosphorylation of the novel phosphoSer666-Pro site was not affected by SB203580, a specific inhibitor of SAPK2a/p38, or U0126, inhibitor of Erk1/2 activation (data not shown).

mTOR mediates the insulin and IGF1-induced phosphorylation of pSer/Thr-Pro motifs on 4E-BP1/PHAS (pT₃₇PGGTLFSTpT₄₆P) [36] and p70S6K (PVDS₃₇₁P) [37]. mTOR signalling (TOS) motifs on these proteins act as docking sites for the mTOR/raptor (TORC1) complex, and are required for the phosphorylation of the Ser/Thr-Pro sites [38]. Intriguingly, the AS160 protein contains a sequence, FEMDI, identical to the TOS motif on 4E-BP1. Consistent with a functional role for this motif, when the FEMDI motif was mutated to AEMDI the IGF1-stimulated phosphorylation of Ser666 was more inhibited by rapamycin, compared with the wild-type HA-AS160 (Fig 7A, last lane). However, neither rapamycin alone, nor mutation of the FEMDI motif alone, had any obvious effect on Ser666 phosphorylation in IGF1-stimulated cells (Fig 7A). The IGF1-induced phosphorylation of a

TOS-dependent site on p70S6K was prevented by rapamycin alone, as expected (Fig 7A, bottom panel).

Amongst other effectors tested, the AMPK activator AICAR abolished both basal and IGF1-stimulated phosphorylation of Ser666 of AS160 (Fig 7B).

DISCUSSION

We identified a specific and reversible interaction between phosphorylated AS160 and 14-3-3 proteins in cells stimulated with IGF1. The affinity of the phosphoAS160/14-3-3 interaction is sufficient for the phosphorylated AS160 to bind rapidly to the 14-3-3s inside the cell. However, it is poised at a point where the phosphorylated AS160 and 14-3-3s dissociate during the washing procedures of immunoprecipitation, unless stabilised by cross-linking. Such reversibility makes physiological sense for a regulatory interaction that must respond dynamically to changes in the stimulation and metabolic status of the cell. Cellular AS160 binds to at least six of the seven human isoforms of 14-3-3s (Fig 1F and Supplementary Table 1). 14-3-3sigma was absent from the AS160 immunoprecipitates, and was not detected in cell extracts using anti-14-3-3sigma antibodies (not shown). The 14-3-3s that interact with AS160 may simply reflect the complement of isoforms in this particular cell type, although isoform selectivity cannot be ruled out.

We identified eight phosphorylated residues in AS160, of which six have been reported previously [22] and the pSer666-Pro and pThr568 sites are novel. However, studies of the responsiveness of AS160 to extracellular stimuli generally use the anti-PAS antibody that primarily detects phosphoThr642 ([27, 30]; Supplementary Fig 2B). Our wider analysis shows how distinctive patterns of multisite phosphorylation of AS160 are induced by incoming signals from the PI 3-kinase pathway, the PKC/Erk/RSK pathway, LKB1/AMPK, and other signalling pathways. By comparing the phosphorylation status of the eight sites in response to extracellular stimuli, the effects of protein kinases inhibitors, and the *in vitro* phosphorylation specificity of protein kinases, we can start to assign candidate AS160 kinases to specific sites. For example, our data are consistent with the possibility that PKB phosphorylates Thr642 in response to IGF1, but the IGF1-activated Ser341 kinase has yet to be identified (Figs 4A, 5 and 6). In contrast, RSK1 is a good candidate for phosphorylating Ser341 in response to PMA (Figs 5 and 6). When more of the gaps have been filled in with

respect to which protein kinases phosphorylate which sites on AS160 in response to which stimuli, we suggest that the signatures detected with the phospho-specific antibodies will provide valuable read-outs of the responsiveness of different signalling pathways and protein kinases to cellular stimuli and drugs.

Subtle interdependencies were observed amongst the phosphorylations. For example, a Ser318Ala mutation decreased phosphorylation of Ser341 (Fig 3B and Supplementary Fig 2A). In the unstimulated state Ser341 is phosphorylated. Although this looks like a perfect mode I 14-3-3-binding motif (<http://scansite.mit.edu/motifsca.seq.phtml>), Ser341 phosphorylation mediates only a low affinity basal interaction with 14-3-3s, which is markedly enhanced when Thr642 is phosphorylated in response to insulin or IGF1. Consistent with an essential role for 14-3-3s in GLUT4 translocation [13], Thr642 is critical for mediating the translocation of GLUT4 and glucose uptake in response to insulin [22]. We suggest that a single 14-3-3 dimer might bind phosphoSer341 and phosphoThr642. Intriguingly, these sites flank the second phosphotyrosine binding domain (PTB) on AS160 (Supplementary Fig 1), which was reported to bind to insulin-responsive aminopeptidase (IRAP; [23]) thereby docking AS160 onto the GSVs [17, 23]. Thus, 14-3-3 binding might disrupt the interaction between IRAP and the second PTB domain of AS160, thereby releasing the AS160 from the GSVs, enabling the Rabs to be loaded with GTP to promote translocation of the GSVs towards the cell surface. However, we have thus far failed to observe the IRAP/AS160 interaction either in vitro or cell extracts (not shown).

The potential for the AMPK to mediate the stimulatory effects of the nucleoside AICAR, as well as exercise, on GLUT4 translocation and glucose uptake into skeletal muscle and adipocytes has been studied [27, 29, 30] using the anti-PAS antibody that primarily detects phosphoThr642 (Supplementary Fig 2B). However, we find that Ser588 is most strongly phosphorylated by AMPK, while Thr642 is a secondary site of phosphorylation by this kinase in vitro (Fig 5) and in cell responses to AICAR (Fig 4A). Intriguingly, Sano et al [22] pinpointed the double Ser588Ala/Thr642Ala-AS160 mutant as having a greater inhibitory effect on insulin-stimulated GLUT4 translocation than the single Thr642Ala-AS160 mutant, suggesting that Ser588 and Thr642 can both influence GLUT4 translocation.

