Serine 474 phosphorylation is essential for maximal Akt2 kinase activity in adipocytes

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ABSTRACT
The Ser/Thr protein kinase Akt regulates essential biological processes such as cell survival, growth, and metabolism. Upon growth factor stimulation Akt is phosphorylated at Ser474, however how this phosphorylation contributes to Akt activation remains controversial. Previous studies, which induced loss of Ser474 phosphorylation by ablating its upstream kinase mTORC2, have implicated Ser474 phosphorylation as a driver of Akt substrate specificity. Here, we directly studied the role of Akt2 Ser474 phosphorylation in 3T3-L1 adipocytes by preventing Ser474 phosphorylation, without perturbing mTORC2 activity. This was achieved by utilising a chemical genetics approach, whereby ectopically expressed Ser474Ala Akt2 was engineered with a Trp80Ala mutation to confer resistance to the Akt inhibitor MK2206, and thus allow for its activation independently of endogenous Akt. We found that insulin-stimulated phosphorylation of four bona fide Akt substrates (TSC2, PRAS40, FOXO1/3a and AS160) were reduced by approximately 50% in the absence of Ser474 phosphorylation. Accordingly, insulin-stimulated mTORC1 activation, protein synthesis, FOXO nuclear exclusion, GLUT4 translocation and glucose uptake were attenuated upon loss of Ser474 phosphorylation. We propose a model whereby Ser474 phosphorylation is required for maximal Akt2 kinase activity in adipocytes.

INTRODUCTION
The serine/threonine kinase Akt (Protein Kinase B; PKB) is a key regulatory node in a range of cell signaling networks that control essential biological processes such as cell survival, proliferation and metabolism (1). Upon growth factor stimulation, there is an increase in plasma membrane phosphatidylinositol (3,4,5) triphosphate (PIP3) (2,3). Akt then binds PIP3 via its pleckstrin homology (PH) domain and accumulates at the plasma membrane (4,5). This interaction causes a conformational change, allowing PDK1 to phosphorylate Akt within its kinase domain at T309 (in Akt2, T308 in Akt1, T305 in Akt3), and mTORC2 to phosphorylate Akt within its hydrophobic motif (HM) at S474 (in Akt2, S473 in Akt1, S472 in Akt3) (6,7). These phosphorylation events enhance Akt kinase activity, allowing Akt to phosphorylate a number of substrates and facilitate several biological processes. For example, phosphorylation of TSC2 and PRAS40 activate mTORC1 and protein synthesis (8-10), phosphorylation of AS160/TBC1D4 facilitates GLUT4 translocation and glucose uptake (11,12), and phosphorylation of FOXO proteins regulate their transcriptional activity via nuclear exclusion (13,14).

While there is consensus that Akt T309 phosphorylation is required for its activation (6,15-17), the role of S474 phosphorylation remains
controversial. Initial studies which assessed Akt activity in vitro reported that loss of S474 phosphorylation severely diminished Akt kinase activity (6,17). Accordingly, Akt crystal structures show that an active kinase conformation is only possible by mimicking S474 phosphorylation with a S474D substitution (18,19). From this, a model arose whereby S474 phosphorylation was integral to a high level of Akt kinase activity.

However, the accuracy of this model has been disputed in more recent studies in cells, where S474 phosphorylation was required for Akt activity towards only specific substrates. These studies abolished S474 phosphorylation through genetic or pharmacological ablation of its upstream kinase, mTORC2. For example, in mouse embryonic fibroblasts lacking SIN1, one of the subunits of mTORC2, there was loss of FOXO1/3a phosphorylation but other Akt substrates (TSC2 and GSK3) and mTORC1 effectors (S6K and 4EBP1) were unaffected (20). Similarly, phosphorylation of FOXO3a and AS160 was impaired, but GSK3β phosphorylation was unaltered, in adipocytes from mice lacking the mTORC2 subunit RICTOR (21). Further, rat adipocytes treated with small molecule inhibitors of mTORC2 exhibited decreased phosphorylation of PRAS40, but not GSK3β (22). These studies have implicated S474 phosphorylation as a driver of Akt substrate specificity. Alternatively, a recent study using RICTOR knockout mice placed S474 phosphorylation as dispensable for maximal phosphorylation of AS160, PRAS40, FOXO1 and GSK3β in adipose tissue (23). Nevertheless, the use of mTORC2 deletion or inhibition to study the role of Akt S474 phosphorylation may be confounded by several factors including: 1) the induction of compensatory mechanisms due to chronic absence of mTORC2 (24); 2) the lack of specificity of mTORC2 inhibitors (25), and; 3) interference from other downstream actions of mTORC2 that are independent of Akt S474 phosphorylation, such as other phosphorylation sites on Akt (26-28).

A chemical genetics approach originally designed by Green et al. overcomes these disadvantages, by facilitating the acute prevention of S474 phosphorylation in cells without perturbing mTORC2 activity (29). An Akt phospho-mutant can be engineered to contain a W80A mutation in its PH domain, which confers resistance to the allosteric pan-Akt inhibitor MK2206, without compromising its activation. Treatment of cells ectopically expressing this mutant with MK2206 acutely eliminates endogenous Akt activity, allowing specific analysis of the W80A Akt mutant (29-31). We have utilised this system to address how loss of S474 phosphorylation influences Akt2 activity towards a number of substrates and insulin-regulated processes in 3T3-L1 adipocytes. We show that insulin-stimulated phosphorylation of four Akt substrates was reduced by approximately 50% in the absence of S474 phosphorylation. Similarly, several insulin-regulated processes were partially impaired following loss of S474 phosphorylation. These findings support a model where S474 phosphorylation is essential for maximal Akt2 kinase activity in adipocytes.

