

Supplementary Materials for

Phosphorylation-Dependent Inhibition of Akt1

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Supplementary Materials and Methods

Affinity column chromatography. The His-tagged proteins were purified using Ni affinity column chromatography. 0.5 ml of Ni-NTA resin (Thermo-Fisher Scientific) was used for 1 l of *E. coli* culture. The cell lysates (see Methods) were loaded into the column (2 ml column volume) and washed with 20 column volumes of wash buffer A (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM Dithiothreitol, 1 mM Na₃VO₄ and 5 mM NaF, 15 mM imidazole) followed by 10 column volumes of wash buffer B (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM DTT, 1 mM Na₃VO₄ and 5 mM NaF, 20 mM imidazole). Protein was eluted using an elution buffer (20 mM Hepes, 150 mM NaCl, 3 mM β -mercaptoethanol, 3mM dithiothreitol, 1 mM Na₃VO₄ and 5 mM NaF) with 75 mM Imidazole. The same protocol was used for Akt1 variants with phosphomimetic mutations except: 1) phosphatase inhibitors were excluded from the buffers, 2) the imidazole concentrations of the wash buffers were 20 - 50 mM, and 3) the imidazole concentration of the elution buffer was 200 mM. Fractions were run on 10% SDS (sodium dodecyl sulfate) poly-acrylamide gels. As detailed previously [1], Akt1 variants were further purified by size exclusion chromatography.

Supplementary References

1. Balasuriya, N.; Kunkel, M.T.; Liu, X.; Biggar, K.K.; Li, S.S.; Newton, A.C.; O'Donoghue, P. Genetic code expansion and live cell imaging reveal that thr308 phosphorylation is irreplaceable and sufficient for akt1 activity. *J Biol Chem* **2018**, *293*, 10744-10756.
2. George, S.; Aguirre, J.D.; Spratt, D.E.; Bi, Y.; Jeffery, M.; Shaw, G.S.; O'Donoghue, P. Generation of phospho-ubiquitin variants by orthogonal translation reveals codon skipping. *FEBS Lett* **2016**, *590*, 1530-1542.

Supplementary Table S1. Plasmids used in this study.

Plasmid ID	Plasmid backbone	Selectable marker	Inserted genes	Genetic modifications	Protein product
(1) WT Akt1	pDS0 (pUC) [2]	Amp	<i>AKT1</i>	None.	Akt1, unphosphorylated. (full-length, inactive) [1]
(2) WT Akt1-PDK1	pCDF-Duet-1	Strep	<i>AKT1</i> <i>PDK1</i>	None.	Akt1, phosphorylated at position T308. (full-length, active) [1]
(3) Akt1 UAG473	pCDF-Duet-1	Strep	<i>AKT1</i>	UAG mutation at position 473 in <i>AKT1</i> .	Akt1, phosphorylated at position S473. (full-length, slightly active) [1]
(4) Akt1-UAG473-PDK1	pCDF-Duet-1	Strep	<i>AKT1</i> <i>PDK1</i>	UAG mutation at position 473 in <i>AKT1</i> .	Akt1, phosphorylated at positions T308 and S472. (full-length, highly active) [1]
(5) pSer-OTS	pDS-pSer2	Kan	tRNA ^{ser} (5x) SepRS9 EFSep21	This system is comprised of the 2 nd generation mutants of the pSer incorporation system.	pSer orthogonal translation system, capable of decoding UAG codons and incorporating pSer into nascent peptides [2]
(6) ΔPH-WT Akt1	pDS0 (pUC) [2]	Amp	<i>AKT1</i>	Deletion of the PH domain from <i>AKT1</i> (Δ residues 2-109).	ΔPH-Akt1, unphosphorylated. (truncated, inactive)
(7) ΔPH-WT Akt1-PDK1	pCDF-Duet-1	Strep	<i>AKT1</i> <i>PDK1</i>	Deletion of the PH domain from <i>AKT1</i> (Δ residues 2-109).	ΔPH-Akt1, phosphorylated at position T308. (truncated, active)
(8) ΔPH-Akt1-UAG473	pCDF-Duet-1	Strep	<i>AKT1</i>	Deletion of the PH domain from <i>AKT1</i> (Δ residues 2-109). UAG mutation at position 473 in <i>AKT1</i>	ΔPH-Akt1, phosphorylated at position S473. (truncated, slightly active)
(9) ΔPH-Akt1 UAG473-PDK1	pCDF-Duet-1	Strep	<i>AKT1</i> <i>PDK1</i>	Deletion of the PH domain from <i>AKT1</i> (Δ residues 2-109). UAG mutation at position 473 in <i>AKT1</i> .	ΔPH-Akt1, phosphorylated at positions T308 and S472. (truncated, highly active)