The high pSer588/pThr642 phosphorylation ratio induced by AICAR (Fig 4A), mirrors the specificity of AMPK towards AS160 *in vitro* (Fig 6), consistent with AMPK mediating the AICAR-induced phosphorylations. AICAR in cells is converted by adenosine kinase into the monophosphorylated nucleotide, ZMP, which mimics the effects of AMP on the AMPK system [39]. However, the AICAR-induced phosphorylation of AS160 occurred with barely detectable AMPK phosphorylation and activation (Fig 4A and not shown). This lack of effect of AICAR on total AMPK in HEK293 cells is consistent with previous findings [40]. AMPK is an alpha/beta/gamma heterotrimer and, theoretically, twelve forms of AMPK could exist comprising combinations of the two alpha, two beta and three gamma subunit isoforms. AICAR activates AMPK alpha2 complexes in mouse muscle [41], whereas AMPK alpha2 and gamma3 subunits are required for AICAR-induced AS160 phosphorylation detected by the PAS antibody [30]. The alpha2 subunit of the AMPK, which is a less major form than alpha1 in HEK293 cells (D.G. Hardie laboratory, personal observations), may therefore be the AS160 kinase. If so, one would have to explain how both metformin and phenformin promoted a marked phosphorylation of the AMPK and its downstream target ACC, and yet have little effect on phosphorylation of AS160, in contrast to the effects of AICAR. Metformin and phenformin inhibit Complex I of the mitochondrial respiratory chain and are thought to activate AMPK by increasing the intracellular AMP/ATP ratio [39]. We considered the possibility that AMPK phosphorylates Ser588 of AS160, but that (being metabolic poisons) metformin and phenformin cause another effect that inhibits AS160 phosphorylation. However, neither metformin nor phenformin blocked the IGF1- or AICAR-induced phosphorylations of AS160. Another possibility is that phenformin activates a phosphatase that can obscure phenformin-induced phosphorylation, but is insufficient to impact on IGF1- and AICAR-induced phosphorylations. We emphasise that the paradoxical effects of AICAR and phenformin do not seem to be unique to HEK293 cells. These were also seen for the AS160 from rat L6 myotubes (not shown), which are commonly used for studying the regulation of glucose trafficking by insulin. However, the majority of extractable AS160 in L6 cells was a truncated form lacking the regulatory phosphorylation sites (not shown).

LKB1 is the upstream kinase that mediates the activation of AMPK by AICAR and metformin/phenformin [39]. LKB1 also activates a number of AMPK-related protein

kinases [39]. We found that Ser588 of AS160 was phosphorylated in HeLa cells containing active LKB1, but not in cells carrying the kinase-dead LKB1 (Fig 4B). Overall, these findings are consistent AS160 being phosphorylated by AMPK or another LKB1-dependent enzyme. Our findings agree with the lack of effect of metformin on basal or insulin-induced AS160 phosphorylation measured with the PAS antibody [42]. To our knowledge, no other researchers have reported effects of metformin/phenformin on AS160. Perhaps, others made the same findings with AICAR and metformin/phenformin as we report here, and have been similarly puzzled by these paradoxical data.

The identity of the kinase(s) that phosphorylates Ser666 is unknown. The IGF1-induced phosphorylation of Ser666 on AS160 was not blocked by rapamycin, unlike the IGF1-dependent phosphorylation of pSer-Pro sites on elongation factor 2 kinase (eEF2K), p70S6K, and 4EBP1 ([43]; Fig 7A). Moreover, phosphorylation of Ser666 was not inhibited by mutation of a putative TOS motif (FEMDI), which mediates mTOR /TORC1-dependent phosphorylation of downstream targets. We cannot rule out a role for mTOR at this stage though, because the combination of mutation of the TOS-like motif and rapamycin together suppressed the IGF1-induced phosphorylation of Ser666 of AS160. The FEMDI sequence is conserved in AS160 proteins from all other mammalian species currently cited in sequence databases. Moreover, the IGF1-induced Ser666 phosphorylation was inhibited by AICAR. Activation of mTOR in the TORC1 complex by insulin and IGF1 initially involves PKB, which inhibits the Rheb-GAP activity of the Tuberous Sclerosis heterodimer (TSC1/TSC2), and thereby increases the RhebGTP/RhebGDP ratio [44]. AMPK inhibits insulin-induced mTOR/TORC1 signalling by mechanisms that include phosphorylation and activation of TSC2 [44-47]. Therefore, the finding that AICAR blocks IGF1-induced phosphorylation of Ser666 of AS160 is consistent with a role for the mTOR family.

Recently, a mutation in the TBC1D1 gene, encoding a protein related to AS160, was linked with severe obesity in females [48]. TBC1D1 was isolated as a 14-3-3-binding protein [3]. Thr596 in TBC1D1, analogous to Thr642 in AS160, is phosphorylated in response to insulin in adipocytes [49] and some other AS160 phosphorylation sites are conserved in TBC1D1 (Supplementary Fig 1), and we found that 14-3-3s bind to TBC1D1 in response to IGF1 and insulin (S. Chen and C. MacKintosh, unpublished). Thus, dissecting further details of the control of AS160 and TBC1D1 by insulin/IGF1, and the respective

roles of these proteins in glucose homeostasis, should give new insights into links between insulin signalling, obesity and other metabolic disorders. AS160 and TBC1D1 are both widely expressed though, and our findings also highlight AS160 as a potential mediator of growth factor-regulated vesicle trafficking beyond insulin regulation of GLUT4.

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Figure legends

Figure 1 Phosphorylation-dependent binding of AS160 to 14-3-3 proteins in response to IGF1

- A. Isolation of AS160 by 14-3-3-affinity chromatography of HeLa cell extracts. Crude extract, flow through, first wash fractions (40 μ g) and 5 μ l of the concentrated last wash, control peptide and final wash fractions (<1 μ g protein) from the 14-3-3 column were loaded on a 4-12% Bis-Tris SDS-gel, alongside 2 μ g of the proteins that were eluted with 14-3-3-binding phosphopeptide, ARAApSAPA (phosphopeptide elution). The gel was transferred to nitrocellulose, and anti-AS160 Western blot performed. Protein stains and 14-3-3 overlay signals for this column were similar to those in [5].
- B. Untransfected HEK293 cells that had been serum-fed, unstimulated, IGF1-stimulated, IGF1 stimulated in the presence of LY294002, and treated with calyculin A, were lysed in the presence of DSP cross-linker. Endogenous AS160 was immunoprecipitated from lysates (6 mg), denatured and run on a 4-12% Bis-Tris SDS-gel, transferred to nitrocellulose, and the 14-3-3-binding ability of AS160 was tested by 14-3-3 overlay (middle panel). Cellular 14-3-3s that co-precipitated with endogenous AS160 were identified by Western blots with pan-14-3-3 antibodies (K-19 from Santa Cruz; bottom panel).
- C. 14-3-3-Sepharose (20 μ l, prepared as in [5]) was incubated with HEK293 lysate (6 mg) from cells that were serum-fed, unstimulated (serum-starved), IGF1-stimulated, IGF1-stimulated in the presence of LY294002, and treated with calyculin A. The 14-3-3-Sepharose was washed three times in a high salt buffer followed by two washes in low salt. AS160 bound to the 14-3-3-Sepharose was identified by Western blotting.
- D. Endogenous AS160 was immunoprecipitated from 4 mg HEK293 cell extract, and incubated for 30 min at 30°C in the presence of 50 milliunits/ml protein phosphatase 2A (PP2A), with or without the inhibitor microcystin-LR (MC-LR; 5 mM), as indicated. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and the AS160 was probed for binding to 14-3-3s in the overlay assay.

