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Development of a Peptidase-Resistant Substrate for Single-Cell Measurement of Protein Kinase B Activation

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Abstract

An iterative design strategy using three criteria was utilized to develop a peptidase-resistant substrate peptide for protein kinase B. Libraries of peptides possessing non-native amino acids were screened for time to 50% phosphorylation, degradation half-life within a lysate, and appearance of a dominant fragment. The lead peptide possessed a half-life of 92 ± 7 and 16 ± 2 min in HeLa and LNCaP cytosolic lysates, respectively, representing a 4.6- and 2.7-fold lifetime improvement over that of the starting peptide. The redesigned peptide possessed a 4.5-fold improvement in phosphorylation efficiency compared to the starting peptide. The same peptide fragments were formed when the lead peptide was incubated in a lysate or loaded into single cells although the fragments formed in significantly different ratios suggesting that distinct peptidases metabolized the peptide in the two preparations. The rate of peptide degradation and phosphorylation was on average 0.1 ± 0.2 zmol pg^{-1} s^{-1} and 0.04 ± 0.08 zmol pg^{-1} s^{-1}, respectively, for single LNCaP cells loaded with 4 ± 8 μM of peptide. Peptidase-resistant kinase substrates should find wide-spread utility in both lysate-based and single-cell assays of kinase activity.

INTRODUCTION

Protein kinase B (PKB, also known as Akt) is responsible for regulation of many cell functions, such as survival under stress, cell proliferation, and apoptosis.[1–3] Interest in PKB has steadily increased due to its role in enabling cells to survive stressful conditions, namely in preventing apoptosis in cells normally targeted for programmed cell death.[4] The PKB cascade and its upstream constituents have been implicated in tumor progression and survival in a wide array of cancers, including pancreatic, breast, and prostate tumors.[5] Strategies to monitor PKB activity in tumor cells are needed to identify which patients would benefit from inhibitors of PKB as well as in identifying optimal therapeutic concentrations. Analysis of PKB activation can be performed on cell lysates using Western blots in which phosphorylated residues on PKB are probed with phosphospecific antibodies to infer PKB activity.[6] A new technology incorporating isolelectric focusing within a capillary followed by immunoblotting permits the analysis of smaller cell numbers than a Western blot.[7] However, neither of these techniques enable measurement of PKB activation

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at the single-cell level. Alternative methods that use phosphospecific antibodies to probe PKB activity at the single cell level include immunocytochemistry, phosphoflow, and cytometry by time of flight (CyTOF).[8–11] The latter two methods are designed for non-adherent cells and all require cell fixation and permeabilization prior to measurement. Additionally, immunocytochemical staining of adherent cells identifies the presence of phosphorylated PKB but is generally considered to be non-quantitative.[12] Indicators employing fluorescent proteins have been developed to measure PKB activity at the single-cell level by monitoring fluorescence resonance energy transfer between two fluorescent proteins.[13, 14] However, these indicators require transfection of DNA into cells, making their use in primary cells challenging. Capillary electrophoresis has also been used to measure PKB activity in single cells; however, this strategy requires use of a fluorescently-tagged substrate peptide which can have a very short lifetime in cells, substantially limiting the technique's utility.[15–17]

Peptide substrates have been used extensively in biomedical research to assay kinase activity due to the ease of peptide design and synthesis and the ability to construct extensive peptide libraries for screening and optimization. As part of these endeavors, peptides serve as assay substrates for kinases obtained in pure form, from cell lysates, and even in single cells.[18–21] However, peptides often possess limited lifetimes in the cellular milieu due to the presence of peptidases which can degrade them. Multiple strategies have been employed to stabilize peptides against degradation, including cyclization, terminus modifications, and inclusion of non-native amino acids. Peptide cyclization yields degradation-resistant peptides, but this often results in poor substrate recognition by the kinase and these constructs can be synthetically challenging.[22, 23] Modifications to the C- and N-terminus are relatively simple to accomplish and provide protection against amino- and carboxy-peptidases, but not endopeptidases.[24] Terminal modifications can be used in combination with non-native residues, including N-methylated and D-amino acids, to impart additional stability to peptide bonds.[16, 25, 26] These non-native residues are readily available commercially and are simply incorporated into peptides utilizing standard solid phase peptide synthesis. Peptides with non-native residues generally suffer greatly diminished kinase-substrate properties and the design of kinase substrates with non-native amino acids remains challenging.[26] However, peptidase-resistant substrates would be of wide utility throughout biomedical research in assays of kinase activity in the presence of cellular constituents.