RESULTS

The Akt2 W80A mutation confers MK2206 resistance and is a powerful tool to study Akt2 mutants

Studying the activity of ectopically expressed mutant proteins in cells can be confounded by the presence of their endogenous counterparts. To circumvent this and examine the role of Akt2 S474 phosphorylation in 3T3-L1 adipocytes (hereafter referred to as adipocytes), we employed a chemical genetics approach originally designed by Green et al. (29). MK2206 is an Akt inhibitor that works by preventing Akt recruitment to the plasma membrane (PM), which is essential for its activation (5,32,33). However, Akt with the W80A mutation is resistant to MK2206 inhibition. Treatment of cells ectopically expressing W80A Akt with MK2206 prevents activation of endogenous Akt, allowing for specific assessment of the W80A Akt mutant upon insulin stimulation (Fig. 1A) (29-31).

To validate the W80A system we assessed insulin-stimulated PM recruitment of TagRFP-T-WT and W80A Akt2 in adipocytes using live cell total internal reflection fluorescence (TIRF) microscopy. There was no significant difference in the kinetics of insulin-stimulated WT and W80A Akt2 PM recruitment (Fig. 1B-C). Aligning with previous studies (34,35), both WT and W80A Akt2 exhibited
an overshoot in PM localisation in response to insulin. However, pre-treatment with 10 µM MK2206 abolished PM accumulation of WT Akt2, whereas robust accumulation of W80A Akt2 was observed under identical conditions (Fig. 1D-E). These data are consistent with the Akt2 W80A mutation conferring MK2206 resistance but having negligible impact on Akt2 recruitment to the PM.

We next generated a series of N-terminally FLAG-tagged Akt2 constructs that were engineered to express Akt2 with T309 or S474 mutated to alanine to prevent phosphorylation; wild type Akt2 (WT), Akt2 with the W80A mutation (W80A), Akt2 with W80A and T309A mutations (W80A-T309A), and Akt2 with W80A and S474A mutations (W80A-S474A) (Fig. 2A). These were stably expressed in adipocytes to similar degrees at approximately 75% of endogenous Akt2 (Fig. 2B-D). Ectopic FLAG-Akt2 could be separated from endogenous Akt2 (Supplementary Fig. 1). As expected, treatment of adipocytes expressing WT Akt2 with 10 µM MK2206 blocked insulin-stimulated phosphorylation of Akt at T309 and S474, and the phosphorylation of its substrates TSC2, PRAS40, FOXO1/3a and AS160 (Fig. 2E-F). MK2206 did not completely block insulin-stimulated phosphorylation of the Akt substrate GSK3β at S9 (Supplementary Fig. 2), suggesting GSK3β is also phosphorylated by another kinase at this site. Therefore, we did not use GSK3β phosphorylation as a read-out of Akt activity in this study since we could not confidently attribute changes in GSK3β phosphorylation to Akt.

In adipocytes expressing W80A Akt2, there was a time-dependent increase in the phosphorylation of all substrates following insulin stimulation (Fig. 3A-B). Distinct phosphorylation kinetics were observed between each substrate, aligning with previous reports of a lack of concordance in the behaviour of Akt substrates (36,38). Nevertheless, in adipocytes expressing W80A-S474A Akt2, substrate phosphorylation plateaued at approximately 50% of that observed in adipocytes expressing W80A Akt2 (Fig. 3A-B). These data suggest that S474 phosphorylation plays an important role in maximising Akt2 kinase activity towards TSC2, PRAS40, FOXO1/3 and AS160 in adipocytes, with no evidence for substrate specificity.

Throughout the insulin time course, total phosphorylation of Akt at T309 was not significantly different between adipocytes expressing W80A and W80A-S474A Akt2 (Fig. 3A-B). This finding is corroborated by previous studies where loss of S474 phosphorylation did not affect T309 phosphorylation (6,20), supporting the
Akt2 S474 phosphorylation is required for maximal insulin-stimulated mTORC1 activation and protein synthesis

We next assessed whether loss of Akt2 S474 phosphorylation alters the activation of the insulin signaling network beyond the immediate phosphorylation of Akt substrates. In response to insulin, Akt activates mTORC1 via phosphorylation of its substrates TSC2 and PRAS40 (8,9). mTORC1 then phosphorylates its substrates, which facilitate a variety of responses including an increase in the rate of protein synthesis (Fig. 4A) (10,39,40). Thus, we first assessed whether loss of S474 phosphorylation affects insulin-stimulated activation of mTORC1 and protein synthesis in adipocytes. We assessed mTORC1 activation by the phosphorylation status of its substrate, P70 S6K. In adipocytes expressing WT or kinase dead W80A-T309A Akt2, MK2206 blocked the ability of Akt2 to activate mTORC1 in response to insulin (Fig. 4B-C). Similarly, following MK2206 treatment, adipocytes expressing WT Akt2 could not facilitate insulin-stimulated protein synthesis (Fig. 4D), aligning with these events being activated downstream of Akt. In the presence of MK2206, adipocytes expressing W80A-S474A Akt2 exhibited partially impaired P70 S6K phosphorylation, S6 phosphorylation and protein synthesis compared to adipocytes expressing W80A Akt2 (Fig. 4B-D). These data, together with diminished TSC2 and PRAS40 phosphorylation (Fig. 3A-B) suggest that Akt2 S474 phosphorylation is essential for maximal insulin-stimulated mTORC1 activation and protein synthesis.