Supplementary Figures

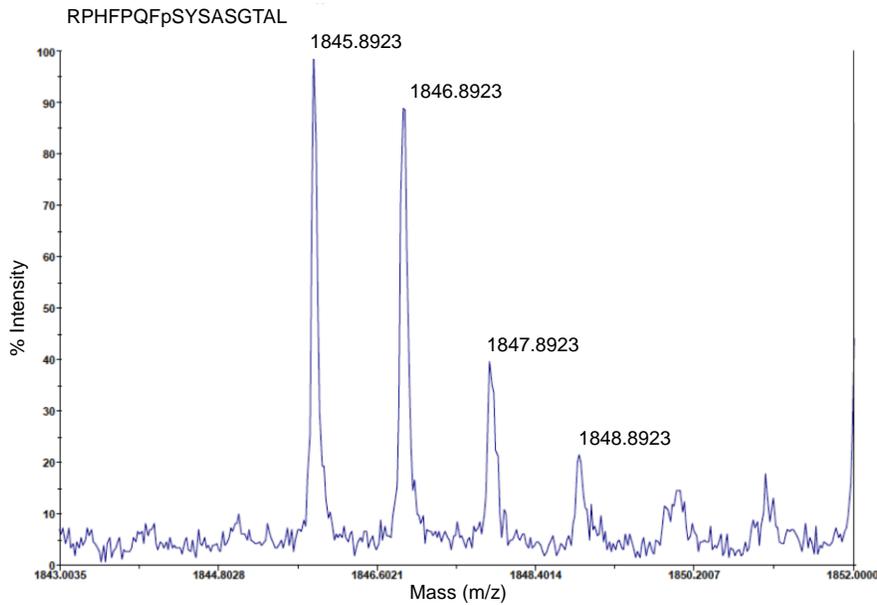


Figure S1. Mass spectra confirming genetically encoded phosphoserine in Δ PH-ppAkt1. Tryptic digested peptides of Δ PH-ppAkt1^{T308,S473} were subjected to MALDI-TOF mass spectrometry. The C-terminal peptide of Δ PH-Akt1 carrying a single phosphorylation was readily detected. The isotopic masses of the peptide carrying the most abundant peak of ^{12}C isotope (observed $m/z = 1845.8923$; expected $m/z = 1845.8378$) and the 3 consecutive peaks with ^{13}C isotopes ($m/z = 1846.8923$, 1847.8923 , 1848.8923 , respectively) are shown.

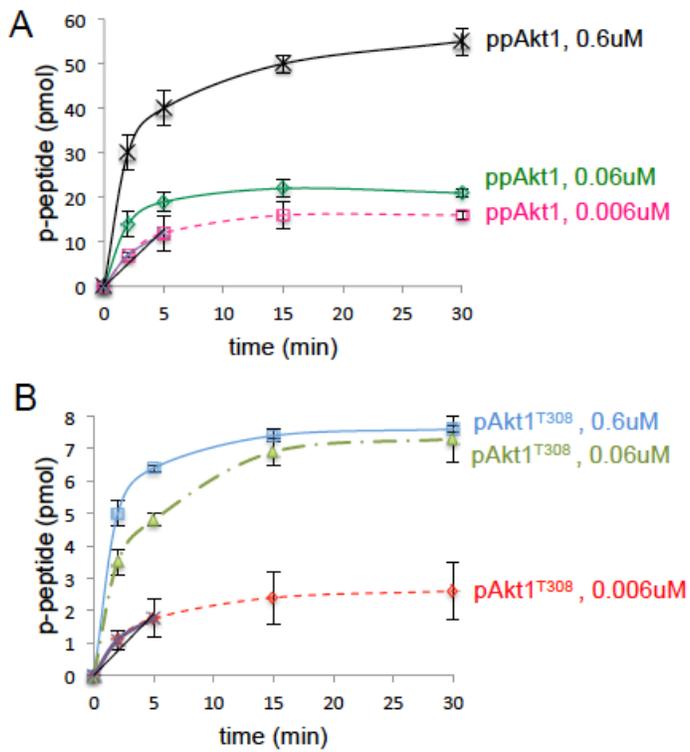


Figure S2. Initial velocity measurements for highly active Δ PH Akt1 variants. (A) Δ PH-ppAkt1^{T308, S473} and (B) Δ PH-pAkt1^{T308} were diluted by 10-fold and 100-fold, respectively, to obtain a linear range of increasing enzyme activity. Initial velocities were calculated using the data points at the linear range of 100-fold diluted enzyme.