- E. HA-tagged AS160A (see Methods) was immunoprecipitated from lysates (3 mg) of transfected HEK293 cells that had been serum-fed, unstimulated, IGF1-stimulated, IGF1-stimulated in the presence of LY294002 and calyculin A treated. The immunoprecipitated protein was analysed by the 14-3-3 overlay. In addition, HA-AS160 was immunoprecipitated from cell lysates (1 mg), denatured in SDS sample buffer and run on a 4-12% Bis-Tris gel, which was stained with Colloidal blue (bottom panel).
- F. HA-AS160A was immunoprecipitated from cell lysates (1 mg) prepared with and without DSP crosslinker, as indicated. The immunoprecipitated protein was run on a 4-12% Bis-Tris SDS-gel, which was stained with Colloidal blue, and the proteins indicated were identified by MS/MS analysis of tryptic digests. (See text and Supplementary Table 1 for details).

Figure 2

Precursor ion scan for the tryptic digest of endogenous AS160 from calyculin A-treated cells By liquid chromatography mass spectrometry on an Applied Biosystems 4000 Q-TRAP, a precursor ion scan identifies those parent masses that yield daughter ions with a m/z of 79kDa (PO₃⁻), that is only the phosphopeptides indicated. The phosphorylated peptides were analysed further to identify the phosphorylated residues (Supplementary Table 2).

Figure 3

Identification of 14-3-3 binding sites on AS160

- A. HEK 293 cells growing in the presence of serum were transfected with vectors to express wildtype HA-AS160A and mutants with the single Ser/Thr to Ala mutations indicated. The cells were unstimulated (U) or stimulated with IGF1 (I), and HA-tagged proteins were immunoprecipitated from 500 μ g extract, and subject to anti-HA, anti-phosphoThr642-AS160, and anti-14-3-3 (K19) Western blots, and 14-3-3 overlays.
- B. HEK 293 cells transfected with wild-type HA-AS160A, Thr642Ala-AS160A, and double mutants were unstimulated (U) or treated with IGF1 (I). The HA-tagged proteins were immunoprecipitated from 500 μ g cell extract, one fifth of each

immunoprecipitate was loaded onto each lane for immunoblotting, and blots were subject to anti-AS160, anti-HA, anti-phosphoThr642-AS160, anti-phospho-Ser341, and anti-14-3-3 (K19) Western blots, and 14-3-3 overlays.

The specificity of the phospho-specific antibodies is demonstrated in Supplementary Fig 2A.

Figure 4

Phosphorylation of seven sites on AS160 in response to different stimuli in HEK293 cells, and effect of LKB1 on the phosphorylation of Ser588

- A. HEK293 cells transfected with HA-AS160A were stimulated as indicated. Crude lysates (60 μ g per lane) were probed for binding to the antibodies as indicated along the left hand side of the upper panels. The cells transfected with empty vector were serum-starved before harvesting. In the lower four panels, extracts (40 μ g) of HEK293 cells that had been stimulated as indicated were analysed by Western blotting with antibodies to monitor the phosphorylation status of the activation loop on AMPK, the AMPK site on ACC (pSer79 on the rat protein, pSer80 in the human), and the activating (pThr308) phosphorylation site on PKB.
- B. Endogenous AS160 was immunoprecipitated from HeLa cells stably transfected with either LKB1 or kinase-dead LKB1 [32] and Western blotting and 14-3-3 overlays performed, as indicated.

Figure 5

In vitro phosphorylation of AS160 by AMPK and the AGC kinases, PKB, SGK1 and RSK1

In the right hand panels, GST-AS160A was expressed and purified from *E. coli*, and phosphorylated in vitro with PKB (1 Unit/ml) and AMPK purified from rat liver (10 Units/ml). The phosphorylated proteins (300 ng per lane) were analysed by Western blots using phospho-specific-AS160 antibodies (Supplementary Fig 3A, C) and 14-3-3 overlay, as indicated. Similarly, in the left hand panels, the GST-AS160A was phosphorylated in vitro by PKB, SGK1 and RSK1 (1 Unit/ml).

Figure 6

Phosphorylation of all eight sites on AS160 in response to IGF1, EGF and PMA in HEK293 cells, and effects of protein kinase inhibitors

HEK293 cells transfected with HA-AS160A were stimulated after pre-exposure to inhibitors, as indicated, and HA-AS160A was analysed by Western blots. In addition, cell lysates (60 μ g per lane) were analysed by Western blotting to detect phosphorylation of Erk1 and Erk2, pThr308 of PKB, and total PKB.

Figure 7

Effect of FEMDI to AEMDI mutation, rapamycin and AICAR on phosphorylation of Ser666

- A. HEK293 cells transfected with HA-AS160A and HA-AEMDI-AS160A were stimulated with IGF1, in the presence or absence of LY294002 and rapamycin, as indicated. Crude lysates (60 μ g) were probed with antibodies against phospho-Thr308-PKB and phosphoThr389-p70S6K. HA-tagged proteins were immunoprecipitated, run on SDS-PAGE, transferred to nitrocellulose and probed with the antibodies indicated.
- B. HEK293 cells transfected with HA-AS160A were stimulated with IGF1, in the presence and absence of AICAR, as indicated. HA-tagged proteins were immunoprecipitated, run on SDS-PAGE, transferred to nitrocellulose and probed with the antibodies indicated.