This work develops an iterative strategy to screen rationally designed peptides for their suitability to act as a kinase substrate in a cell lysate. Three criteria, time to 50% phosphorylation, half-life within a lysate, and appearance of a dominant fragment over time, were utilized to screen small libraries to design a degradation-resistant peptide substrate for PKB activity. Cytosolic lysates and purified kinase assays were utilized to rapidly assess synthetic peptides for their peptidase resistance as well as their ability to act as a substrate. Cleavage sites in peptides incubated in a cell lysate were characterized using capillary electrophoresis. Fluorescent peptide fragments were identified by comparison of their migration times with those of synthesized standards while the amount of each fragment was quantified from its fluorescence. Identification of cleavage sites permitted iterative replacement of amino acids with either non-native residues or more suitable native residues. Substitution success was then reassessed using the three criteria above and new substitutions incorporated based on these results until a peptidase-resistant PKB substrate was identified. This designed substrate was used to assay PKB activity in single prostate cancer cells in the presence and absence of a PKB inhibitor. The heterogeneity of substrate phosphorylation, kinase inhibitor performance, and peptidase activity in single cells was also evaluated.

EXPERIMENTAL SECTION

Materials

2-(6-chloro-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), 9-fluorenylmethoxycarbonyl (Fmoc) amino acids and resins were obtained from ChemPep or Novabiochem and 6-carboxyfluorescein (6FAM), N-Hydroxybenzotriazole (HOBt), Fmoc-Nα-Me-Arg(Mtr)-OH and were purchased from AnaSpec. Fmoc-β-HArg(Pbf)-OH was obtained from AAPPTec. Fmoc-α-MeAla-OH, and Fmoc-Phe(F5)-OH were purchased from Advanced ChemTech. All other peptide synthesis reagents were purchased from Aldrich or Fisher. Active PKBα enzyme was purchased from Millipore. Wortmannin was obtained from LC Laboratories. Bovine serum albumin was procured from Calbiochem. Roswell Park Memorial Institute Media (RPMI-1640) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Cellgro. Penicillin/streptomycin was obtained from Gibco and fetal bovine serum (FBS) was purchased from Atlanta Biologicals. All other chemicals used in assays were purchased from Fisher or Sigma.

Peptide Synthesis

Full-length substrate peptides amidated on the C-terminus were synthesized via standard Fmoc peptide synthesis (Prelude Peptide Synthesizer, Protein Technologies, Tucson, AZ) on TGR resin (supplemental information). Peptide fragment standards with a free carboxylic acid on the C-terminus were synthesized using the same strategy but starting with a 2-chlorotrityl chloride resin. The N-terminus of all peptides was labeled with fluorescein.

in vitro Kinase Assay

Protein kinase assays were performed at 30 °C in assay buffer [8 mM MOPS (3-(N-morpholino) propanesulfonic acid), pH 7.0, 0.2 mM EDTA, 4 mM $MgCl_2$, 1 mM ATP] with PKBα kinase (6 nM) and substrate (25 μM). Aliquots were removed from the reaction mixture at various times and the reaction was halted by heating at 90 °C for 4 min. Replicates were performed with different purified enzyme aliquots at different times. The average and standard deviation of triplicate measurements were reported. The amount of peptide phosphorylation was measured using capillary electrophoresis coupled with laser-induced fluorescence detection (CE-LIF).

Measurement of Peptide Degradation in a Cell Lysate

A HeLa or LNCaP cell lysate was prepared as previously described.[16] Assessment of peptide degradation was performed by mixing peptide substrate (1 μM) with cell lysate (3 mg mL^{-1} total cell protein) and incubating at 37 °C. Aliquots were removed and stopped by adding HCl to a final concentration of 100 mM. A 0 min timepoint was prepared by adding the HCl to the lysate prior to the addition of the peptide. Reaction mixtures were then separated and detected with CE-LIF. Peptide fragments were identified by adding standards (250 nM) to the HCl-terminated aliquots and comparing the electropherograms with and without the added standard. Replicates were performed with different cytosolic lysates at different times. The average and standard deviation of triplicate measurements were reported. The average initial degradation and fragmentation rates were calculated using the initial and 5 min time points by monitoring the change in peptide quantity divided by the change in time per amount of cytosolic protein. The units are defined as zmol of peptide per pg of total cytosolic protein per s (zmol pg^{-1} s^{-1}).

Measurement of Kinetic Parameters

Protein kinase assays were performed as described in the *in vitro* kinase assay section with the following exceptions: substrate concentrations ranged from 0.5 to 30 μM; and PKBα

enzyme concentration was 1 nM for peptide I and 6 nM for peptide VI-B. The immobilized metal ion affinity-based fluorescent polarization (IMAP) assay (Molecular Devices Corporation, Sunnyvale, CA) was used to measure the amount of phosphorylated peptide in reaction mixtures.