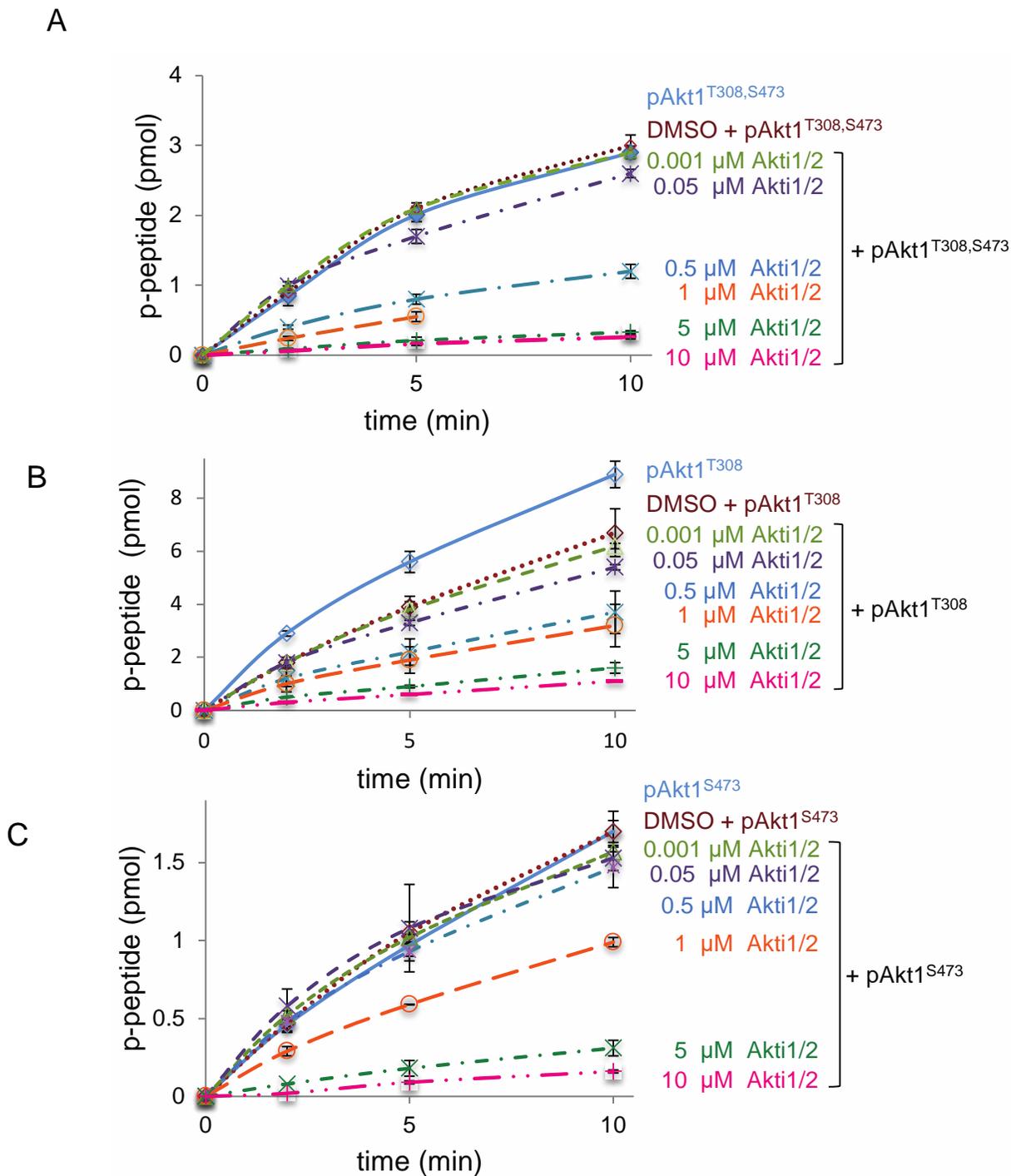


Figure S3. Inhibition of the full length Akt1 variants incubated with Akti-1/2 inhibitor VIII. (A) ppAkt1^{T308, S473}, (B) pAkt1^{T308}, (3) pAkt1^{S473} variants were incubated with increasing concentrations (0.001 uM to 10 uM) of Akti1/2. Error bars indicate 1 standard deviation based on triplicate experiments.