Fig 1A, B, C

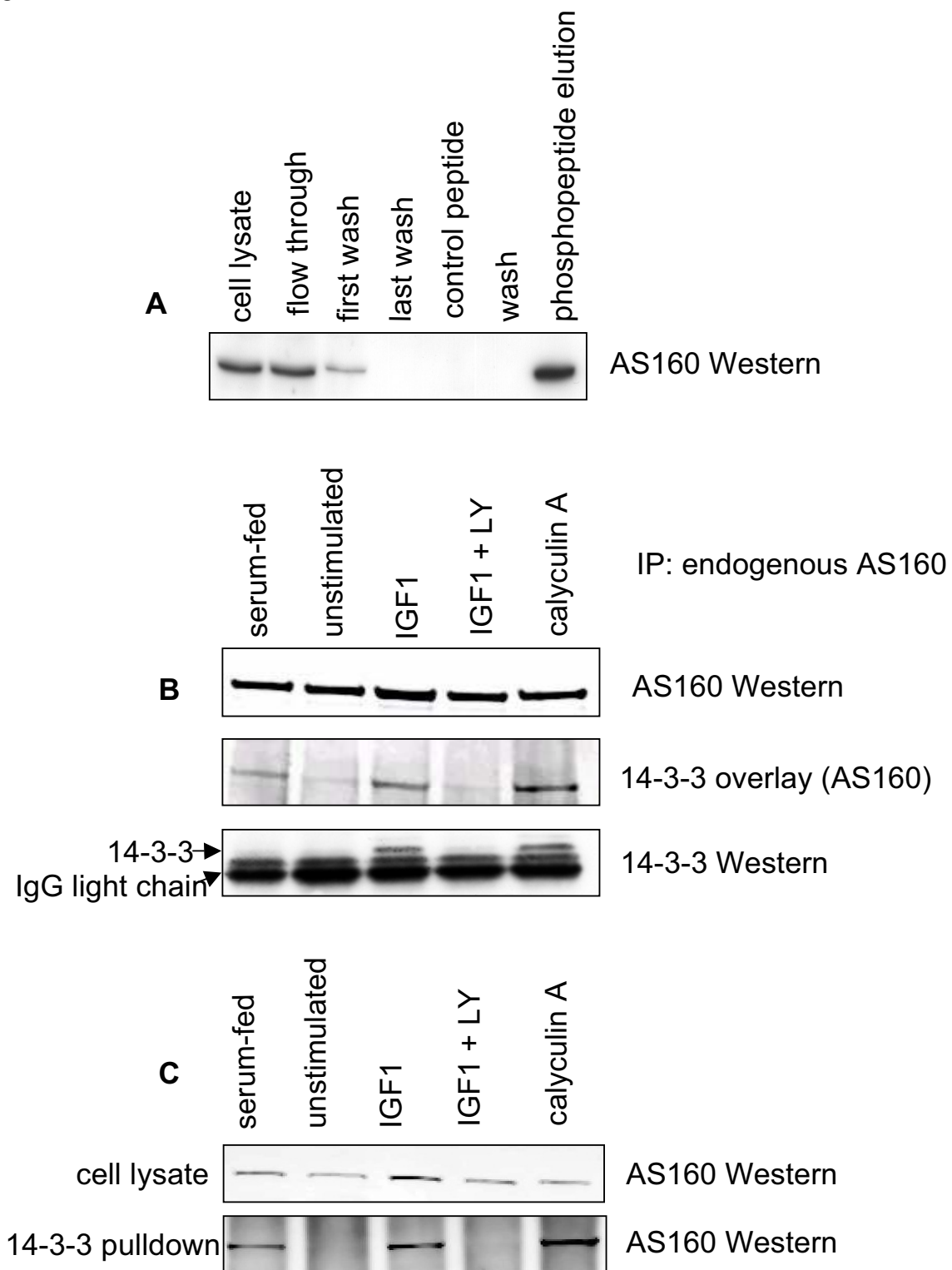


Fig 1D,E,F

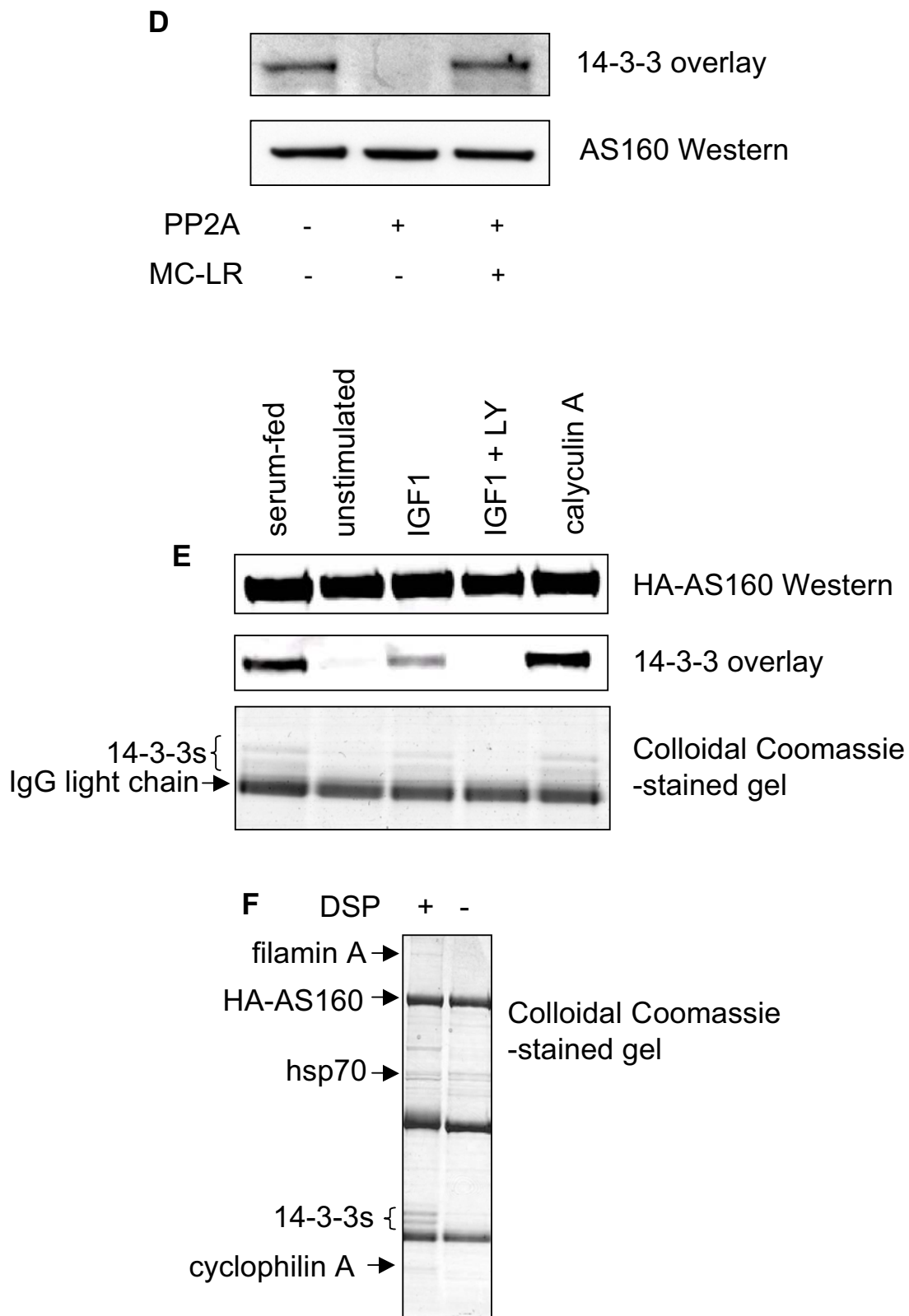