Akt2 S474 phosphorylation is required for maximal insulin-stimulated FOXO nuclear exclusion

Akt phosphorylates FOXO proteins to regulate their transcriptional activity via nuclear exclusion (Fig. 4A) (13,14). Therefore, we next assessed the functional consequence of Akt-mediated FOXO phosphorylation by examining whether Akt2 S474 phosphorylation is required for insulin-stimulated FOXO1 nuclear exclusion. In adipocytes expressing WT Akt2, MK2206 inhibited insulin-stimulated FOXO1 nuclear exclusion (Fig. 4E-F), consistent with it being Akt-dependent. In adipocytes expressing W80A-S474A Akt2, FOXO1 nuclear exclusion was reduced by approximately 50% when compared to adipocytes expressing W80A Akt2 (Fig. 4E-F). These observations, together with impaired FOXO1/3a phosphorylation (Fig. 3A-B), suggest that Akt2 S474 phosphorylation is required for maximal insulin-stimulated FOXO nuclear exclusion.

Akt2 S474 phosphorylation is required for maximal insulin-stimulated GLUT4 translocation and glucose uptake

Finally, we assessed whether Akt2 S474 phosphorylation is required for insulin-stimulated GLUT4 translocation and glucose uptake in adipocytes. Akt activation is both necessary and sufficient for insulin-stimulated glucose uptake (41). This is predominantly achieved through the phosphorylation of AS160, which is required for translocation of GLUT4 to the PM (Fig. 4A) (30,42). Consistent with this, MK2206 blocked insulin-stimulated GLUT4 translocation and glucose uptake in adipocytes expressing WT Akt2 (Fig. 4G-I). In the presence of MK2206, both GLUT4 translocation and glucose uptake were activated by insulin in adipocytes expressing W80A Akt2, however only partial activation was achieved in adipocytes expressing W80A-S474A Akt2 (Fig. 4G-I). These observations, together with impaired AS160 phosphorylation (Fig. 3A-B), suggest that Akt2 S474 phosphorylation is essential for maximal insulin-stimulated GLUT4 translocation and glucose uptake.

DISCUSSION

Akt is a major regulatory node for numerous biological processes, yet the mechanism by which it is activated remains controversial. It is widely accepted that T309 phosphorylation is essential for Akt activation (6,15-17), however the role of S474 phosphorylation is unclear. Here, we have examined how loss of S474 phosphorylation influences Akt2 activity towards a number of substrates and insulin-regulated processes in 3T3-L1 adipocytes. Insulin-stimulated phosphorylation of four canonical Akt substrates was reduced by approximately 50% in the absence of S474 phosphorylation. Accordingly, we measured reduced efficiency of several insulin-regulated
Regulation of Akt2 kinase activity by Ser474 phosphorylation

Overall, our findings suggest that S474 phosphorylation regulates the full activation of Akt2 towards a cadre of substrates and biological processes in adipocytes.

Previous studies have proposed an alternate model whereby S474 phosphorylation is required for Akt activity towards only select substrates. Evidence for this model is largely derived from studies where S474 phosphorylation was prevented via ablation of its upstream kinase, mTORC2 (20-22). However, by interfering with the upstream kinase of S474 these studies have several disadvantages such as the possible induction of long-term compensatory mechanisms, and interference with other downstream actions of mTORC2, independent of Akt S474 phosphorylation. MK2206 resistant W80A Akt offered a valuable tool to overcome these disadvantages and interrogate the role of Akt2 S474 phosphorylation in cells through direct mutation of S474. Despite examining a cadre of Akt substrates and downstream biological processes, many of which were identical to those examined in prior studies, our data does not illustrate selective signaling in the absence of S474 phosphorylation – but rather a global decrease in Akt2 activity. We cannot exclude that these differences between our data and previous reports are due to cell specific effects, as each study was undertaken in a different cell line, or that the role of S474 phosphorylation differs for each Akt isoform. However, we speculate that these disparities are more likely reflective of other events downstream of mTORC2 conferring Akt substrate specificity, rather than S474 phosphorylation itself. For example, mTORC2 has been shown to phosphorylate other sites on Akt, such as T450 on its turn motif (27,28), and S477/T479 on its hydrophobic motif (26). This, as well as other reported mechanisms of Akt substrate specificity (43), deserve further investigation largely due to their applicability to cancer treatments. Akt inhibitors have been tested in clinical trials, however while they can successfully inhibit cancer growth, hyperglycaemia often accompanies treatment (44,45). This is not surprising considering Akt controls glucose metabolism. To design cancer therapeutics without these metabolic toxicities, it is of interest to therapeutically target specific actions of Akt, rather than using pan-Akt inhibitors.

A major advantage of the W80A system used in this study is that it allowed the investigation of Akt mutants in cells rather than in vitro. Our work in cells suggests that Akt can achieve half of its maximal kinase activity without S474 phosphorylation, which is in striking contrast to in vitro studies showing no or very little kinase activity in the absence of S474 phosphorylation (6,17,49). This difference is likely reflective of in vitro systems not reproducing complex cellular processes in the absence of S474 phosphorylation.
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control points, comprising signal amplification, macromolecular crowding, interacting proteins, protein localisation, and positive/negative feedback (36,49,50). As substantial Akt activation occurs without S474 phosphorylation in the cell, our data aligns with studies reporting that S474 phosphorylation poorly correlated with Akt activity (51). Yet, S474 phosphorylation is used in countless studies as a proxy of Akt activity. Rather, we suggest using the phosphorylation of Akt at T309 or its substrates as more accurate indices of Akt activation (52).

