Phosphorylation-dependent substrate selectivity of protein kinase B (AKT1)

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Protein kinase B (Akt or AKT1) is a central node in a signaling pathway that regulates cell survival. The diverse pathways regulated by AKT1 are communicated in the cell via the phosphorylation of perhaps more than 100 cellular substrates. AKT1 is itself activated by phosphorylation at Thr-308 and Ser-473. Despite the fact that these phosphorylation sites are biomarkers for cancers and tumor biology, their individual roles in shaping AKT1 substrate selectivity are unknown. We recently developed a method to produce AKT1 with programmed phosphorylation at either or both of its key regulatory sites. Here, we used both defined and randomized peptide libraries to map the substrate selectivity of site-specific, singly and doubly phosphorylated AKT1 variants. To globally quantitate AKT1 substrate preferences, we synthesized three AKT1 substrate peptide libraries: one based on 84 “known” substrates and two independent and larger oriented peptide array libraries (OPALs) of ~10^11 peptides each. We found that each phospho-form of AKT1 has common and distinct substrate requirements. Compared with pAKT1 308, the addition of Ser-473 phosphorylation increased AKT1 activities on some, but not all of its substrates. This is the first report that Ser-473 phosphorylation can positively or negatively regulate kinase activity in a substrate-dependent fashion. Bioinformatics analysis indicated that the OPAL-activity data effectively discriminate known AKT1 substrates from closely related kinase substrates. Our results also enabled predictions of novel AKT1 substrates that suggest new and expanded roles for AKT1 signaling in regulating cellular processes.

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This article contains supporting information.

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Using active preparations of AKT1 from Sf9 insect cells over-expressing PDK1 and human AKT1, the target motif was further investigated through oriented peptide array library (OPAL) screens. The results indicated selectivity for Arg residues at positions 3, 5, and 7 as well as Thr at position 2. AKT1 also showed moderate preferences for aromatic residues at positions 1 and 1 and for small residues able to induce tight turns (Gly, Ser, Asn, or Thr) at the position 2(13). Because Sf9 produced AKT1 normally contains a mixture of active AKT1 phospho-forms (8, 14), these data could not identify substrate preferences associated with differentially phosphorylated AKT1.

Peptide library screening is a gold standard method that has been widely used to determine specific amino acid preferences of the kinase substrate recognition motif. Degenerate peptide libraries or OPAL screens provide a systematic approach to identify the important residues surrounding the phosphorylation site on the substrate (13, 15). Here we designed and synthesized OPALs to define the substrate requirements for differentially phosphorylated AKT1 variants. We discovered that the phosphorylation status of AKT1 has a significant impact on substrate selectivity and on the specific residue preferences at each location in the consensus motif. Kinase activity data derived from our OPAL experiments was able to differentiate each AKT1 phospho-form from another. The OPAL data were also able to effectively discriminate known AKT1 substrates from closely related substrates in database searches, which also suggested high confidence putative AKT1 targets were enriched in pathways linked to RNA metabolism.

Results

For preparation of active and purified AKT1, the use of phosphoinositide-dependent kinase (PDK1) leads to efficient phosphorylation of AKT1 at Thr-308. Although in the cell mTORC2 is recognized as the major upstream kinase for Ser-473 (11, 16), incorporation of phosphate at Ser-473 is challenging in the test tube. Previous approaches relied on phosphorylation of Ser-473 by MAPKAPK-2, which was pre-activated by p38 in the presence of phosphatidylinositol (3,4,5)-trisphosphate and lipid vesicles (17). In contrast, our approach leads to recombinant and site-specifically phosphorylated AKT1 variants produced in Escherichia coli without the need to purify or activate additional kinases. Using a combination of genetic code expansion to encode phosphoserine at Ser-473 and co-expression of PDK1, we were able to produce active full-length and PH domain-truncated AKT1 variants in E. coli with either or both Thr-308 and Ser-473 sites phosphorylated. We previously characterized the activity and phosphorylation status of the enzyme preparations biochemically and with MS (8, 9). In that...
work, we discovered that phosphomimetic substitutions are incapable of replacing or approximating the functionality of phosphorylated residues at the activation sites of AKT1. We further found that alanine substitutions at Thr-308 and Ser-473 inactivated the enzyme (8). One strength of our approach, therefore, is the ability to generate pAKT1 and ppAKT1 variants without the need to mutate the enzyme, which greatly alters enzyme function.

Our previous studies on pAKT1 and ppAKT1 variants focused on their differential activity with a single substrate peptide derived from GSK-3β (8). Here we quantified the substrate preference for phosphorylated AKT1 variants using a synthetic peptide library of known AKT1 substrates, and two independent oriented peptide arrays. We found that deletion of the PH domain abolishes its autoinhibitory activity and leads to a more soluble enzyme (9) appropriate for larger scale screening studies presented here.

**AKT1 phosphorylation status alters activity among known substrates**

A set of “known” AKT1 substrates have been identified and in some cases validated using multiple experimental approaches, including in vitro biochemical (18) and cell-based activity assays (19), proteomic analyses (20), as well as phospho-specific immunoblotting of AKT1 substrates (21) from cells (22) and animals (23). Our study allowed the evaluation of these separately reported substrates using a systematic approach to quantify AKT1 kinase activity. The doubly phosphorylated AKT1 was capable of phosphorylating most, but not all, of the substrate peptides in our peptide library representing known substrates (Fig. S1). The ppAKT1 enzyme showed significantly above background activity with 95% of the peptides tested (Fig. S1C). In our activity assays, the initial reaction velocity varied over an 80-fold range. We found that ppAKT1 produced between 0.1 and 8.0 pmol/min of phosphorylated peptide.