Fig 2

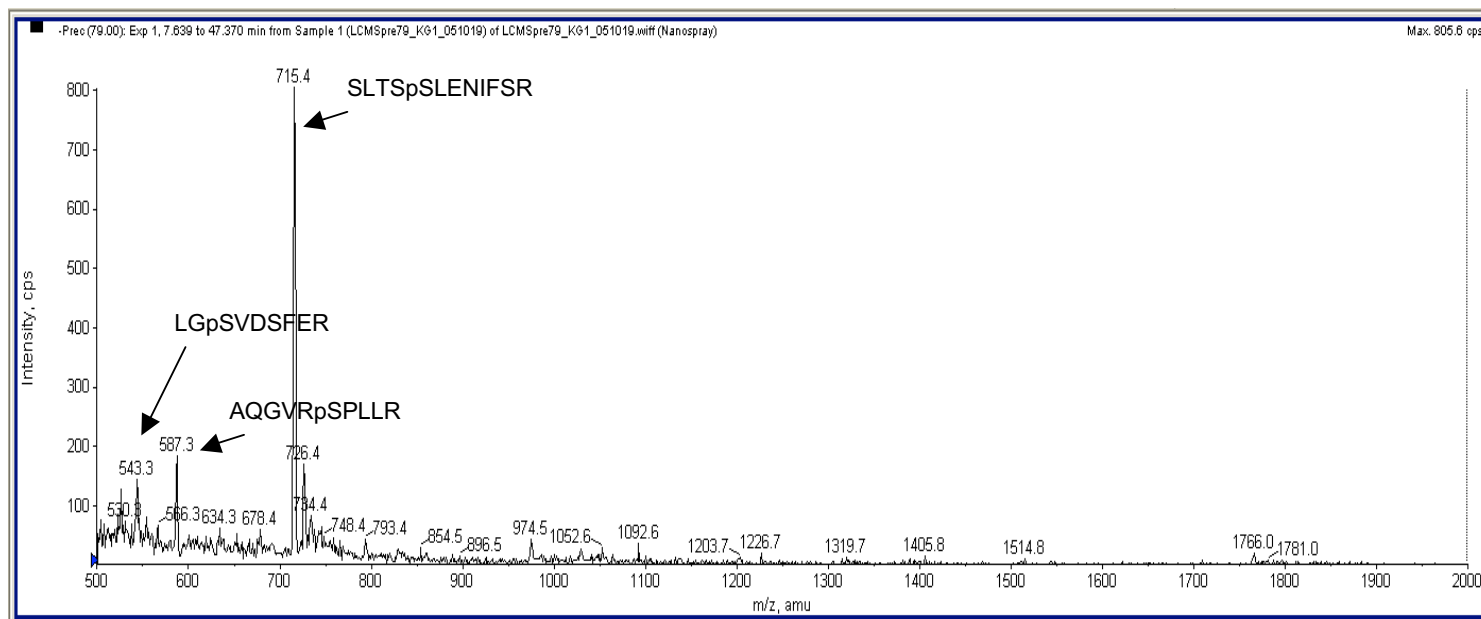


Fig 3A