While we show a global defect in insulin-stimulated Akt2 activity upon loss of S474 phosphorylation, a recent study by Beg et al. using the same W80A system in 3T3-L1 adipocytes reported that insulin-stimulated GLUT4 translocation and mTORC1 activation were unaffected by loss of Akt2 S474 phosphorylation (30). There are differences between our studies which may explain these disparities. Most importantly, Beg et al. used a dose of MK2206 (1 µM) that they, and others have shown is insufficient to completely impair substrate phosphorylation by endogenous Akt (30,53). Having residual endogenous Akt activity will likely alter the behaviour of the ectopic W80A Akt mutant, as Akt activity is extremely sensitive to temporal changes in its activation (54-56). Even a minute amount of residual endogenous Akt activity may skew signaling outcomes, as only a small percentage of the total Akt pool is required to achieve a maximal response for many downstream processes (36,37). In contrast, we utilised 10 µM MK2206 as it was sufficient to block endogenous Akt activation, allowing for specific analysis of ectopic W80A Akt2 (Fig. 1D-E, Fig. 2E-F). Furthermore, to control for endogenous Akt inhibition, Beg et al. used untransfected 3T3-L1 adipocytes (30). However, based on our studies it is important to use adipocytes expressing FLAG-WT Akt2, to ensure effective comparison with adipocytes expressing FLAG-W80A Akt2. Nevertheless, Beg et al. also reported that insulin-stimulated GLUT1 translocation requires Akt2 S474 phosphorylation (30), which we support (Supplementary Fig. 4). These data suggest that further inquiry may reveal Akt substrates that control GLUT1 translocation and require S474 phosphorylated Akt for activation. However, an alternate explanation is that maximal Akt activity may be required to trigger insulin-stimulated GLUT1 translocation - this result may not reflect Akt substrate specificity per se, but rather a difference in the dose-response relationship between Akt activation and various downstream processes.

The mechanism by which substantial Akt activity is possible in the cell without S474 phosphorylation remains elusive. Akt crystal structures suggest that hydrophobic motif (HM) binding to the N-lobe of the kinase domain is required for an active conformation, and that S474 phosphorylation is essential for this interaction (18,19). We speculate that this interaction can occur in the absence of S474 phosphorylation in cells - this weak interaction could be more favourable in cells than in vitro due to macromolecular crowding, or stabilisation by interacting proteins (50). Intriguingly, other AGC kinases such as Protein Kinase A lack a phosphosite within their HM, yet it retains the ability to interact with its kinase domain and induce activity (18,19,57). Thus, S474 phosphorylation may increase the affinity of the HM for the kinase domain, but not be a prerequisite for the interaction nor Akt activation. Alternatively, it has recently been found that the HM interacts with the PH-kinase domain linker to relieve PH domain-mediated autoinhibition and increase Akt activity (58). Whether this interaction can occur without S474 phosphorylation remains to be elucidated. Uncovering the mechanism by which substantial Akt kinase activity is possible without S474 phosphorylation is essential as there is interest in developing therapeutics which inhibit Akt by targeting its hydrophobic motif (59).

EXPERIMENTAL PROCEDURES

Cloning
TagRFP-T-Akt2 has been previously described (34). TagRFP-T-Akt2 with the W80A mutation was generated using site directed mutagenesis (60). For FLAG-Akt2 constructs, Akt2 was amplified by PCR from human cDNA, with the addition of attB sites either end to be compatible with gateway cloning. Akt2 cDNA was inserted into pDONR221 (Thermo Fisher Scientific) using Gateway™ BP Clonase™ II Enzyme Mix (Invitrogen) and then into the retroviral vector pMIG-t-sapphire-
puromycin (pMIG-tsap-puro) using Gateway™ LR Clonase™ II Enzyme Mix (Invitrogen). A FLAG tag was then added to the N-terminus of Akt2 using Gibson assembly cloning (61). Site directed mutagenesis (60) was used to create FLAG-tagged W80A, W80A-T309A, and W80A-S474A Akt2 plasmids. For FOXO1-mNeonGreen, the N-terminal region of FOXO1 (1-380) containing the nuclear localisation and nuclear export sequences was codon optimised and synthesised as a gene block (IDT technologies). An mNeonGreen tag was placed at its C-terminus, and this was cloned into the mammalian expression vector pCDNA3.1. The mutations S209A, H212A and S215A were introduced to impair DNA binding and phosphorylation by STK4 as previously described (62). pHluorin-GLUT4-mRuby3 was generated from pHluorin-GLUT4-tdTomato (63) by replacing tdTomato with mammalian expression optimised mRuby3 synthesised as a gene block (IDT technologies). Primer and construct sequences are available upon request. All constructs were confirmed by Sanger Sequencing.