Overall, the level of phosphorylation observed was markedly lower with pAKT1T308,S473 compared with pAKT1T308 and ppAKT1T308,S473 (Fig. S1). Compared with the singly phosphorylated variants (Fig. S1, A and B), ppAKT1T308,S473 (Fig. S1C) usually displayed the greatest activity over the widest range of substrate peptides. Indeed, we observed a clear trend in activity over the library with pAKT1T308 showing the most restricted substrate range. The ppAKT1T308 enzyme showed low activity (<0.1 pmol/min) with more than 50% of the substrates. The variants phosphorylated at Thr-308 showed activity above this same relative level with >90% of the library.

Interestingly, some substrates representing interleukin-1 receptor-associated kinase 1 (IRAK1) and B-cell CLL/lymphoma protein 10 (BCL10), showed no significant activity with any of the AKT1 phospho-forms. Although BCL10 was found in association with AKT according to immunoprecipitation experiments (24), the ability of AKT1 to directly phosphorylate BCL10 was not demonstrated. Indeed, further studies in T cells found that AKT1 is not required to phosphorylate BCL10 (25). The suggestion of IRAK1 as an AKT1 substrate was based on work in human embryonic kidney cells showing that over-expression of calcium/calmodulin-dependent protein kinase kinase (CaMKKc) or AKT lead to increased phosphorylation at Thr-100, but a direct link between AKT and IRAK1 was not established (26). It is possible that AKT1 is not a direct kinase for these substrates or that the substrates require the complete protein structure or other co-incident modification to become active substrates for AKT1.

**AKT1 phosphorylation status re-wires substrate selectivity**

The three AKT1 phospho-forms have distinct preferences for particular peptides. The highest active substrates for the pAKT1T308 variant were completely orthogonal to or distinct from the most active substrates with pAKT1T308 or ppAKT1T308,S473 (Figs. S1 and S2). To visualize these distinct substrate preferences, we generated a heat map showing the relative activity of each AKT1 phospho-form over the library of 84 known substrates (Fig. 2). The substrates were categorized into groups based on shared biochemical function. Inside each functional group, the substrates were ranked according to decreasing ppAKT1 activity, and the functional groups were also listed in order of decreasing average activity with ppAKT1.

The ppAKT1 enzyme is most active on average with the Forkhead box O (FOXO) family of transcription factors, which are well-established substrates of AKT1 (21, 27). Peptides representing the FOXO family were among the most active substrates with ppAKT1 and AKT1 phosphorylated only at Thr-308 (Fig. S1, B and C). Certain substrates, including FOXOs and TSC2, have more than one AKT1-dependent phosphorylation site, which we represented with distinct synthetic peptides. Each AKT1 phospho-variant showed different preferences for the multiple sites in these substrates. For example, with pAKT1T308, Ser-256 and Ser-319 of FOXO1 showed relatively high activity, but this enzyme showed low activity with a peptide derived from FOXO1 Thr-24. In contrast, for both pAKT1T308 and ppAKT1T308,S473 FOXO1 Ser-319 was the least preferred compared with Ser-256 and Thr-24 (Fig. 2).

**Impact of Ser-473 phosphorylation on active AKT1**

The accepted view that ppAKT1 is more active than singly phosphorylated AKT1 is not always true at the resolution of individual substrates. To better understand the impact of Ser-473 phosphorylation on the activity of the doubly phosphorylated AKT1 for our library of known substrates, we plotted kinase activity data of pAKT1T308 and ppAKT1T308,S473 together (Fig. 3). We analyzed these comparative data for statistically significant differences in activity. Interestingly, we observed that additional phosphorylation at Ser-473 significantly increased AKT1 activity for many (49%, 41 of 84) but not all AKT1 substrates (Fig. S1, and Fig. 3). In the context of AKT1 phosphorylated at Thr-308, for nearly half of the substrates (45%, 38 of 84), Ser-473 phosphorylation did not change activity, and for 6% (5 of 84) of the substrates, phosphorylation at Ser-473 actually decreased kinase activity (Table 1, Fig. 3). Phosphorylation at Ser-473 reduced activity for two peptides derived from distinct sites in the FOXO4 transcription factor (Thr-32, Ser-262), as well as sites in 14-3-3Z, Grb2-associated binding protein (Gab2), and TSC complex subunit 2 (TSC2). Together the data show that both regulatory phosphorylation sites in AKT1 have a significant and unexpectedly complex...
Figure 2. Relative activity of AKT1 variants across functional clusters of known substrates. The heatmap is colored according to high (red), medium (white), and low (blue) relative activity for pAKT1 S473, pAKT1 T308, and ppAKT1 T308,S473. The substrates were categorized into functional categories. Inside each group, the substrates are ranked according to decreasing ppAKT1 activity, and the functional groups are listed in order of decreasing average activity with ppAKT1.
impact on substrate selectivity over a library of known AKT1 substrates.

Phosphorylation-dependent changes in the AKT1 target motif

Based on the activity data in Fig. S1, we computed sequence logos for each AKT1 phospho-form. The logos (Fig. S2) represent the relative importance of residues at each site for AKT1-dependent phosphorylation based on the activity level recorded for each substrate peptide (Fig. S1). The sequence alignments used to generate the logos were, therefore, populated with each of the 84 peptides such that the number of occurrences of each peptide in the alignment was linearly related to the enzyme activity (see “Materials and methods”).