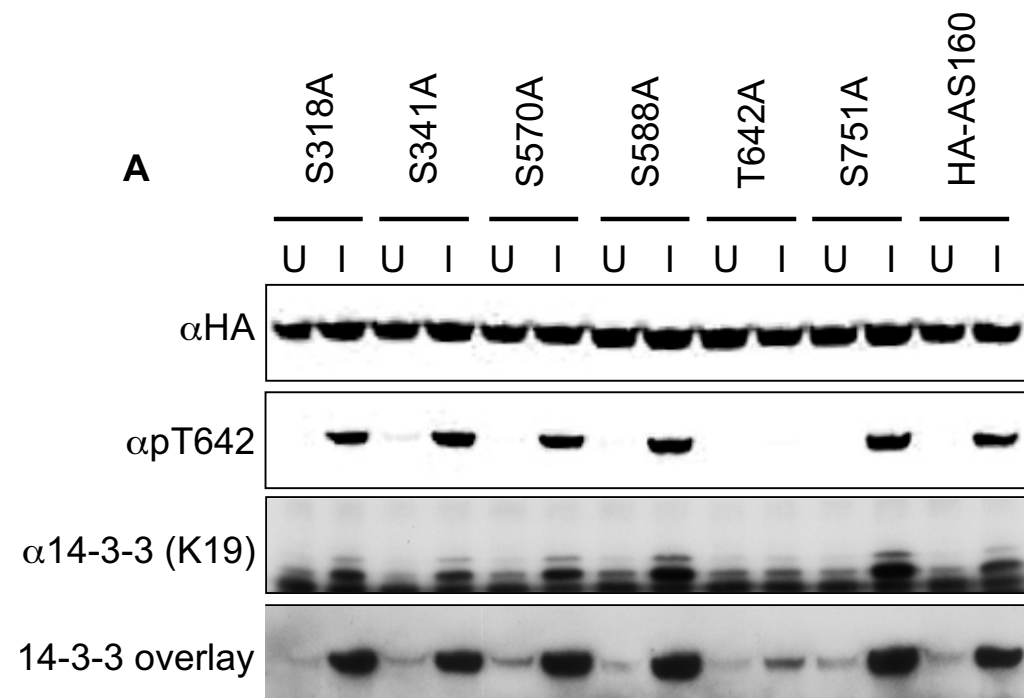


Fig 3B

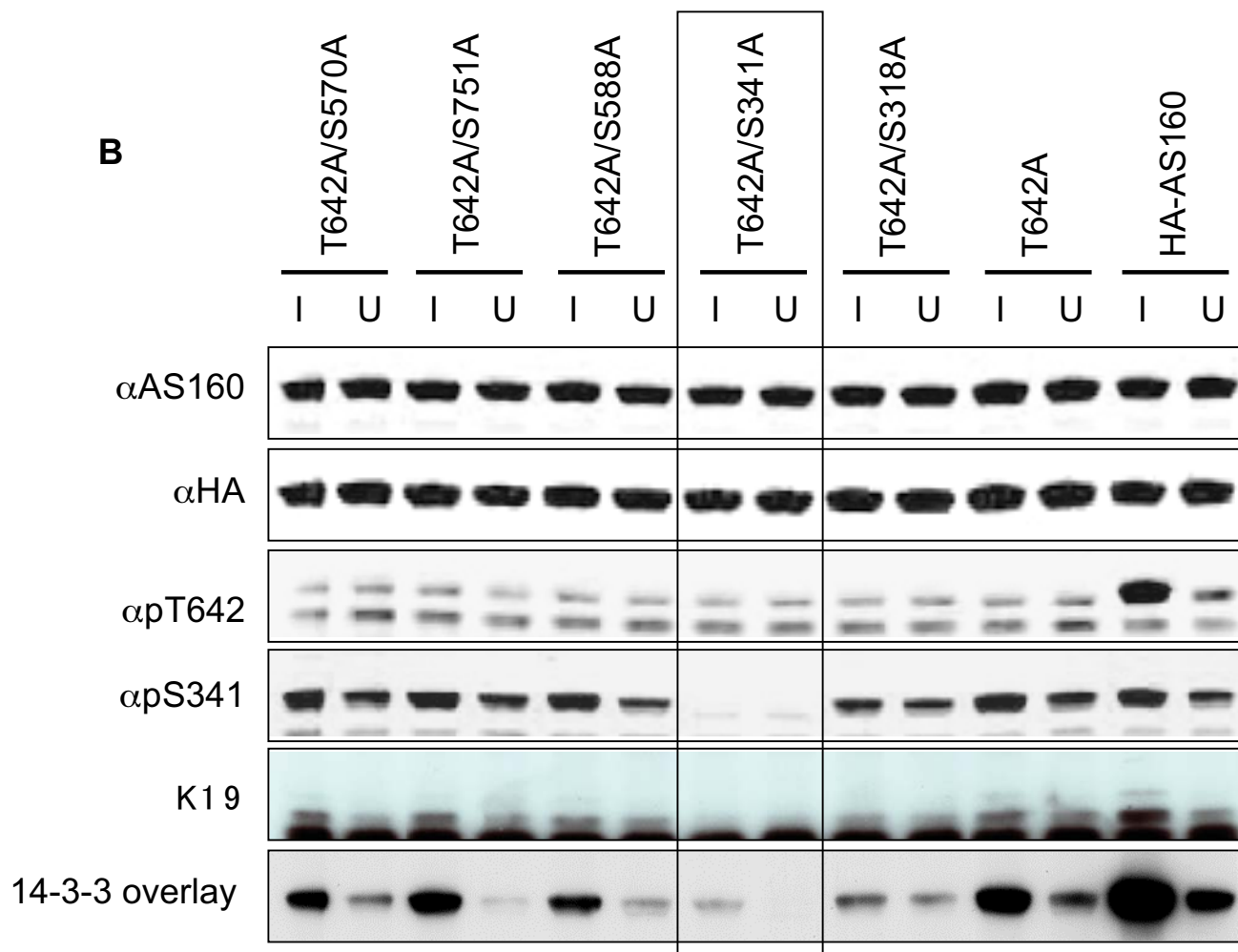


Fig 4A

