MALDI-MS analysis of synthetic Akt C-tail peptides that were used in this work.
Purification of semisynthetic Akt1 proteins. Size exclusion chromatography (SEC) with a Superdex 75 column was for Δ143 Akt1 and Δ122 Akt1 semisynthetic proteins, and with a Superdex 200 column for full length Akt1s. The red trace shows MW standards corresponding to 670 kDa (thyroglobulin), 158 kDa (gamma-globulin), 44 kDa (ovalbumin), and myoglobin 17 kDa. The black traces demonstrated that each semisynthetic Akt protein was monomeric. (a-c)
Chromatograms and coomassie stained SDSPAGE of fractions for Δ143-Akt1-pThr^{308}/pSer^{473}, Ser^{477}, Thr^{479}, Δ122-Akt1-pThr^{308}/pSer^{473}, Ser^{477}, Thr^{479}, and FL-Akt1-pThr^{308}/pSer^{473}. 
Size exclusion chromatograms of semisynthetic full length Akt proteins.
SDSPAGE gel analysis shows purified semisynthetic Akt1 proteins with phospho-Thr308 obtained by co-expression with GST-PDK1 in okadaic acid (25 nM)-treated insect cells.
SDSPAGE gel analysis of purified semisynthetic Akt proteins (a-e) that were used for activation assays with GST-PDK1, and Akt-PH domain (aa 1-121) that was used for binding assays. (f) Western blot analysis of various FL-Akt phospho forms, lane 1: non-p Thr<sup>308</sup>, non-p C tail Akt(2-480), lane 2: pThr<sup>308</sup>, non-p C tail Akt(2-480), lane 3: nonpThr<sup>308</sup>, pSer<sup>473</sup> Akt(2-480), lane 4: pThr<sup>308</sup>, pSer<sup>473</sup> Akt(2-480), lane 5: pThr<sup>308</sup>, pSer<sup>477</sup>/pThr<sup>479</sup> Akt(2-480), M: protein marker.
SDS-PAGE gel analysis of semisynthetic full length Akt proteins with unmodified Ser473 or pSer473, and site-specific C-terminal fluorescein labeling with coomassie staining (a) and fluorescence imaging (b). These semisynthetic FL-Akt1 proteins were used for binding affinity assays with PDK1.
GSK3-ATP bisubstrate analog and GSK3 peptide substrate. (a) Schematic representation of the synthetic approach to the bisubstrate analog and the MALDI-MS for bromo-GSK3 peptide penultimate precursor. (b) MALDI-MS analysis of the biotinylated GSK3 peptide substrate.
a) 

b) 

c) 

<table>
<thead>
<tr>
<th>Semisynthetic truncated Akt(144-480) constructs</th>
<th>ATP Km (µM)</th>
<th>GSK3 Km (µM)</th>
<th>kcat (min⁻¹)</th>
<th>kcat/Km² (min⁻¹, µM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔPH, pThr³⁰⁸, pSer⁴⁷³</td>
<td>86±13</td>
<td>3.2±0.7</td>
<td>26±1</td>
<td>0.31</td>
</tr>
<tr>
<td>ΔPH, pThr³⁰⁸, S473D</td>
<td>273±60</td>
<td>3.5±0.7</td>
<td>132±4.0</td>
<td>0.48</td>
</tr>
<tr>
<td>ΔPH, pThr³⁰⁸, pSer⁴⁷³/pThr⁴⁷⁹</td>
<td>48±15</td>
<td>3.8±1.0</td>
<td>13±1.0</td>
<td>0.26</td>
</tr>
<tr>
<td>ΔPH, pThr³⁰⁸, non-p C tail</td>
<td>n.d.</td>
<td>7.3±2.3</td>
<td>n.d.</td>
<td>0.21</td>
</tr>
</tbody>
</table>

d) 

\[ \text{IC}_{50} = 37.5 \pm 1.4 \, \mu\text{M} \]

\[ K_i = 3.6 \, \mu\text{M} \]
(a-c) Kinase assays for semisynthetic N-terminally truncated Akt(144-480) proteins. (a) Steady-state kinetic plots for V vs [ATP] with 10 mM Mg\(^{2+}\), 20 \(\mu\)M GSK3 peptide substrate (left), and V vs [peptide] with 10 mM MgCl\(_2\), 20 \(\mu\)M ATP (right). Enzyme concentrations are 3 nM for pThr\(^{308}\), pSer\(^{473}\) Akt; and pThr\(^{308}\), pSer\(^{477}\)/pThr\(^{479}\) Akt, and 5 nM pThr\(^{308}\), S473D Akt. The reactions were carried out at 30°C for 10 minutes. (b) Steady-state kinetic plots for V vs [ATP] with 10 mM Mg\(^{2+}\), 20 \(\mu\)M GSK3 peptide substrate (left), and V vs [peptide] with 10 mM MgCl\(_2\), 20 \(\mu\)M ATP (right) for 2 nM of pThr\(^{308}\), non-p C tail Akt(144-480). (c) Enzymatic parameters of semisynthetic truncated Akt(144-480) constructs. The kcat and kcat/Km values were obtained from kinetic plots for V vs [ATP] (*), values shown +/- standard error (n=2). (d) Kinase inhibition assays for full length Akt1-pThr\(^{308}\), pSer\(^{473}\)/pSer\(^{477}\)/pThr\(^{479}\) with ATP-GSK3 bisubstrate peptide. Inhibition assays were run with 250 \(\mu\)M ATP, 20 \(\mu\)M GSK3 peptide substrate, 10 mM MgCl\(_2\) and 1 nM enzyme. Variable concentrations of ATP-GSK3 bisubstrate peptide (0, 0.1, 0.2, 0.5, 10, 50, 250, 500 \(\mu\)M) were preincubated with enzyme for 20 minutes on ice, and the reaction proceeded for 10 minutes, 30°C.

**Phospho-Ser473 interacts with Arg144.** Steady-state kinetic plots of V vs [ATP] for full length Akt1-R144A-pThr\(^{308}\)/pSer\(^{473}\).
Electron transfer dissociation spectrum of RPK$_{\text{Bio}}$FPQBSYSASGTA (peptide 1) crosslinked to DGATMK (peptide 2) in the activation loop of Akt. Ions of type c from peptide 1 and peptide 2 are highlighted in blue and green, respectively. Similarly, ions of type z from peptide 1 and peptide 2 are shown in red and yellow. Non-crosslinked product ions are additionally labeled with parent sequence (“1” or “2”) followed by the charge state of the ion (i.e. 2+). For example, z$_1$ (2/1+) corresponds to the z$_1$ ion of peptide 2 singly charged. Crosslinked product ions are indicated with a bracket notation that lists each peptide along with the corresponding cleavage. For example, [p1/c$_{14}$]-[p2] 3+ indicates a cleavage to produce c$_{14}$ from peptide 1, crosslinked to peptide 2, that is triply charged. K$_{\text{Bio}}$, biotinylated lysine; B, benzozyphenylalanine; CR, charge reduced precursor ion; *, water loss.