The preference for conserved consensus sequence residues Arg<sup>H11002</sup> and Arg<sup>H11002</sup> among known AKT1 substrates (R<sup>H11002</sup>XR<sup>H11002</sup>XX(S/T)) was observed with all three variants. As reported previously (12), the presence of a large hydrophobic residue following the phosphorylation site (+1 position) was observed among the peptides with all three AKT1 variants. Pro most frequently occupied the +2 position among highly active peptides for the pAKT<sup>T308</sup> and ppAKT<sup>T308,S473</sup> variants. These data are in agreement with the finding that the +2 position prefers amino acids that enable tight turns in protein structure (13). Interestingly, peptide substrates showing high activity with a pAKT<sup>T308</sup> variant were dominated by those peptides with a Ser rather than a Thr at the phosphorylation site. Conversely, for pAKT<sup>T308</sup> and ppAKT<sup>T308,S473</sup>, highly active peptides were approximately equally likely to have Ser or Thr at the phosphorylation site.

Determination of high-resolution AKT1 target specificity

Our analysis of known substrates revealed several new facets of AKT1 enzymology. These data, however, are biased by our current and perhaps limited knowledge of AKT1 substrates. We designed a far larger, unbiased oriented library of peptides (OPAL1) to test the most preferred amino acid residues at each site of the AKT1 substrate consensus motif. OPAL1 used 17 different amino acids at each of 8 variable positions in the synthetic peptides. We synthesized 136 different pools of peptide sublibraries to cover the complete library of ~10<sup>10</sup> peptides (see “Materials and methods”). Using each phospho-form of AKT1, we conducted kinase assays with each of the substrate sublibraries (Fig. 4A, Figs. S4–S7 and Data File S1). In each sublibrary a single amino acid is held constant at a single position in the consensus motif (Fig. 4A), whereas all other variable positions are allowed to cover the complete sequence space with the exception of Cys, Thr, and Ser. Because Arg<sup>H11003</sup> is normally required for AKT1 activity (13), we held this position fixed in OPAL1.

The AKT1 activity for singly and doubly phosphorylated AKT1 variants over each of the peptide sublibrary pools are shown in terms of absolute activity (Fig. S7) and overlaid as a heat map indicating relative changes in activity (Fig. 4A). To visualize the amino acid preferences and anti-determinants we converted the activity data into position-specific score matrices (PSSMs, see “Materials and methods”). We used the PSSMs to generate sequence logos (28) representing the OPAL1 data (Fig. 5). The results demonstrate that the substrate selectivity of pAKT<sup>T308</sup> and ppAKT<sup>T308,S473</sup> is distinct from the profile we observed for the more similar pAKT<sup>T308</sup> and ppAKT<sup>T308,S473</sup> enzymes. This observation is confirmed by principle component analysis (PCA) of the 3 OPAL1 matrices (Fig. 4C). PCA plots the variance between the OPAL matrices such that the first component explains most of the variance between the data sets. The principle components are linear combinations of the observed variables, which are the activity values at each location in the oriented array. The magnitude and direction of variance at each
position in the array is shown as a vector. For example, the preference of pAKT1S473 for Trp at positions 2 (W2) and 3 (W3) most distinguishes this enzyme from the variants phosphorylated at Thr-308 (Fig. 4C).

Although consensus patterns are evident (Table 2), specific amino acids significantly preferred at each position show distinguishable profiles for each AKT1 phospo-form (Fig. 4, A and C) in concordance with the unique substrate selective pro-
files we recoded over the library of known substrates (Fig. S1, and Fig. 2). By varying the Arg−3 position, we found that basic residues are highly favored and Arg−5 is most preferred or nearly so for the enzymes phosphorylated at Thr-308. The pAKT1S473 enzyme is active with basic residues at the −5 position yet prefers aromatic residues at this site. Both ppAKT1T308,S473 and pAKT1T308 preferred large hydrophobic residues (Phe and Trp or Tyr) at the +1 position in the target peptide. In contrast, pAKT1S473 prefers basic residues (Lys, Arg, His) at +1. For the pThr-308 containing enzymes, basic amino acids (Arg, Lys) are among the most active residues at most of the variable positions (−5, −4, −2, −1, +1, +2, +3); this same preference is also seen at positions −4, −1, +1 for the pAKT1S473 enzyme.

In terms of anti-determinants to kinase activity, Ile at −1 and +2 positions were disfavored for both pAKT1T308 and ppAKT1T308,S473 variants; Phe and Pro are least preferred at −1 and +2 positions for pAKT1S473, ppAKT1 was least selective at position +2, showing high activity with a large variety of amino acids. As observed before (12), we found Pro at +1 significantly inhibited activity in all of our OPAL matrices.

To confirm the generality of these findings, we conducted independent OPAL2 experiments based on a distinct peptide design (Fig. 4B, and Fig. S8). In contrast to OPAL1, this library probed the −3 position, which was fixed as Arg in OPAL1, included phosphorylated Thr and Tyr residues, and varied the +4 but not the −6 position. OPAL2 used 22 different amino acids at each of 9 positions leading to a library size of \(10^9\). OPAL2 used a 15-residue peptide, rather than 13 in OPAL1, and the biotin tag was placed on the C rather than N terminus. The results confirm the dominant preference of Arg−3 for all AKT1 phospho-forms, yet significant activity is observed with Lys−3 and to a lesser extent His−3. The OPAL2 data were also able to clearly discriminate each phospho-form of AKT1 from the other (Fig. 4D). We observed that the relative differences between the OPAL2 matrices were not as great as that observed in OPAL1 (Fig. 4), suggesting the OPAL1 peptide design may discriminate between the phospho-forms more effectively.