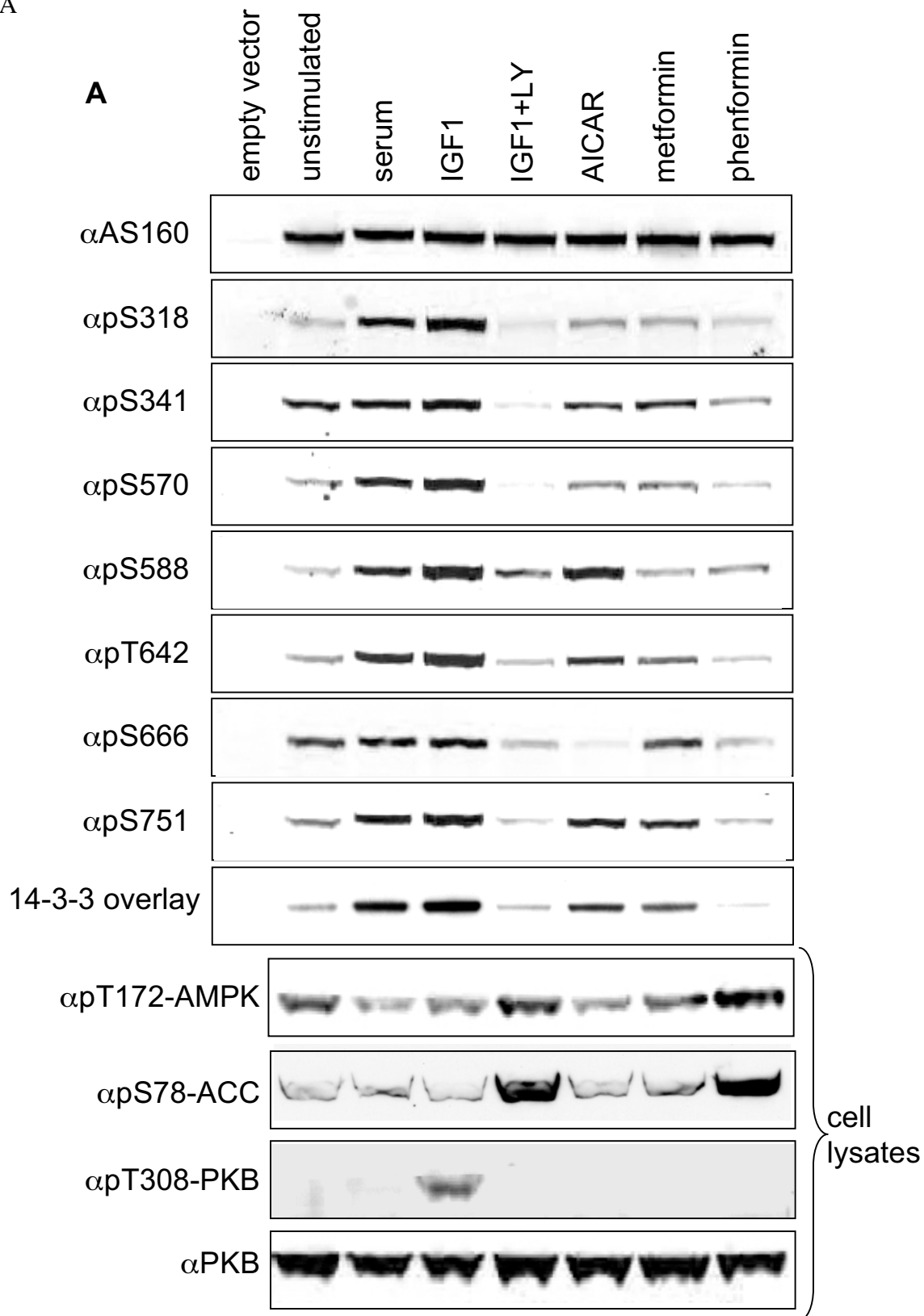


Fig 4B

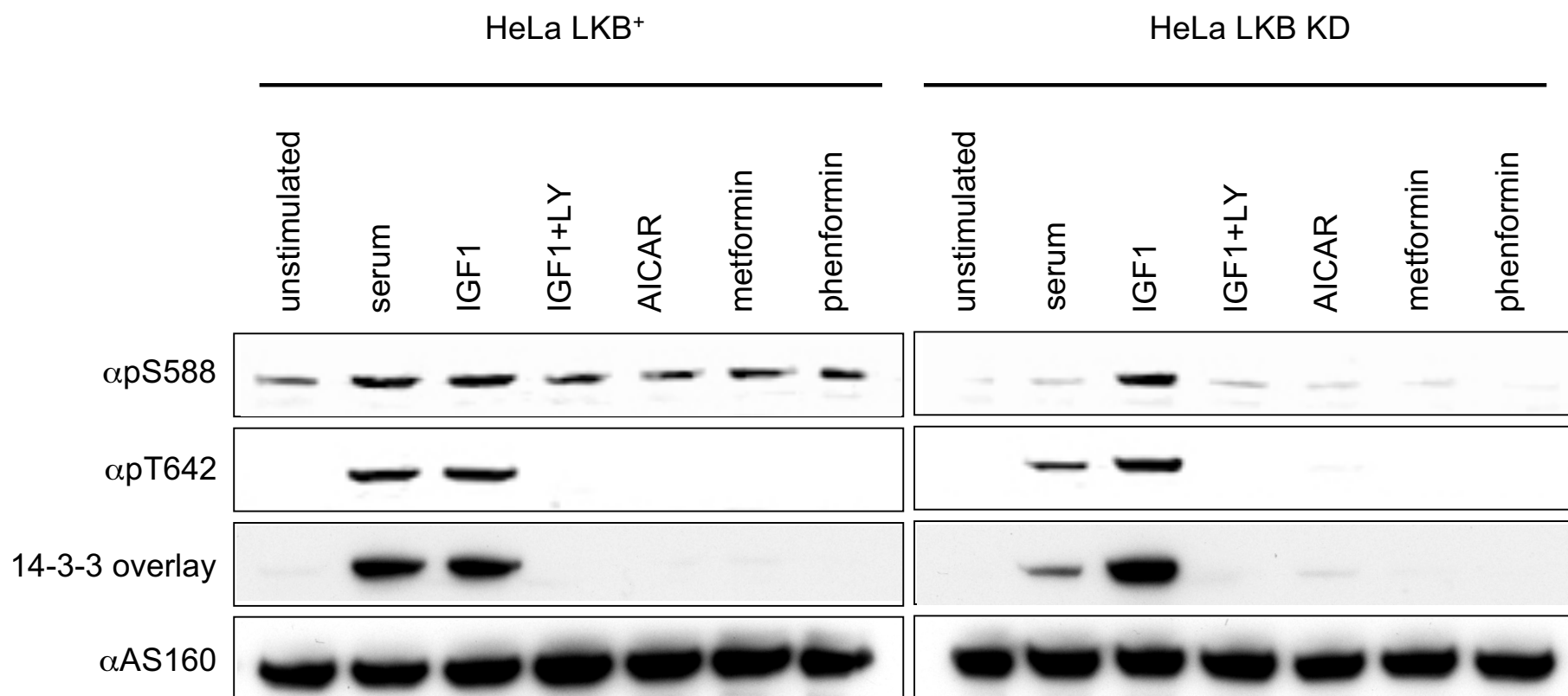


Fig 5