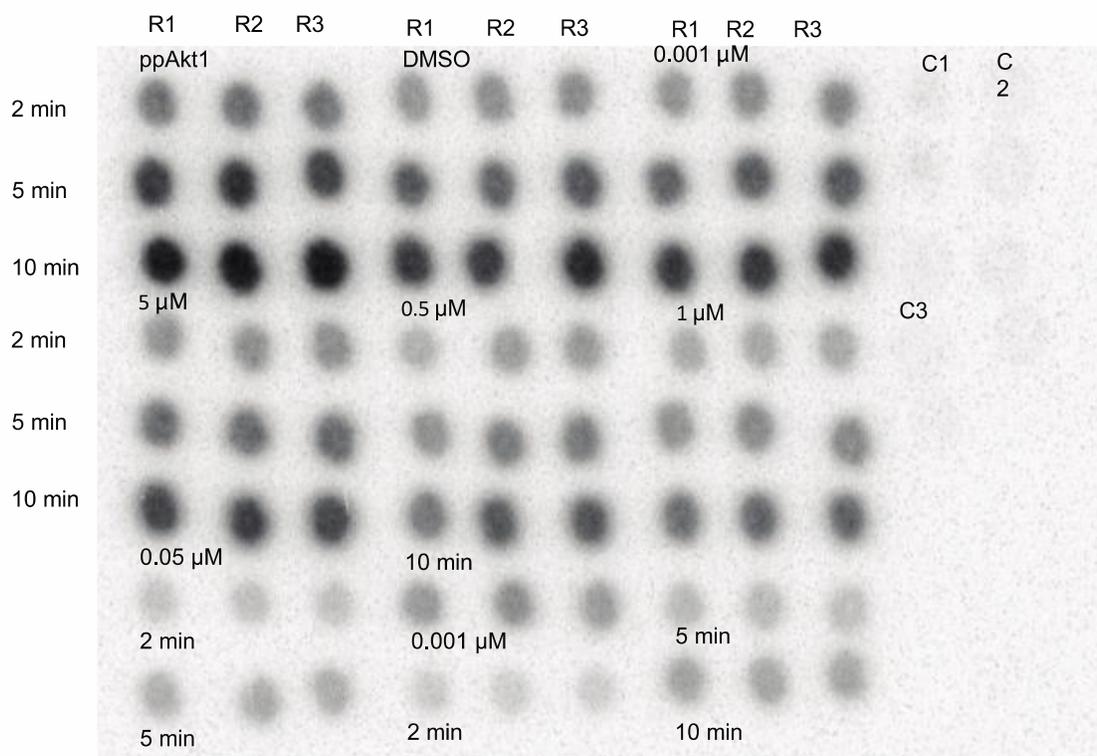


Figure S4. Autoradiographs of Akt1 inhibitor assays with ppAkt1^{T308, S473}. Time courses are shown for the reaction catalyzed by ppAkt1^{T308, S473} with the indicated concentrations of inhibitor Akti1/2. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 without substrate peptide, C2 without enzyme, C3 only kinase assay buffer.