OPAL2 also confirmed that the pThr-308 containing enzymes, including AKT1 produced in insect cells (Sf9), were all far more active than pAKT1S473. The finding is consistent with the data here (Figs. S1, S7, and S8) as well as our previous findings (8, 9). The Sf9 prepared AKT1 showed many commonalities with the AKT1 variants prepared in E. coli, however, the PCA indicates the Sf9 enzyme was distinguishable from the others with the majority of the variance explained by preferences for Ser/Thr−2, Asn−1, Ser/Thr−2, and Pro4. Overall, the PCA indicates that although the Sf9 AKT1 is most similar to ppAKT1, it is a distinct AKT1 enzyme and not a linear combination of the pSer-473 or pThr-308 containing enzymes. Together the data further support our hypothesis that each AKT1 phospho-form has distinct substrate preferences.

Despite these differences, similarities among the OPAL data are also apparent. We summarized the consensus residues that are most or least favorable at each position across experiments and AKT1 variants (Table 2). His−1 is among the most preferred for all phospho-forms, whereas Val and Ile are least preferred for all variants at −1. The addition of a basic residue, normally Arg or Lys but also His, at nearly any position will lead to increased activity with any AKT1 variant. The only clear exception to this rule is at +1 where Phe is commonly favored. Conversely, introduction of a negatively charged amino acid (Asp, Glu, as well as pTyr and pThr, Fig. 4B) at any position will lead to significantly reduced AKT1 activity (Fig. 5). The intro-
duction of Pro or small hydrophobic residues (Ile, Val) is disfa-
vored at the majority of positions for all AKT1 variants.

Identifying known and putative AKT1 substrates

We derived PSSMs from the OPAL1 data according to an
approach established previously (29) and detailed under "Mate-
rials and methods." To begin to estimate the predictive value of
these PSSMs, we plotted the activity data we generated for the
84 known substrates (Fig. S1) against the PSSM score that
results from that particular peptide according to the OPAL1-
derived PSSMs for each AKT1 phospho-form (Fig. S10). Inter-
estingly, these data show statistically significant correlations
($p < 10^{-5}$) for the pAKT1\textsuperscript{T308} and ppAKT1\textsuperscript{S473,T308} enzymes,
suggesting PSSMs derived from the OPAL1 data can be used to
identify known and predict novel targets. The data, however,
did not correlate for the pSer-473 enzyme (Fig. S10A). For this
enzyme only, our data suggests that either the OPAL1-derived
PSSM is not a good predictor, or the 84 known substrates are
not true substrates for pAKT1\textsuperscript{S473}. The latter conclusion is sup-
ported by our data showing low activity of the pAKT1\textsuperscript{S473}
enzyme (Figs. S1, S4, S7 and S8) as well as our previous work
showing that AKT1 phosphorylated at Ser-473 alone was not

| position | pAKT1\textsuperscript{S473} | pAKT1\textsuperscript{T308} | ppAKT1 | AKT1 Sf9 | Consensus |
|----------|-------------------|-------------------|--------|----------|-----------|
| -6\textsuperscript{a} | + | - | + | - | + | - |
| -6\textsuperscript{b} | -- | -- | -- | -- | -- | -- |
| -5\textsuperscript{a} | F | IVP | RKH | Y | HRK | DYV | -- | -- | RK | IDE |
| -5\textsuperscript{b} | RK | NED | RKSH | IDE | RK | EID | RK | DEV | RK | IDE |
| -4\textsuperscript{a} | QK | AGP | RKH | FYW | KRH | DILF | -- | -- | RK | D |
| -4\textsuperscript{b} | RK | IDE | RK | EID | RK | DFI | RK | DVI | RK | D |
| -3\textsuperscript{a} | -- | -- | -- | -- | -- | -- | -- | -- | -- | -- |
| -3\textsuperscript{b} | R | NDE | RK | IVDE | RK | VFED | RK | DEVLI | RK | DEV |
| -2\textsuperscript{a} | LYFVW | PN | KLV | WPDE | HVR | WPDE | -- | -- | RK | PWDE |
| -2\textsuperscript{b} | RK | PWNED | RK | WPDE | RKST | PWDE | RSTK | WEID | RK | PWDE |
| -1\textsuperscript{a} | DHLK | F | H | I | KH | IV | -- | -- | KH | VIE |
| -1\textsuperscript{b} | YMHN | VIE | KRN | VEI | NRGKYH | IV | NPK | VI | KH | VIE |
| 1\textsuperscript{a} | HRKF | POND | FYMW | EDPG | FYMW | GEDP | -- | -- | FM | PDE |
| 1\textsuperscript{b} | MFI | PDE | FMIV | PDE | MF | PDE | SFM | ED | PDE |
| 2\textsuperscript{a} | WI | EP | HKR | I | GMH KR | EI | -- | -- | RHK | DP E |
| 2\textsuperscript{b} | SRH | DE | STRHK | PDE | RHK | DEI | STG | MLI | RHK | DP E |
| 3\textsuperscript{a} | W | EPD | IHR | DEYA | KRH | DPE | -- | -- | RK | EP D |
| 3\textsuperscript{b} | RKH | PDE | RSKH | LVED | KR | DE | SPG | LVI | RK | EP D |
| 4\textsuperscript{a} | -- | -- | -- | -- | -- | -- | -- | -- |
| 4\textsuperscript{b} | RKP | VDE | KRP | FYE | KR | EFD | KH | LIV | K | E |

Table 2
Consensus of positive (•) and negative (–) AKT1 activity determinants from OPAL data

The table is based on sequence logo analysis in Fig. 5 (OPAL1) and Fig. S9 (OPAL2). Bold residues indicate residue identities that are a consensus between AKT1 phospho-forms and experiments. Positive (black) and negative (red) activity determinants are listed in order of decreasing entropy. Data not determined (–).