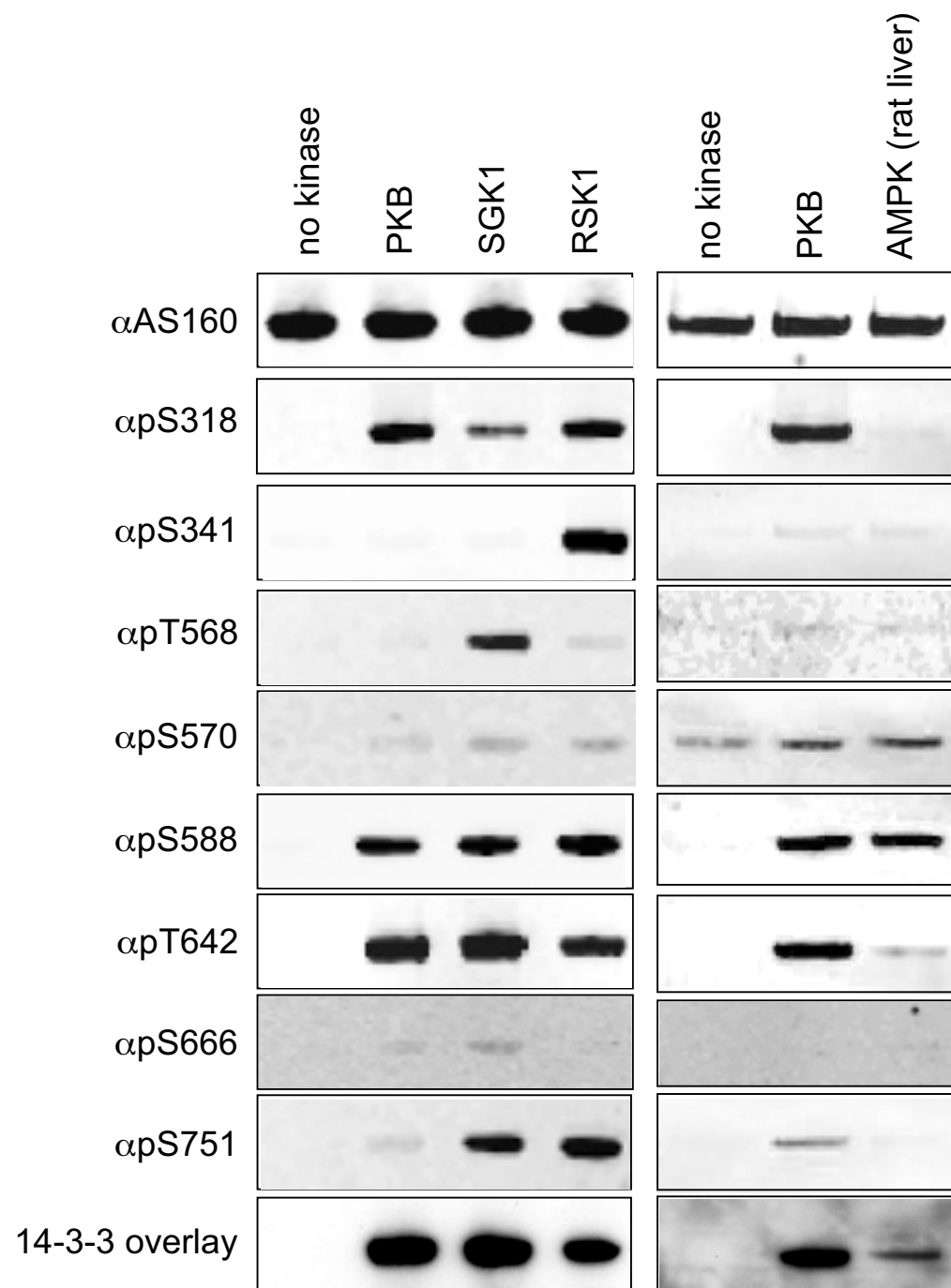


Fig 6

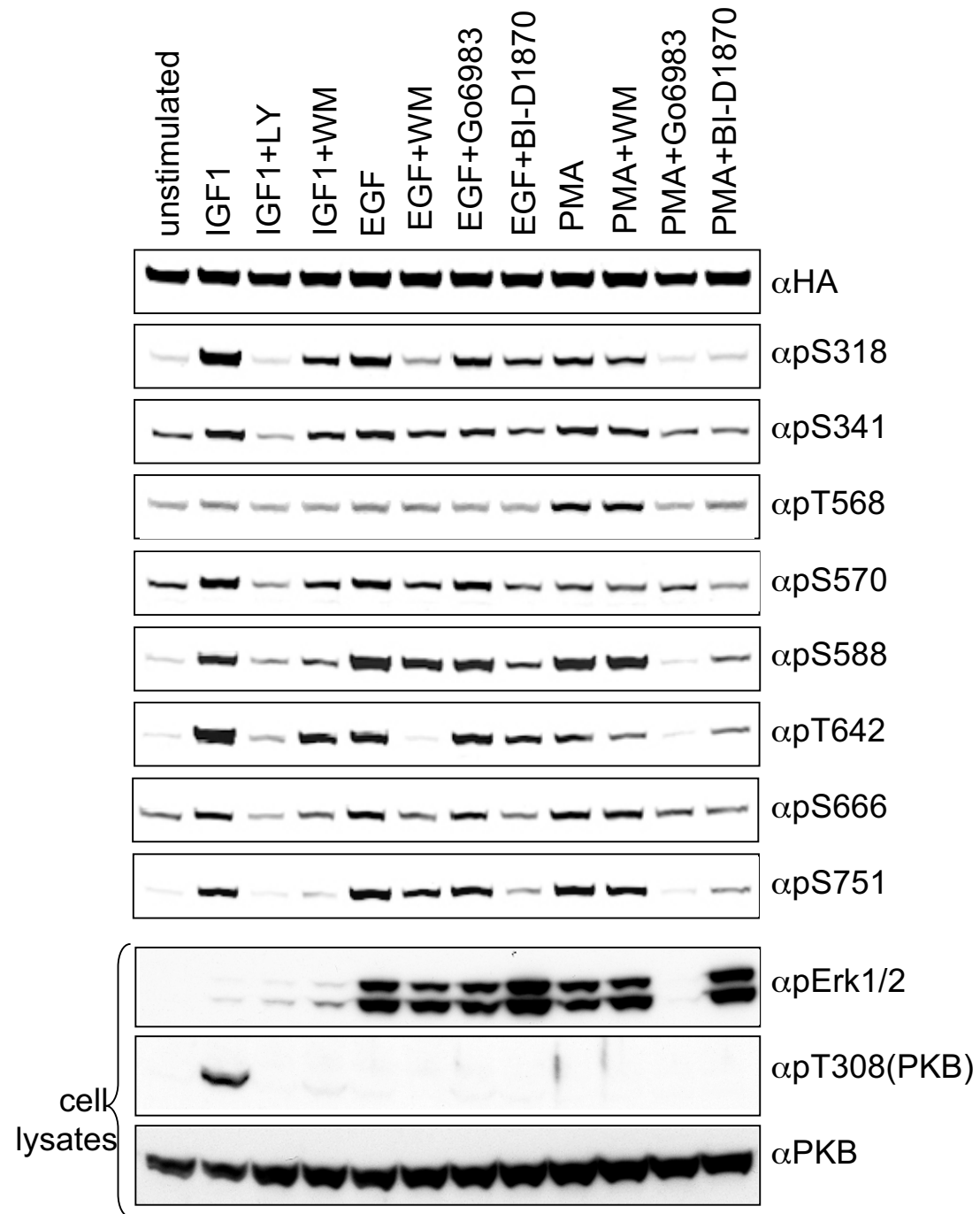


Fig 7