**Cell culture**

3T3-L1 fibroblasts obtained from the Howard Green Laboratory (Harvard Medical School) were cultured in high glucose Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco by Life Technologies) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco by Life Technologies), and 1x GlutaMAX (Gibco by Life Technologies) at 37°C and 10% CO₂. All cell lines were mycoplasma free. For the generation of 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants, fibroblasts were transduced in the presence of 8 µg/mL hexadimethrine bromide (polybrene) (Sigma-Aldrich) with pMIG-tsap-puro (empty vector control), or pMIG-tsap-puro-FLAG-Akt2 retrovirus, generated from Plat-E cells (Cell Biolabs San Diego) as previously described (64). Transduced cells were then selected using 2 µg/mL puromycin. Cells were differentiated into adipocytes as previously described (65), and used for experiments 7-12 days after the initiation of differentiation. To minimise differences between cells expressing each Akt2 mutant, retroviral transduction was performed simultaneously using the same population of 3T3-L1 fibroblasts. Cells were infected with high virus titres such that there was minimal cell loss upon antibiotic selection. Then, to minimise differences in genetic drift each cell population was subject to identical culturing conditions, used for experiments at the same passage, and not passaged more than ten times. Macroscopically, we observed no morphological differences between cell populations, at any stage throughout their differentiation into adipocytes. Furthermore, we have performed the central experiments comprising Fig. 3A-B and Fig. 4B-D with three independently derived cell populations. Each achieved identical results, whereby S474 phosphorylation was required for maximal Akt2 kinase activity.

**Western blotting**

3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were serum starved with DMEM containing 1x GlutaMAX and 0.2% BSA (w/v) for 2 h. Cells were then exposed to 10 µM MK2206 (Selleckchem) or DMSO (Sigma-Aldrich) vehicle control for 5 min, followed by 1 nM insulin. We utilised 10 µM MK2206 to inhibit endogenous Akt, as we have previously shown that this dose is required to completely inhibit Akt activation (53). Cells were then placed on ice, washed with cold phosphate buffered saline (PBS), lysed with 1% (w/v) SDS in PBS containing protease inhibitors (Roche Applied Science) and phosphatase inhibitors (2 mM Na₃VO₄, 1 mM Na₄O₇P₂, 10 mM NaF), and tip probe sonicated. Lysates were centrifuged at 13,000 x g for 15 min at 4 °C. The lipid layer was removed, and protein content quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). 10 µg of lysate was then resolved by SDS-PAGE, transferred to PVDF membranes, and immunoblotted as described previously (39). All primary antibodies were from Cell Signaling Technology, except 14-3-3 was from Santa Cruz Biotechnology. Densitometry analysis was performed using ImageStudioLite version 5.2.5 (LI-COR). Band intensities were normalised to the loading control (14-3-3). Statistical tests were performed using GraphPad Prism version 7.0.

**Live cell total internal reflection fluorescence (TIRF) microscopy**

Matrigel coated coverslips were prepared as previously described (34). To assess insulin-stimulated GLUT4 translocation to the plasma membrane, 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were electroporated 7 d post-
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differentiation with 6 µg pHluorin-GLUT4-mRuby3. To assess insulin-stimulated Akt2 recruitment to the plasma membrane, 3T3-L1 adipocytes were electroporated 6-8 d post-differentiation with 6 µg WT or W80A TagRFP-T-Akt2. Then, cells were placed onto the Matrigel coated coverslips as previously described (34). 24-48 h post electroporation, adipocytes were serum starved for 2 h and then incubated at 37 °C with Krebs-Ringer-Phosphate-HEPES (KRPH) buffer (0.6 mM Na₂HPO₄, 0.4 mM NaH₂PO₄, 120 mM NaCl, 6 mM KCl, 1mM CaCl₂, 1.2 mM MgSO₄, 12.5 mM HEPES, pH 7.4) supplemented with 10 mM Glucose, 1x MEM amino acids (Gibco by Life Technologies), 1x GlutaMAX, and 0.2% (w/v) BSA. The cells were then treated with 10 µM MK2206 and/or 1 nM insulin using a custom made perfusion system. Images were acquired using the Nikon Ti-Lapps H-TIRF module angled to image ~90 nm into cells. Temperature and humidity were maintained using an Okolab cage incubator and temperature control. The change in pixel intensity over time was determined for each individual cell, using Fiji (66). The average pixel intensity over time for each cell was normalised to its average intensity over the basal period.

³H-2-deoxyglucose uptake assay
3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were serum starved in leucine-free DMEM supplemented with 0.2% BSA and 20mM HEPES (pH 7.4) for 2 h. Cells were then treated with 10 µM MK2206 for 5 min, followed by 5 µCi/mL ³H-Leucine (Perkin-Elmer) and 1 nM insulin for 1 h. To determine background radiation, 10 µM cycloheximide, a translation inhibitor, was added 30 min before ³H-Leucine to a single well. Leucine incorporation was terminated by placing the plate on ice, washing with cold PBS, and then incubating cells with cold 10% (v/v) TCA for 10 min to lyse cells and precipitate protein. Pellets were washed 3 times in cold 10% (v/v) TCA to remove free ³H-Leucine. Pellets were resuspended in 50 mM NaOH with 1% (w/v) Triton X-100 in PBS at 65 °C for 30 min, and then added to a scintillation vial with 3mL Ultima Gold™ XR liquid scintillation cocktail (Perkin-Elmer). The radioactivity of each sample in DPM was determined using a Tri-Carb liquid scintillation counter (Perkin-Elmer). The protein content of each sample was assessed using the Pierce BCA Protein Assay Kit (Thermo Scientific). Assays were performed in triplicate, and the average of the triplicate was considered as 1 biological replicate. The DPM for each condition was converted to nmol leucine, and data was normalised to protein levels. The value for cycloheximide treated cells were subtracted from all other values, to account for background radiation. Statistical tests were performed using GraphPad Prism version 7.0.