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sufficient to propagate AKT1-dependent signaling in cells (8). The OPAL1 data (Fig. 4A) also suggests that the pAKT1S473 enzyme may prefer rather different substrates (Fig. 5A) than those included in the library of known substrates (Fig. S2).

We then used PSSMSearch (30) to rank potential AKT1 substrates across the entire human proteome with PSSMs derived from OPAL1 (Data File S2). We refined these data to include only hits with experimental evidence for phosphorylation at the predicted S\textsubscript{473}/T\textsubscript{473} site (Data File S2). Collectively, the search identified both known and potentially novel AKT1 substrates. The known hits were significantly enriched in GO biological processes related to cell signaling and apoptosis, whereas the top novel hits among all AKT1 variants were enriched in processes linked to RNA processing and metabolism (Fig. S11).

To gauge the reliability of the PSSMs, we performed specificity and selectivity analysis (Table S1, Fig. 6, Fig. S12). The PSSMs were used with PSSMSearch over a database of known AKT1 substrates with a specified outgroup of kinase substrates as defined by PhosphoELM (31). Several outgroups were tested, including those lacking known AKT1 substrates (such as Not_PKB or Not Basic\textsubscript{S473}) and other groups that do contain AKT1 (SGK, Arg\textsubscript{T308}). In all cases, the PSSMs scored known AKT1 substrates higher than other groups (Fig. S12), however, greater discrimination between known AKT1 substrates and the specific outgroup is characterized by the area under the curve (AUC), which has an inverse correlation with the PSSM score of the outgroup (Table S1). The ROC plot shows the OPAL1 data are effective in discriminating known AKT1 substrates (true positives) from groups that do not contain AKT substrates. As expected, there is less discrimination and reduced AUC values for outgroups that contain known AKT1 substrates, e.g. Arg\textsubscript{T308} group. We observe better discrimination using OPAL data for the pThr-308 containing enzymes compared with pAKT\textsubscript{S473}. In all cases, the OPAL data also provide discrimination against closely related protein kinase C (PKC) substrates and to a lesser extent protein kinase A (PKA) as well. The OPAL2 data performed similarly well in database searches (Fig. S13).

Next, we used an alternate and independent approach to score the known human phospho-proteome to identify potential new AKT1 substrates. We converted the OPAL1 AKT1 activity data into PSSMs based on calculating Z-scores (see “Materials and methods”) as before (15). The known human phospho-proteome was scored based on the amino acid preferences at each position in the Z-score PSSM (Data File S3). The scoring of peptides was performed for data based on each phospho-form of AKT1. We then selected the top scoring substrates with a normalized sum of Z-scores \(>3\). We included all of these high confidence hits in a Search Tool for Retrieval of Interacting Genes/Protein (STRING) (32) diagram to identify potential protein interactions and functional clusters among the known and predicted substrates (Fig. 7).

The searches identified both known and unknown AKT1 substrates among the high confidence hits. Interestingly, the prediction suggested the presence of substrates not previously
known to be regulated by AKT1. These include clusters of proteins that participate in translation, transcription, cell division, cytoskeletal organization, and lipid signaling (Fig. 7A). The putative AKT1 substrates are significantly enriched in gene ontology (GO) (33) molecular functions related to protein kinases, cytoskeletal organization, and RNA binding (Fig. 7B). Putative AKT1 substrates include centromeric proteins involved in cellular mitosis. Chromosome-associated protein G (NCAPG2), borealin (CDCA8), opa protein 5 (OIP5), and centromere protein O (CENPO) were the identified strong candidates for activity with each phospho-form of AKT1. Another novel cluster of putative AKT1 targets includes nucleolar proteins involved in rRNA processing. RNA-binding motif protein 34 (RBM34), nucleolar protein 10 (NOL10), RB-binding protein 6 ubiquitin ligase (RBBP6), and a DEAD box RNA helicase (DDX27) are also included in this functional cluster.

**Figure 7. Identifying optimal phosphospecific AKT1 substrates.** A, STRING diagram shows functional interactions of known (indicated by nodes with blue inner filled circles) and putative AKT1 substrates. The diagram includes high confidence hits to the known human phospho-proteome (Z-score > 3) using OPAL1 data generated with pAKT1T308 (green), pAKT1S473 (red), and ppAKT1T308,S473 (yellow) variants. Novel clusters of substrates are involved in cell division (orange), transcription (brown), translation (blue), cytoskeleton (purple), RNA processing, and lipid signaling. B, GO enrichment analysis with the high confidence hits shown in A. The analysis suggests statistically significant enrichment in protein kinases and RNA-binding proteins.
the results from our independent PSSMSearch (Fig. S1A), the top putative AKT1 substrate hits are strongly enriched in biological processes associated with RNA metabolism.