Single Cell Capillary Electrophoresis

Single cell CE was performed using a custom made CE system with LIF detection, as described previously.[16] Fused silica capillaries [30 μm inner diameter, 360 μm outer diameter, (Polymicro Technologies, Phoenix, AZ)] had a total length of 38 cm and an effective length of 21.5 cm. For peptide I, the electrophoretic buffer was 100 mM borate and 100 mM SDS, pH 7.7 and a field strength of 315 V cm^{-1} was applied. For peptide VI-B, the electrophoretic buffer was either 300 mM borate, pH 7.5 or 100 mM borate and 7 mM SDS, pH 7.7, with a field strength of 260 V cm^{-1}. In all instances, electrophoresis was initiated by application of a negative voltage to the outlet while the inlet reservoir was held at ground. Cells were perfused with extracellular buffer (ECB; 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM HEPES, pH 7.4, 37 °C) during experiments, with the flow turned off immediately prior to lysis and loading into the capillary. Laser-based lysis was achieved with a focused Nd:YAG laser as previously described[27] and the cell contents were electrokinetically loaded into the capillary by applying −3 kV to the capillary outlet while holding the inlet at ground. To identify the peptide fragments formed in a cell, standards of peptide fragments (100 nM) were hydrodynamically loaded into the capillary following the contents of a cell previously microinjected with the full-length peptide. A voltage was then applied across the capillary to initiate electrophoretic separation. These electropherograms were compared to electropherograms with no standard co-injected to determine if that peptide fragment was formed. To calibrate the amount of peptide on the electropherograms, a known concentration of standard of each intact peptide was hydrodynamically loaded into the capillary, electrophoresed, and the area under the peak calculated. Poiseuille's equation was utilized to estimate the amount of peptide injected.[28]

RESULTS AND DISCUSSION

Selection of the Starting Peptide

The starting peptide I (*6FAM*-GRPRAATFAEG) was based on the consensus sequence RPRAATF, determined in a library screen to be phosphorylated by PKB, but not phosphorylated by two other closely-related kinases, p70 S6 Kinase and MAP kinase activated protein kinase-1, the only peptide screened in the library with these properties.[29] Furthermore, in a large multiplexed assay to analyze substrate specificity of 26 kinases, peptide I was found to be relatively selective for PKB.[30] Peptide I was efficiently phosphorylated by purified PKB *in vitro* (Supplementary Figure S1). Two cell types with upregulated PKB activity were chosen for this proof-of-principle work. LNCaP cells, a line derived from a human metastatic prostate adenocarcinoma, have decreased PTEN, a phosphatase with actions that lead to increased PKB activation.[31, 32] HeLa cells, a line derived from a human cervical adenocarcinoma, overexpress upstream components of PKB, resulting in increased PKB activity.[33]

In order to identify the initial peptide bond hydrolyzed in a cellular environment, peptide I was incubated in a HeLa or LNCaP cell lysate. Aliquots were removed over time and CE-LIF was utilized to quantify the peak area of fluorescent species over time. At time zero in both the HeLa and LNCaP cytosolic lysates, a single peak with a migration time identical to that of the intact substrate was seen (Figure 1A). However, as the incubation time was increased, peptide I was rapidly metabolized with a lifetime of 20 ± 2 and 6 ± 0.9 min in the HeLa and LNCaP lysate, respectively (Figure 1, Supplementary Figures S1 and S2). Under

these conditions and when normalized for total cytosolic protein, peptide I was broken down at an average initial rate of 0.12 ± 0.01 and 0.4 ± 0.06 zmol pg^{-1} s^{-1} in the HeLa and LNCaP lysates, respectively. In both the HeLa and LNCaP lysate, multiple additional peaks formed over time with similar initial rates. This suggested that in these lysates, several different peptide bonds could be utilized as the initial site of hydrolysis. By 60 min, six peaks other than that due to the starting peptide were present in the lysate and the original peptide had decreased to approximately 10% and 0% of all species present in the HeLa and LNCaP lysate, respectively. Since the cytosolic lysates did not possess peptidase or phosphatase inhibitors or added ATP, it was likely that the six additional peaks were proteolytic fragments of the starting peptides. The significantly shorter lifetime of the peptide in the LNCaP lysate relative to the HeLa lysate was most likely due to the large amounts of proteases synthesized by cells of the prostate lineage.[34]

To identify which peptide fragments were formed in the cell lysates, standards of all possible fluorescent fragments of peptide I were synthesized and the migration time of each standard compared to that resulting from the cell lysates. Under the electrophoretic conditions utilized, all fragment standards were present as a single major peak and each migrated at a unique time. Each of the peaks generated in the HeLa and LNCaP lysates matched to a single standard fragment (Figure 1B, C, F). After incubation in the HeLa lysate for 60 min, two peptide fragments (peptides k and i, Figure 1F) each totaled approximately 20% of all species present, while the remaining four peptide fragments (peptides h, g, f, and e) each comprised less than 10% of all species present. After 60 min in the LNCaP lysate, the same 6 peptide fragments were present although in different amounts and peptide k was present as the dominant species. From these data, no clear initial bond was identified as a primary site of hydrolysis in either cell lysate.