³H-Leucine incorporation assay for protein synthesis
3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were serum starved in leucine-free DMEM supplemented with 0.2% BSA and 20mM HEPES (pH 7.4) for 2 h. Cells were then treated with 10 µM MK2206 for 5 min, followed by 5 µCi/mL ³H-Leucine (Perkin-Elmer) and 1 nM insulin for 1 h. To determine background radiation, 10 µM cycloheximide, a translation inhibitor, was added 30 min before ³H-Leucine to a single well. Leucine incorporation was terminated by placing the plate on ice, washing with cold PBS, and then incubating cells with cold 10% (v/v) TCA for 10 min to lyse cells and precipitate protein. Pellets were washed 3 times in cold 10% (v/v) TCA to remove free ³H-Leucine. Pellets were resuspended in 50 mM NaOH with 1% (w/v) Triton X-100 in PBS at 65 °C for 30 min, and then added to a scintillation vial with 3mL Ultima Gold™ XR liquid scintillation cocktail (Perkin-Elmer). The radioactivity of each sample in DPM was determined using a Tri-Carb liquid scintillation counter (Perkin-Elmer). The protein content of each sample was assessed using the Pierce BCA Protein Assay Kit (Thermo Scientific). Assays were performed in triplicate, and the average of the triplicate was considered as 1 biological replicate. The DPM for each condition was converted to nmol leucine, and data was normalised to protein levels. The value for cycloheximide treated cells were subtracted from all other values, to account for background radiation. Statistical tests were performed using GraphPad Prism version 7.0.

Live cell spinning disk confocal microscopy
Matrigel coated coverslips were prepared as previously described (34). To assess insulin-stimulated FOXO nuclear exclusion, 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were electroporated 6 d post-differentiation with 6 µg FOXO1(1-380; S209A, H212A, S215A)-
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mNeonGreen. Then, cells were placed onto the Matrigel coated coverslips as previously described (34). 24 h post electroporation, adipocytes were serum starved for 2 h and then stained with Hoechst 33342 for 10 min. Adipocytes were then incubated at 37 °C with KRPH buffer supplemented with 10 mM Glucose, 1x MEM amino acids, 1x GlutaMAX, and 0.2% (w/v) BSA. The cells were then treated with 10 µM MK2206 and 1 nM insulin using a custom made perfusion system. Images were acquired on a Nikon Ti inverted confocal with an Andor Discovery spinning disk system and an Andor Ixon 888 EmCCD camera (Andor). Temperature and humidity were maintained using an Okolab cage incubator and temperature control. For each individual cell, the median pixel intensity of the cytosol was divided by the median pixel intensity of the nucleus using Fiji (66).

Lambda protein phosphatase assay
3T3-L1 adipocytes were serum starved for 2 h, and then treated with 100 nM insulin for 10 min. Cells were placed on ice, washed with cold PBS and harvested in 1% (w/v) Triton X-100 in PBS. Cells were homogenized by passing through a 22-gauge needle 6 times and a 27-gauge needle 6 times prior to centrifugation at 12,000 x g for 15 min. 100 µg of the supernatant was treated with 1 mM MnCl₂ (New England Biolabs), 1x NEBuffer for Protein MetalloPhosphatases (New England Biolabs), and either 800 U of Lambda Protein Phosphatase (New England Biolabs), or phosphatase inhibitors (10 mM Na₃VO₄, 5 mM Na₂O-P₃, 50 mM NaF) at 30 °C for 15 min. Reactions were stopped with 2% (w/v) SDS, and then resolved by SDS-PAGE.

Subcellular fractionation
3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were serum starved for 2 h, and then exposed to 10 µM MK2206 for 5 min, followed by 0.5 nM insulin for 20 min. Cells were placed on ice, washed with cold PBS and harvested in cold HES buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, pH 7.4) containing phosphatase and protease inhibitors. All subsequent steps were carried out at 4 °C as previously described (67). Briefly, cells were homogenised using a dounce tissue grinder prior to centrifugation at 500 x g for 10 min. The supernatant was centrifuged at 13,550 x g for 12 min to pellet the plasma membrane and mitochondria/nuclei. This pellet was resuspended in HES buffer and again centrifuged at 13,550 x g for 12 min. The pellet was then resuspended in HES buffer, layered over high sucrose HES buffer (20 mM HEPES, 1 mM EDTA, 1.12 M sucrose, pH 7.4), and centrifuged at 111,160 x g for 60 min in a swing-out rotor. The PM fraction was collected at the interface between the sucrose layers, and pelleted by centrifuged at 235,000 x g for 75 min. The PM pellet was resuspended in HES buffer containing phosphatase and protease inhibitors. Protein concentrations for each fraction was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific).
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CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest with the contents of this article.