Discussion

We recently developed a new method to produce AKT1 with programmed phosphorylation at either or both key regulatory sites (8). This breakthrough enabled resolution of the contribution of each phospho-site to the activation (8) or inhibition (9) of AKT1. Because this approach was only recently developed, there are still very few studies that report on the function or impact of each phosphorylation site on AKT1 substrates (34). This situation occurs despite the fact that both phospho-sites are current and routine clinical markers for multiple cancers, e.g. see Refs. 35–37.

Prior investigations into the substrate specificity of AKT have focused on characterizing the isozyme-specific substrate preferences that differ between AKT1, AKT2, and AKT3 (1, 38). Indeed, the previous lack of tools to isolate the function of each AKT1 phospho-form inhibited studies to characterize the impact of each phosphorylation site on substrate specificity.

**Phosphorylation-dependent substrate specificity of AKT1**

In our previous work (8), we found that phosphorylation at Thr-308 was necessary and sufficient for maximal AKT1 activity in mammalian cells. The addition of Ser-473 phosphorylation increased kinase in the test tube, but did not influence phosphorylation of AKT-specific live cell fluorescent reporter in COS-7 cells (8). These observations led to our hypothesis that phosphorylation of Ser-473 may function to tune rather than activate the AKT1. Using peptide library approaches here, we mapped the substrate preferences for each key phospho-form of AKT1. The data presented revealed that Ser-473 phosphorylation does indeed impact substrate specificity. More generally, we found that the phosphorylation status of AKT1 has a global impact on substrate preference as determined by kinase activity assays with multiple independent peptide libraries.

A multitude of studies (39, 40), including our own (Figs. S1, S4–S6 and S8) (8), have presented evidence indicating that AKT1 activity increases monotonically with ppAKT1S473 < ppAKT1T308 < ppAKT1. Based on all of our assays, it remains clear that the ppAKT1S473 enzyme is far less active than the other two. Of the 84 known substrates we tested, this rule only holds completely true for half of the peptides (49%). When comparing ppAKT1T308 with ppAKT1T308,S473, we found that many substrates (45%) show the same level of activity with ppAKT1T308 and ppAKT1, whereas a minority (6%) are actually more active with the singly phosphorylated ppAKT1T308 compared with the doubly phosphorylated enzyme.

Our current study further revealed that when only Ser-473 is phosphorylated, the substrate preference profile is distinct compared with that observed for AKT1 with Thr-308 phosphorylation. This observation was supported by data generated from known substrates as well as the much larger data set generated using the OPAL approach. Interestingly, several of the most active known substrates for pAKT1S473, including MDM2 (Ser-166), FOXO1, and FOXO3, showed reduced activity in the absence of Ser-473 phosphorylation (Fig. 3).

Additional studies have revealed correlation between AKT1 phosphorylation status and substrate selectivity in conditions of endoplasmic reticulum (ER) stress. Upon ER stress, the ER chaperone protein GRP78 is down-regulated and this leads to increased phosphorylation of AKT1 at Ser-473 but not Thr-308 in human cells (JEG3). As a result, increased AKT1-dependent phosphorylation of MDM2 (at Ser-166), FOXO1 (at Ser-319), and GSK3-β (at Ser-9) were observed by Western blotting (42). In our study, we found the additional of pSer-473 significantly increases activity with the GSK-3β peptide (Fig. 3). The FOXO3 319 site is one of the most active with pAKT1S473 (Fig. S1).

Our data clearly indicate that ppAKT1 is normally (but not always) more active and has a broader substrate profile than either of the singly phosphorylated enzymes. In alignment with the essentiality of pThr-308 in the activation of AKT1 in vitro and in cells (8, 40), we found that the two variants phosphorylated at Thr-308 showed greatest similarities in substrate preferences compared with the pAKT1S473 enzyme. Previous work (10) found that Ser-473 was not essential for phosphorylating the non-N-terminal phosphosites of the FOXO transcription factors, TSC2 or GSK-3β. These observations are in close agreement with and supportive of our findings. The ppAKT1T308,S473 enzyme showed high activity with FOXO1 C-terminal phosphorylation sites (Ser-319, Ser-256), TSC2 and GSK-3β (Fig. S1, Fig. 3). For ppAKT1T308, all three FOXO1 phosphosites ranked among the highest active substrates, suggesting that Ser-473 is not a critical factor in FOXO1 phosphorylation. In the clinic, phosphorylation of Thr-308 was associated with poor cell survival with nonsmall cell lung cancer (43) and acute myeloid leukemia (44). According to our data here and previously (8), we suggest that phosphorylation of Thr-308 alone may be capable of switching-on downstream and disease-linked signaling to promote cell survival by inhibiting the FOXO group of transcription factors.

**Phosphorylation dependent changes in the AKT1 substrate motif**

As observed for AKT (13) and many other kinases (45–47), the molecular basis for kinase substrate selectivity can depend critically on the residues neighboring the phosphorylation site. Yet it is important to understand that not all substrates identified for AKT isozymes include the same exact consensus motif
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In the context of the cell, even with an optimal substrate according to the consensus motif requirements, phosphorylation by AKT1 will depend on multiple factors, including accessibility, cellular localization, substrate expression level, scaffolding interactions, and phosphatase activity (11). The binding affinity between AKT1 and full-length protein substrates may also contribute to the degree of substrate phosphorylation.