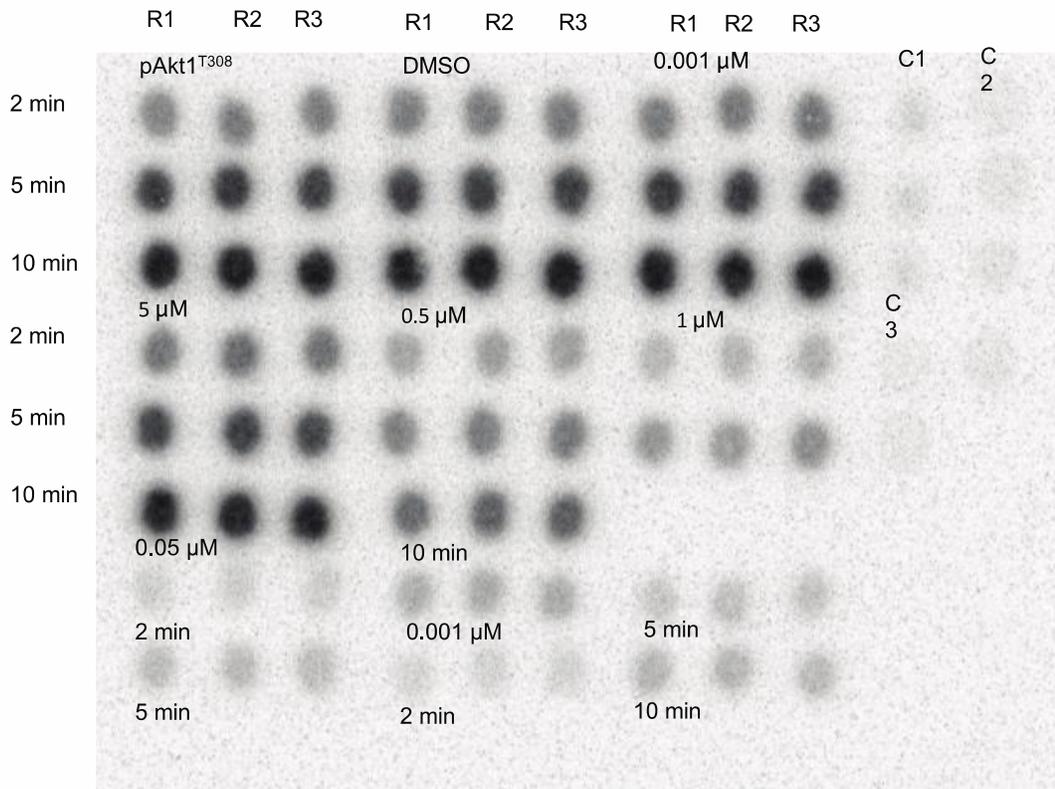


Figure S5. Autoradiographs of Akt1 inhibitor assays with pAkt1^{T308}. Time courses are shown for the reaction catalyzed by pAkt1^{T308} with the indicated concentrations of inhibitor Akti1/2. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 without substrate peptide, C2 without enzyme, C3 only kinase assay buffer.

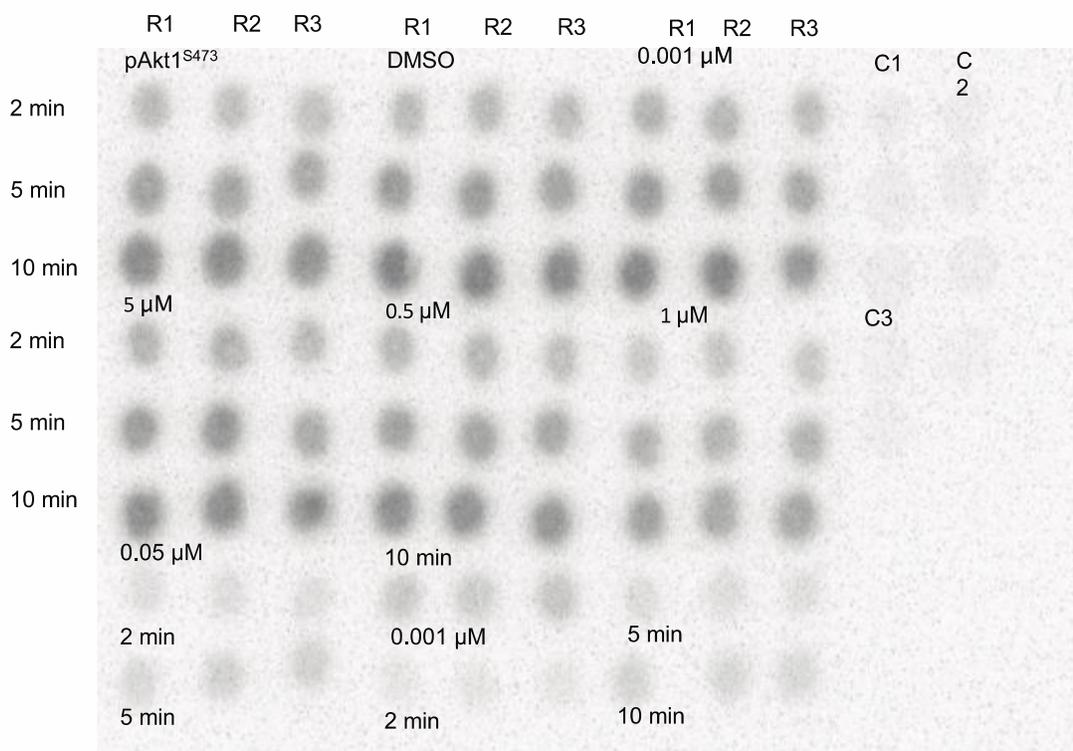


Figure S6. Autoradiographs of Akt1 inhibitor assays with pAkt1^{S473}. Time courses are shown for the reaction catalyzed by pAkt1^{S473} with the indicated concentrations of inhibitor Akt1/2. Assays were performed in triplicate (as indicated by R1-R3). Controls include: C1 without substrate peptide, C2 without enzyme, C3 only kinase assay buffer.