Strategy for Design of Peptidase-Resistant PKB Substrate

Since there was no clear initial peptidase cleavage site on peptide I for either cell lysate, a strategy to repeatedly generate a small library incorporating altered peptide residues followed by screening was utilized. Each library targeted a single residue for modification and was screened for the time for 50% phosphorylation by PKB ($t_{50\%P}$) as well as for the peptide lifetime ($t_{1/2}$) and the appearance of a dominant fragment in a HeLa cell lysate (Figure 2). The appearance of a dominant hydrolysis site narrowed the choice of residues for targeted modifications and offered a rational next step in peptide modification. After identification of the optimal peptide sequence in the library, all possible fluorescent fragments were synthesized to identify cleavage sites to target for subsequent alterations. A second small library was generated followed by rescreening for $t_{50\%P}$ and $t_{1/2}$ and dominant fragment formation. Successive iterations of this process led to identification of multiple cleavage locations that were stabilized to yield a longer-lived substrate for PKB.

Characterization of Peptides Following Arginine Replacement

Peptide modification initially targeted the amino-terminal alanine but this library failed to produce peptides that could act as PKB substrates (supplementary information). Consequently the arginine carboxy-terminal to the proline was targeted since cleavage at this site yielded peptide i, a three-residue peptide and the most abundant fragment formed in the HeLa lysate after 45 min of incubation with peptide I (Figure 1). Mono- and di-methyl-substituted, β-, and D-arginine are readily available derivatives that can impart stability against proteolysis.[25] These modified arginine residues were utilized to synthesize peptides III-A through III-D (Table 1) and $t_{1/2}$ and $t_{50\%\,P}$ were measured for each peptide. These peptides showed no improvement in $t_{1/2}$ over peptide I, with values ranging from 2 to 17 min. Three of the four peptides showed no evidence of phosphorylation, while the N-methylated arginine-substituted peptide (III-B) possessed a $t_{50\%\,P}$ of 1013 min, more than

50X longer than peptide I. When the fragment standards of peptide III-B were generated and utilized to identify the fragments formed in the lysate, the cleavage at the 3-mer site or Arg-Pro bond was eliminated. After 60 min, the 6-residue fragment generated by hydrolysis of the alanine-threonine bond was the dominant fragment, comprising 92% of all peptide present within 15 min. Although III-B possessed poorer properties than the starting substrate, it did yield a dominant fragment upon incubation with a cell lysate and a single preferred hydrolysis site that could be targeted for modification. For this reason, peptide III-B was used as the template in all subsequent optimization rounds.

Characterization of Peptides Following the Replacement of the Carboxy-Terminal Alanine

The alanine residue located at the alanine-threonine cleavage site of III-B was targeted for modification since hydrolysis of this peptide bond yielded the dominant fragment when III-B was incubated in a HeLa cell lysate. Non-native derivatives of alanine, β- and D-alanine, were substituted for the alanine (peptides IV-A and IV-B, Table 1). Furthermore, since the active site of PKB possesses a hydrophobic pocket near the phosphorylatable threonine and is adjacent to a localized region of positive potential,[35] two hydrophobic residues (peptides IV-D and IV-E) and a glutamic acid (peptides IV-F) were also inserted in place of the alanine (Table 1). The $t_{1/2}$ and $t_{50\%\ P}$ were measured for each of the peptides IV-A through IV-E. Four peptides displayed longer $t_{1/2}$ values than III-B, and one peptide, IV-D, demonstrated a $t_{50\%\ P}$ of 30 min, on the same order of magnitude as that for peptide I and over 30X faster than that of peptide III-B. Additionally, when the fragments of peptide IV-D were analyzed, the dominant initial fragment in the HeLa lysate was the 8-residue fragment generated when the phenylalanine-alanine bond was hydrolyzed. Thus peptide IV-D was selected as the next lead peptide.