Our data obtained from the OPAL study for ppAKT1T308S473 are in accord with but extended beyond previously reported results (12, 13). According to our results, all AKT1 phospho-forms showed a preference for Arg at position −5. ppAKT1T308S473 and pAKT1T308 showed a preference for hydrophobic amino acids at position +1. These amino acid preferences are shared by other AGC family kinases including protein kinase C. Obata et al. (13) used degenerative peptide library studies to determine the most relevant substrate motif for active AKT1. In this previous work, active AKT1 was derived from S9 insect cells and the impact of AKT1 phosphorylation status on substrate selectivity could not be probed. The authors reported the amino acid preferences derived from a set of known substrates and a degenerative peptide library. Because the peptide design was identical between Obata et al. (13) and our OPAL2 library, the results are similar. We also observed a strong correlation with the previous results and the OPAL1 at the positions −5 and +1 with pAKT1T308 and ppAKT1T308S473. In Obata et al. (13), the most preferred amino acid at the −5 position was Arg and preferred amino acids at +1 were large hydrophobic residues. Also, both pAKT1T308 and ppAKT1T308S473 enzyme variants showed preference for Phe at position −6 in agreement with the previous study (13). At the +2 position, as before (13), we also observed a preference for amino acids that enable tight turns, especially for pThr−308 containing enzymes: Gly2 in OPAL1, and Ser2 or Thr2 in OPAL2. In addition, Ser and Thr at +2 were the favorable amino acids according to our known library data (Figs. S1 and S2). Finally, in previous work, Pro at +1 inhibited AKT1 activity similarly to p70 S6 kinase (12). We also observed significantly reduced activity in all OPAL experiments for Pro at +1. The +1 position provides some level of orthogonality between AKT1 and proline-directed kinases, such as MAP kinases and cyclin-dependent kinases that select substrates with Pro at +1 (48).

Expanding the AKT1-dependent phospho-proteome

Validation of predictions based on our biochemical data using cellular or ultimately animal models is warranted in future studies beyond the scope of this work. Our initial investigations, based on independent database searches, found that AKT1 may be involved in regulating RNA metabolism (Fig. 7B, Fig. S11). In a recent report published with our collaborators, we validated one of these predicted AKT1 targets, the terminal nucleotidyltransferase Gld2 (49). Our data suggests the pAKT1T308 enzyme would phosphorylate Gld2 at Ser-116 (p value ~10−4) (Data File S2). Gld2 has been implicated in the stabilization and maturation of the tumor suppressor microRNAs miR-122 and let-7 (50, 51). We found Gld2 was indeed specifically phosphorylated by AKT1 at Ser-116, which abolished Gld2 activity in 3′-terminal addition of adenine to miRNA and mRNA substrates (49). As hyperactivity of AKT1 is common in many cancers (52), our results suggest that phosphorylation of Gld2 by AKT1 would be beneficial for cancer cells by decreasing the levels of miRNAs that act as tumor suppressors (49). Our data, thus, suggest a novel link between RNA regulation and oncogenic signaling by AKT1.

Conclusion

The phosphorylation status of AKT1 is a commonly used biomarker for human cancer (43). Here we found that AKT1 phosphorylation status has a global impact on substrate selectivity. Fascinatingly, ppAKT1T308S473 showed higher activity with less than half of the known substrates we tested. We determined that the addition of Ser-473 phosphorylation did not lead to a significant change in activity for nearly half of the substrates tested, and a minority of substrates was significantly more active with pAKT1T308 than with ppAKT1. The pAKT1S473 variant had a distinct substrate preference profile compared with AKT1 variants phosphorylated at Thr-308. Because our data show the impact of Ser-473 phosphorylation on AKT1 activity is substrate-dependent, we conclude pSer−473 may tune substrate selectivity rather than truly activate the kinase. Indeed, whether AKT1 with only Ser-473 phosphorylated is active in the cell or what its true substrates might be remains unknown (8, 40). Our findings have major implications for the use of AKT1 phosphorylation status as a clinical diagnostic (43, 53) or a marker for cancer biology (52).

Materials and methods

**Bacterial strains and plasmids**

A codon optimized human AKT1 gene lacking the PH domain (residues 109−480, 45 kDa) was synthesized at ATUM (Newark, CA, United States) previously (9) and subcloned, as follows: (NcoI/NotI) into an isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible T7lac promoter driven pCDF-Duet vector with CloDF13-derived replicon and streptomycin/spectinomycin resistance (pCDF-Duet1-APHAKT1). The human PDPK1 gene, which was acquired from the Harvard PlasmidID repository service (HsCD00001584, Boston, MA, United States), was subcloned (KpnI/Ndel) into the second multiple cloning site of pCDFDuet-1. The codon for the residue in AKT1 at position Ser-473 was mutated to the amber (TAG) codon in the synthetic gene from ATUM. Successful cloning was verified by DNA sequencing (London Regional Genomics Centre, London, ON, Canada, and Genewiz, Cambridge, MA, United States).

**Protein and phosphoprotein production**

Phosphorylated forms of AKT1 (pAKT1S473, pAKT1T308, and ppAKT1T308S473) were produced according to previously described methods (8, 9).

**Library of known AKT1 substrate peptides**

Based on reports from the literature collated at the Phosphosite database (6), we selected 84 different peptides to generate a library of known AKT1 substrates (Data File S1). The substrate motifs derived from these AKT1 substrates were synthesized in...
the following form: biotin-AXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐS₀/T₀-
X₁X₂X₃X₄X₅X₆YRR. For this library, X does not indicate ran-
domization, rather the nature of the X residues is defined by the
known sequence of AKT1 substrates. The phosphorylation site
(Ser or Thr) is located at the 0 position. Each individual in-
solution peptide was synthesized according to previously
reported methods (54). For Fig. S1, multiple statistical tests
were used to identify a group of peptide substrates that were not
significantly different from the background measurements
determined in experiments containing only kinase enzyme and
buffer but lacking substrate. The statistical significance be-
tween enzyme activities was calculated using one-way anal-
ysis of variance in Minitab statistical analysis software.
The level of significance was adjusted based on Bonferroni
correction.