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**ABBREVIATIONS**

2DOG: 2-deoxyglucose
4EBP1: eukaryotic translation initiation factor 4E binding protein 1
AS160: Akt substrate of 160kDa
AU: arbitrary unit
DMEM: Dulbecco’s modified eagle’s medium
DPM: disintegrations per minute
EV: empty vector
FOXO: forkhead box O
GLUT1: glucose transporter type 1
GLUT4: glucose transporter type 4
GSK3: glycogen synthase kinase 3
HES: HEPES-EDTA-sucrose
HM: hydrophobic motif
IR: insulin receptor
KD: kinase domain
KRPH: Krebs-ringer-phosphate-HEPES
mTORC1: mammalian target of rapamycin complex 1
mTORC2: mammalian target of rapamycin complex 2
P70S6K: ribosomal protein S6 kinase
PDK1: phosphoinositide-dependent kinase 1
PH: pleckstrin homology
PIP3: phosphatidylinositol (3,4,5) triphosphate
PM: plasma membrane
pMIG-tsap-puro: pMIG-t-sapphire-puromycin
PRAS40: Akt substrate of 40kDa
RFP: red fluorescent protein
TBC1D4: TBC1 domain family member 4
TIRF: total internal reflection fluorescence
TSC2: tuberous sclerosis complex 2
FIGURE LEGENDS

Figure 1. The Akt2 W80A mutation confers MK2206 resistance and has negligible impact on Akt2 activation at the plasma membrane in 3T3-L1 adipocytes. (A) The Akt W80A mutation confers resistance to the Akt inhibitor MK2206. Treatment of cells ectopically expressing W80A Akt with MK2206 prevents the activation of endogenous Akt, allowing for specific activation of the W80A Akt mutant upon insulin stimulation (PM; plasma membrane, PIP3; phosphatidylinositol (3,4,5) triphosphate, IR; insulin receptor, P; phosphorylation site). (B) 3T3-L1 adipocytes were electroporated with TagRFP-T-WT or W80A Akt2, and plasma membrane recruitment assessed using live cell TIRF microscopy. Adipocytes were exposed to 1 nM insulin. Representative images for 3-7 independent experiments are presented. (C) Quantification of panel B (WT; 41 cells from 3 independent experiments, W80A; 60 cells from 7 independent experiments, mean ± SEM, PM; plasma membrane). (D) 3T3-L1 adipocytes were electroporated with TagRFP-T WT or W80A Akt2, and plasma membrane recruitment assessed using live cell TIRF microscopy. Adipocytes were exposed to 10 µM MK2206 for 5 min followed by 1 nM insulin. Representative images for 2 independent experiments are presented. (E) Quantification of panel D (WT; 15 cells from 2 independent experiments, W80A; 59 cells from 2 independent experiments, mean ± SEM, PM; plasma membrane).

Figure 2. W80A Akt2 is resistant to MK2206 and can be used to study Akt2 mutants in 3T3-L1 adipocytes independently of endogenous Akt. (A) FLAG-tagged Akt2 constructs were generated; WT, W80A, W80A-T309A, and W80A-S474A Akt2. Each consisted of mutations in the pleckstrin homology (PH) domain, kinase domain (KD) and hydrophobic motif (HM). (B) Lysates from 3T3-L1 adipocytes stably expressing empty vector (EV), WT, W80A, W80A-T309A, or W80A-S474A Akt2 were immunoblotted using antibodies as specified, with 14-3-3 as a loading control. A representative blot for 3 independent experiments is shown. (C) Quantification of FLAG expression in panel B (n=3, mean ± S.E.M, AU; arbitrary unit). (D) Quantification of Akt2 expression in panel B relative to EV cell line, which represents endogenous Akt2 (n=3, mean ± S.E.M). (E) 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were treated with 10 µM MK2206 for 5 min followed by 1 nM insulin for 10 min. Lysates were immunoblotted with antibodies as specified, with 14-3-3 as a loading control. A representative blot for 3 independent experiments is presented (‘Band 1’ and ‘Band 2’ indicate shifts in Akt2 gel migration, p; phosphorylated). (F) Quantification of panel E (n=3, mean ± S.E.M, AU; arbitrary unit, p; phosphorylated).

Figure 3. Akt2 S474 phosphorylation is required for maximal insulin-stimulated phosphorylation of PRAS40, AS160, FOXO1/3a and TSC2 in 3T3-L1 adipocytes. 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were treated with 10 µM MK2206 for 5 min, followed by 1 nM insulin for the times specified. (A) Lysates were immunoblotted with antibodies as specified, with 14-3-3 as a loading control. A representative blot for 3-4 independent experiments is presented (‘Band 1’ and ‘Band 2’ indicate shifts in Akt2 gel migration, p; phosphorylated). (B) Quantification of panel A (n=3-4, mean ± S.E.M, two-way ANOVA; * p < 0.05, ns; not significant, AU; arbitrary unit, p; phosphorylated).

Figure 4. Akt2 S474 phosphorylation is required for maximal insulin-stimulated mTORC1 activation, protein synthesis, FOXO nuclear exclusion, GLUT4 translocation and glucose uptake in 3T3-L1 adipocytes. (A) Akt facilitates insulin-stimulated GLUT4 translocation, glucose uptake, mTORC1 activation, protein synthesis, and FOXO nuclear exclusion. (B) 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were treated with 10 µM MK2206 for 5 min, followed by 1 nM insulin for 10 min. Lysates were immunoblotted with antibodies as specified, with 14-3-3 as a loading control. A representative blot for 3 independent experiments is presented (‘Band 1’ and ‘Band 2’ indicate shifts in Akt2 gel migration, p; phosphorylated). (C) Quantification of panel B (n=3, mean ± S.E.M, two-tailed paired t-test; * p < 0.05, AU; arbitrary unit, p; phosphorylated). (D) 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were treated with 10 µM MK2206 for 5 min, followed by 1 nM insulin for 1 h and assessed for 3H-Leucine incorporation for protein synthesis (n=4, mean ± S.E.M, two-tailed paired t-test; * p < 0.05). (E) 3T3-L1
adipocytes stably expressing FLAG-Akt2 mutants were electroporated with FOXO1-mNeonGreen. Cells were treated with 10 µM MK2206 for 5 min, followed by 1 nM insulin and nuclear exclusion assessed using live cell spinning disk confocal microscopy. Representative images for 3 independent experiments is presented. (F) Quantification of panel E (WT; 45 cells from 3 independent experiments, W80A; 63 cells from 3 independent experiments, W80A-S474A; 63 cells from 3 independent experiments, mean ± SEM, PM; plasma membrane). (G) 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were electroporated with pHluorin-GLUT4-mRuby3. Cells were treated with 10 µM MK2206 for 5 min, followed by 1 nM insulin and GLUT4 translocation to the plasma membrane assessed using live cell TIRF microscopy. Representative images for 3 independent experiments is presented. (H) Quantification of panel G (WT; 78 cells from 3 independent experiments, W80A; 138 cells from 3 independent experiments, W80A-S474A; 135 cells from 3 independent experiments, mean ± SEM, PM; plasma membrane). (I) 3T3-L1 adipocytes stably expressing FLAG-Akt2 mutants were treated with 10 µM MK2206 for 5 min, followed by 1 nM insulin for 20 min and assessed for 3H-2-deoxyglucose uptake (n=4, mean ± S.E.M, two-tailed paired t-test; * p < 0.05).
Figure 1