Characterization of Peptides Following Phenylalanine or Alanine Replacement

The next residues targeted for modification were the two amino acids flanking the cleavage site of the most abundant fragment of peptide IV-D. Pentafluorophenylalanine (Supplementary Figure S4) was substituted in place of the phenylalanine to impart additional hydrophobicity to the position (peptide V-A).[36] In addition, the non-native derivatives D-alanine and sarcosine (Supplementary Figure S4) were inserted in place of the alanine to investigate substrate suitability (peptides V-B and V-C). Two peptides possessed $t_{1/2}$ values 5X greater than that of peptide IV-D while peptide V-B displayed the greatest resistance to proteolysis ($t_{1/2}$ of nearly 900 min, over 60X greater than that of IV-D). Of these peptides, only peptide V-C was phosphorylated at a rate ($t_{50\%\ P}$ of 21 min) similar to that of IV-D. Furthermore, V-C displayed upon lysate incubation a single fragment which formed over time, the 9-mer peptide generated by hydrolysis of the sarcosine-glutamic acid bond (40% of the total peptide present after 60 min). Based on this single hydrolysis position, peptide V-C was selected for further modifications.

Characterization of Peptides Following Truncation of the Substrate

As illustrated by the consensus sequence, RPRAATF (where T is the site of phosphorylation), the amino terminal sites play the predominant role in PKB substrate recognition. Thus removal of the two carboxy-terminal residues from the lead peptide V-C might not significantly alter the suitability of the substrate for PKB. Since the only fragment formed when peptide V-C was incubated in the cytosolic lysate was the 9-residue fragment, this truncated peptide (peptide VI-A) was assessed for its stability over time in a HeLa cell lysate. Additionally $t_{1/2}$ and $t_{50\%\ P}$ for a methyl-alanine substituted 9-mer (peptide VI-B) was also measured. The $t_{1/2}$ of peptide VI-A indicated that this peptide was significantly less stable than the parent peptide V-C. However, peptide VI-B possessed a $t_{1/2}$ of 92 ± 7 min, similar to that of peptide V-C. When the peptides were incubated with PKBα, $t_{50\%\ P}$ for VI-

B was similar to that of peptide V-C. Based on these combined properties, peptide VI-B was selected for further characterization.

Characterization of Lead Peptide VI-B in Cytosolic Lysates

Although the lead peptide VI-B was more stable than peptide I in a HeLa cell lysate, it was still hydrolyzed into four fragments (Supplementary Figure S5). The latter were identified by synthesizing and electrophoresing all possible fluorescent fragments. Each peak in the lysate mixture co-migrated with a single standard peak (Figure 3B). Peptide p, which was the fragment present in the highest amount, comprised 20% of all species after 60 min and formed at an average initial rate of 0.02 ± 0.002 zmol pg^{-1} s^{-1} (Figure 3D). Three additional peaks also formed in the HeLa lysate, but on much slower timescales than peptide p (Figure 3D). The parent peptide was degraded at an average initial rate of 0.03 ± 0.004 zmol pg^{-1} s^{-1}, and approximately 60% remained after 60 min.

Peptide VI-B was also incubated in a LNCaP cytosolic lysate, a more aggressive proteolytic environment, and fragment formation over time was assessed (Figure 3E, Supplementary Figure S6). Only three fragments were seen in addition to the intact parent peptide, with peptide p making up 90% of all peptide present after 60 min. The rate of breakdown of the intact parent in the LNCaP lysate was 0.16 ± 0.03 zmol pg^{-1} s^{-1}, over 5X faster than breakdown in the HeLa lysate. Peptide p formed at a rate essentially as fast as the parent was degraded, at 0.14 ± 0.02 zmol pg^{-1} s^{-1}. Two additional fragments, peptides o and n, formed at much slower rates, similar to the result obtained in the HeLa lysate. As expected in the cell line with more the aggressive proteolytic environment, peptide bond hydrolysis proved to be much faster, although the primary cleavage location remained the same.

The kinetic parameters K_M and k_{cat} were determined for peptide I and the lead peptide VI-B in order to compare the substrate efficiency of these peptides. Varying concentrations of each peptide were incubated with PKB and the initial reaction velocity was calculated for each substrate concentration. Peptide I exhibited a better K_M than peptide VI-B (3 μM compared to 23 μM) but a poorer k_{cat} (3,400 min^{-1}) relative to that of VI-B (5,800 min^{-1}) leading to a 4.5-fold better efficiency for VI-B over peptide I.