**OPAL AKT1 activity screen**

An oriented peptide array library (OPAL1) was designed as a
series of soluble sublibrary pools according to previously
reported methods (15). The known consensus sequence for
AKT1 phosphorylation was used as the design for library syn-
thesis: biotin-AGGXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐS₀. In each of the
136 sublibraries, one of the X positions is held fixed to a
specific amino acid. X represents any amino acid other than Ser,
Thr, or Cys. The phosphorylatable residues Ser and Thr were
excluded from the screen to avoid false-positive signals when
probing position S₀. Each of the 17 other canonical amino acids
were tested in the X positions. The −3 and 0 positions were
fixed to Arg and Ser, respectively, where the 0 position is the
phosphorylation site and Arg−3 is important for substrate phos-
phorylation by AKT1 (42). The total library contains ~10¹⁹
different peptides. Accurate synthesis of the library was con-
firmed (Fig. S3) using a series of test peptides analyzed by
MALDI-TOF MS as before (55).

The independent OPAL2 was based on a peptide design of
YAXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐXₐS₀/GKK-biotin and was
synthesized and spotted exactly as described previously (56).
AKT1 protein was produced from Sf9 cells and kinase activity
assays were performed and processed as before (56); reaction
products were spotted onto avidin-coated filter sheets (Pro-
mega SAM² biotin capture membrane) and visualized by phos-
phorimaging. PCA analysis was conducted to compare the
OPAL matrices for each AKT1-phosphform. These were com-
puted in Matlab (MathWorks, Natick, MA, United States)
using custom scripts and default settings.

**Kinase assays with peptides and libraries**

Kinase assays with peptides or OPAL1 sublibraries were con-
ducted using radiolabeled [γ³²P]ATP according to previously
reported methods (8) with modifications as following: AKT1
activity was determined using 400 μM substrate peptide for
each peptide in the known substrate library. Peptides sublib-
raries were dissolved in 10 μl of dimethyl sulfoxide (DMSO) to
a final total peptide concentration of 1 mM. Each reaction
was conducted in kinase assay buffer (25 mM MOPS, pH 7.0), 12.5
mM β-glycerophosphate, 25 mM MgCl₂, 5 mM EGTA, pH 8.0, 2
mM EDTA, 20 μM ATP, and 0.4 μCi of (33 nM) [γ³²P]ATP.
The indicated AKT1 variant was added to a final concentration
of 6.5 nM to initiate the reaction. Each reaction was assayed
using 3 independent enzyme reaction replicates. Reactions
were spotted and quantified as before (8).

**Sequence logos and database searching**

Sequence logos corresponding to substrate preferences for
each AKT1 variant were generated using WebLogo version
2.8.2 (57) or Seq2Logo 2.0 (28) as indicated. Using the activity
data over our known substrate library (Fig. S1), we generated an
alignment of peptides in which the population of each peptide
was linearly related to its activity level. The resulting peptide
alignment was, thus, most populated by the highest active pep-
tides and least populated by the low active peptides. This
allowed us to convert the activity data over known peptides into
a sequence logo using WebLogo (Fig. S2). The sequence logos
based on the OPAL data were statistically analyzed by compar-
ing the overall average enzyme activity against the enzyme
activities of all tested amino acids at each position (i.e. −6 to
+3) using pairwise t test analysis. Thus, activity that is not sig-
nificantly different from the average activity is highlighted in red
(Fig. 5).

Using the OPAL1 and OPAL2 data we generated PSSMs
according to a similar method used in Scansite (29). For each
data set, a selectivity matrix was calculated by dividing all activ-
ity values by the average value for the complete array, i.e. a
selectivity factor. A scoring matrix was computed as the natural
log of the selectivity matrix. Finally, the raw scoring matrices
were scaled by a factor of 10 multiplied by the selectivity factor
for each data set. The resulting PSSMs based on each OPAL
data set were used to produce sequence logos with the program
Seg2Logo 2.0 (28), where the PSSM-Logos were computed
during default values. We then used the program PSSMSearch
(30) to identify potential AKT1 substrates based on PSSMs cre-
ated with the OPAL1 data.

As an alternate approach to generate a PSSM, we also
converted the OPAL1 activity data to a set of Z-score matri-
ces as before (58). Z-scores were calculated with the formula
Zᵢⱼ = (xᵢⱼ − μ)/s, i refers to the ith peptide sublibrary, and j
refers to the jth randomized position in the target sequence.
There is then a Z-score (Zᵢ) associated with each amino acid
at each position in the consensus motif. The score is calcu-
lated by subtracting the average (μ) kinase activity over the
library from the activity observed (xᵢ) normalized by the
standard deviation (s) of activity values over the entire
library. The preference rank of any potential substrate can
then be calculated as a score based on the sum of Z-scores at
each position in the test peptide. The known human phos-
pho-proteome was scored based on the amino acid prefer-
ences at each position in the Z-score PSSM (Data File S3).

**Data availability**

All of the data described in Figs. 1–7 and Tables 1 and 2 as well as
in supporting information Figs. S1–S13, Table S1, and Data Files
S1–S3 are available on-line.

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