A) Schematic diagram showing the activation of Akt by insulin and MK2206. 1) MK2206 induces the phosphorylation of Akt (WT) and the ectopic W80A Akt mutant. 2) Insulin activates endogenous WT Akt.

B) Fluorescence images of TagRFP-T-Akt2 in WT and W80A cells under basal and insulin treatment conditions.

C) Graph showing the fold change in PM Akt over basal for WT and W80A cells treated with 1nM insulin.

D) Fluorescence images of TagRFP-T-Akt2 in WT and W80A cells treated with MK2206 and MK2206 + insulin.

E) Graph showing the fold change in PM Akt over basal for WT and W80A cells treated with MK2206 and 1nM insulin.
Figure 2

A

WT | FLAG | PH | KD | HM
---|-----|----|----|----
W80A | PH | PH | KD | HM
W80A | PH | PH | KD | HM
W80A T309A | PH | PH | KD | HM
W80A S474A | PH | PH | KD | HM

B

EV | WT | W80A | W80A T309A | W80A S474A
---|----|-------|-------------|--------
FLAG |       |       |             |        
Akt2 |       |       |             |        
14-3-3 |       |       |             |        

C

Relative FLAG Expression (AU)

D

Relative Akt2 Expression (Relative to endogenous)

E

| Treatment | WT | W80A | W80A T309A |
|-----------|----|------|-------------|
| Insulin (1nM) | - | + | - | + |
| MK2206 (10μM) | + | + | + | + |

pAkt T309

pAkt S474

Akt2

FLAG

pTSC2 T1462

TSC2

pPRAS40 T246

PRAS40

pFOXO1/3a T24/32

FOXO1

pAS160 T642

AS160

14-3-3

F

Relative pAkt T309 (AU)

Relative pAkt S474 (AU)

Relative pPRAS40 T246 (AU)

Relative pFOXO1/3a T24/32 (AU)

Relative pAS160 T642 (AU)

Relative TSC2 T1462 (AU)
Figure 3

A

| Protein                | W80A          | W80A S474A |
|------------------------|---------------|------------|
| pAkt T309              | Band 1 60     | Band 1 60  |
| pAkt S474              | 60            | 60         |
| FLAG                   | 60            | 60         |
| pTSC2 T1462            | 260           | 260        |
| TSC2                   | 260           | 260        |
| pPRAS40 T246           | 40            | 40         |
| PRAS40                 | 40            | 40         |
| pFOXO1/3a T24/32       | 80            | 80         |
| FOXO1                  | 80            | 80         |
| pAS160 T642            | 160           | 160        |
| AS160                  | 160           | 160        |
| 14-3-3                 | 30            | 30         |

B

Relative pAkt T309 (AU) W80A vs. W80A-S474A

Relative pAkt S474 (AU) W80A vs. W80A-S474A

Relative pTSC2 T1462 (AU) W80A vs. W80A-S474A

Relative pPRAS40 T246 (AU) W80A vs. W80A-S474A

Relative pFOXO1/3a T24/32 (AU) W80A vs. W80A-S474A

Relative pAS160 T642 (AU) W80A vs. W80A-S474A

ns: not significant

*: statistically significant
Figure 4

A. 

GLUT4 Translocation
Akt
mTORC1 activation
FOXO nuclear exclusion
Protein Synthesis

B. 

|                  | WT | W80A | W80A T309A | W80A S474A |
|------------------|----|------|------------|------------|
| Insulin (1nM)    |    |      |            |            |
| MK2206 (10μM)    |    |      |            |            |
|                  | +  | -    | +          | +          |
|                  | -  | +    | -          | -          |
|                  | +  | -    | +          | +          |
|                  | -  | +    | -          | -          |
|                  | +  | -    | +          | +          |
|                  | -  | +    | -          | -          |
|                  | +  | -    | +          | +          |
|                  | -  | +    | -          | -          |

C. Relative p70 S6K T389 (AU)

D. Leucine incorporation into protein (pmol/mg/hr)

E. WT Akt2

F. Cytosolic/Nuclear FOXO1

G. GLUT4-mRuby3

H. PM GLUT4 (Fold over basal)

I. Glucose Uptake (pmol/mg/min)
Serine 474 phosphorylation is essential for maximal Akt2 kinase activity in adipocytes
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