Characterization of Peptide Degradation in Single LNCaP Cells

Peptides I and VI-B were microinjected into single LNCaP cells and incubated for 90 s prior to lysis and electrophoresis to characterize peptide behavior. Peptide I was degraded into 6 fragment peaks while peptide VI-B was degraded into 2 fragment peaks (Supplementary Figure S7). Thus, peptide VI-B was selected over peptide I for further characterization in single cells. To quantify the amount of peptide VI-B degradation and phosphorylation within intact cells, the peptide was microinjected into single LNCaP cells (n = 19) and incubated for 5 min prior to cell lysis and CE-LIF. Four peaks were observed, with intact, non-phosphoryated, parent peptide accounting for 15 ± 5% of all species present. The parent peptide was degraded into one of two fragment peaks (peptides m or p) or converted to phosphorylated product (Figure 4A). In addition, the major peptide fragment identified in single cells was peptide m (40% of total peptide present), the 8-residue fragment seen only in small amounts in the LNCaP lysate studies (Figure 3E). The major fragment seen in the LNCaP lysate studies, peptide p, was present only as a minor species in the single cells. Assuming a cell volume of 1 pL and a total protein concentration of 100 mg mL^{-1},[37] the intact peptide was eliminated at an average rate of 0.1 ± 0.2 zmol pg^{-1} s^{-1}, similar to the breakdown rate measured in LNCaP cytosolic lysates. The rate of degradation of the intact parent peptide was linearly dependent on the peptide concentration (4 ± 8 μM) in the cell (Figure 4C), with a linear regression analysis yielding an R^2 value of 0.9899.

Phosphorylation of Peptide VI-B in Single LNCaP Cells

Five min after introduction of VI-B into single cells, the percentage of intact peptide that was phosphorylated ranged from 0 – 80% (58 ± 26%). The amount of phosphorylated peptide present varied linearly with respect to the amount of peptide microinjected into the cell (Figure 4D). A linear regression analysis of the rate of phosphorylation yielded an R^2 value of 0.9401, indicating greater cell-to-cell variability in the amount of peptide phosphorylated relative to that degraded in cells even after correction for the amount of peptide loaded into cells. This may be due to a greater variation in the concentration of active kinase molecules in single cells relative to that of the peptidases. Flow cytometry-based measurements suggest that the numbers of protein molecules per cell for enzymes involved in proteolytic degradation are tightly regulated and thus have relatively little cell-to cell variation.[38] By contrast, the copy number for molecules acting as components of signaling cascades responsive to environmental stimuli generally have significant noise or much greater variation in the cell-to-cell copy numbers.

Inhibition of PKB Activity in Single Cells

Wortmannin is a selective inhibitor of PI3-Kinase when used in nanomolar concentrations, which leads to decreased PKB activation.[39] LNCaP cells were pre-incubated with 500 nM wortmannin (10 min) and then loaded with VI-B (n = 10). After 5 min, cells were individually lysed and their contents separately electrophoresed. Four peaks (intact parent, phosphorylated substrate, and fragments m and p) were identified in these cells as in the cells without wortmannin (Figure 4B). There was no statistical difference in the amount of degradation observed when compared to cells with no pre-treatment (p-value of 0.77; Figure 4C). Formation of phosphorylated product was substantially decreased (13% ± 12%) compared to that for cells not pretreated with wortmannin (p-value of 1.1×10^{-6}). While wortmannin also inhibits mTOR, myosin light chain kinase (MLCK), and phosphatidylinositol 4-kinase (PI4K) but at micromolar concentrations,[39] inhibition of substrate phosphorylation by these low concentrations of wortmannin suggested that the peptide was predominantly acting as a substrate for PKB.

CONCLUSION

An iterative design and screening process was used to identify and stabilize locations susceptible to proteolysis in a kinase substrate peptide. Each round of redesign yielded a substrate with more favorable properties until a peptide resistant to lysate peptidases, yet suitable as a PKB substrate, was obtained. The optimized peptide displayed similar fragmentation patterns in lysates from cervical (HeLa) *vs* prostate (LNCaP) cancer cell lines, but the rates of hydrolysis were dramatically different. The relatively high proteolytic rates observed in the prostate-derived cells was likely due to the fact that normal prostate produce an abundance of proteases. The rate of proteolysis in the LNCaP lysate was similar to that seen in the single LNCaP cells. Remarkably, the primary cleavage locations differed between lysates and single cells, suggesting that different peptidases may access the peptide in these two conditions, thereby indicating that lysates do not always provide an accurate mimic of the intact cell. Although substantial variability existed in the percentage of peptide degraded in single cells, this variability was a consequence of the variation in the amount of peptide loaded into cells *i.e.* the final intracellular peptide concentration. With regard to phosphorylation, substantial variability existed between single cells, indicating differing amounts of kinase activity in cells within the same population. These measurements are consistent with other measures of single cell variability, such as that seen in flow cytometry and microscopy.[40, 41]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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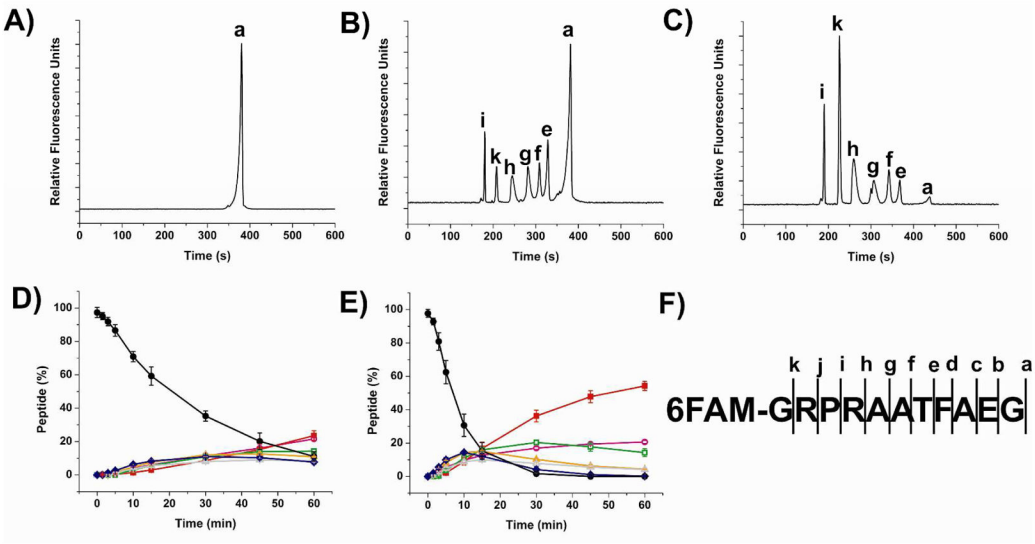

Figure 1.

Degradation of the starting peptide I in cytosolic lysates. (A) – (C) Electropherograms of peptide I after incubation in a HeLa or LNCaP lysate for 0 min (A) for 30 min in HeLa (B) or for 30 min in LNCaP (C) cytosolic lysate. (D) Formation of peptide fragments over time in HeLa lysate. (E) Formation of peptide fragments over time in LNCaP lysate. The symbols are defined as: black closed circle (peptide a or starting peptide); pink open circle (peptide i); red closed square (peptide k); green open square (peptide h); orange closed triangle (peptide g); black open triangle (peptide f); and blue closed diamond (peptide e). (F) The uppercase letters are the single amino acid abbreviations for the starting peptide sequence. The lowercase letters represent cleavage locations that form the indicated fluorescent peptide fragments.

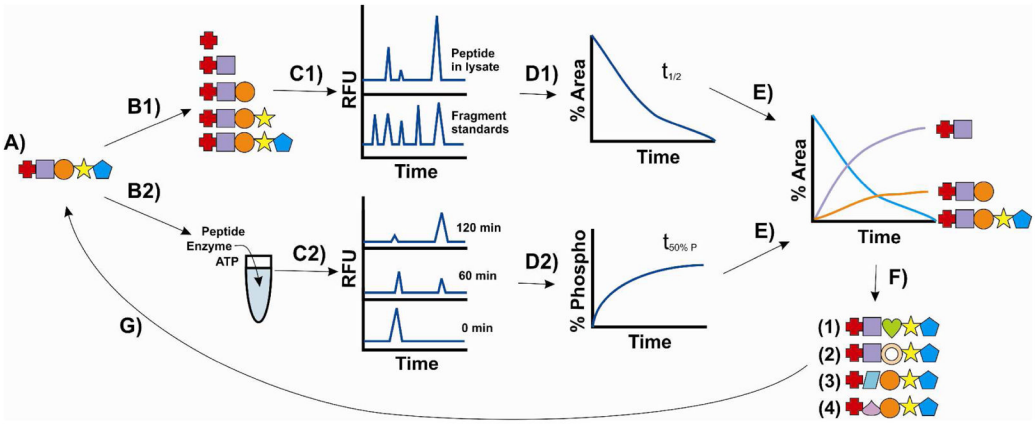

Figure 2.

Schematic of rational peptide substrate design. (A) Synthesize starting peptide. The different geometrical shapes represent different amino acids. (B) Synthesize all possible fluorescent peptide fragments of starting peptide (B1) and incubate starting peptide with active enzyme and ATP (B2). (C) Use CE-LIF to analyze degradation and identify peptide fragments formed (C1) and to assess phosphorylation over time (C2). (D) Determine the half-life of the peptide in the lysate (D1) and the time required for the peptide to reach 50% phosphorylation (D2). (E) Of the peptides showing favorable $t_{1/2}$ and $t_{50\% P}$ values, identify whether a preferred cleavage site exists in the peptide. (F) Synthesize a library of new peptide substrates with non-native amino acids adjacent to the most susceptible bond in the lead peptide. (G) Reiterate through steps (B) – (F) until a suitable, long-lived substrate is obtained.

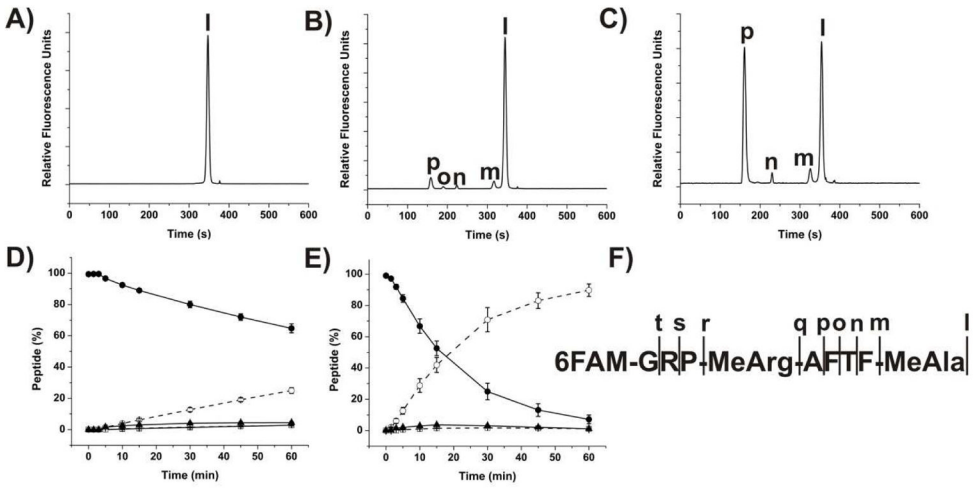

Figure 3.

Degradation of the lead peptide VI-B in cytosolic lysates. (A) – (C) Electropherograms of peptide I after incubation in a HeLa or LNCaP lysate for 0 min (A) for 30 min in HeLa (B) or for 30 min in LNCaP (C) cytosolic lysate. (D) Formation of peptide fragments over time in HeLa cell lysate. (E) Formation of peptide fragments over time in LNCaP cell lysate. The symbols are defined as: closed circle (peptide l); open circle (peptide p); closed square (peptide o); open square (peptide n); and closed triangle (peptide m). (F) The uppercase letters are the single amino acid abbreviations for the lead peptide sequence, MeArg is N-methylated arginine, and MeAla is N-methylated alanine. The lowercase letters represent cleavage locations that form the indicated fluorescent peptide fragments.

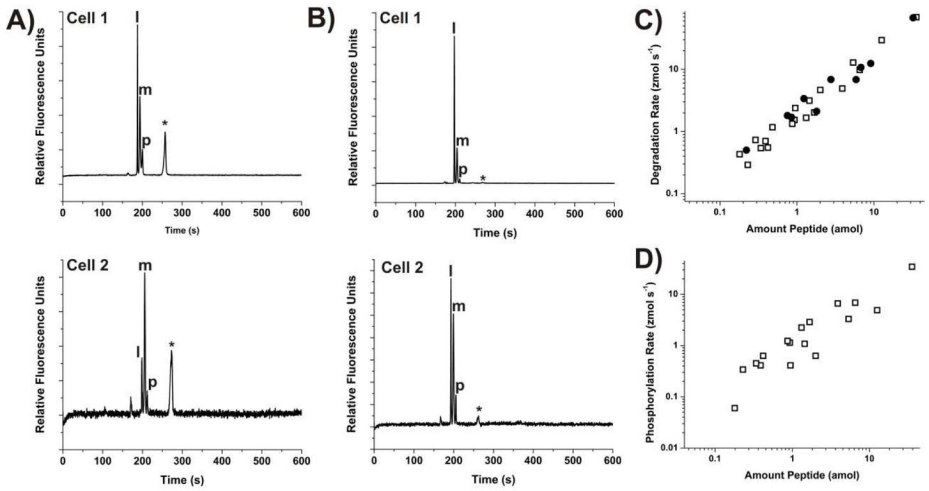

Figure 4.

Electropherograms of single LNCaP cells 5 min after microinjecting the lead peptide QW-VII-48F without (A) or with pre-treatment with wortmannin (B). The lowercase letters mark the peaks due to the intact peptide or peptide fragments identified in Figure 3F and the * labels the peak due to the phosphorylated peptide product. Two representative cells are shown for (A) and for (B). (C) Rate of degradation of parent peptide as a function of amount microinjected in single cells with (open squares, n = 19) and without (closed circles, n = 10) pre-treatment with wortmannin. (D) Rate of phosphorylation as a function of amount microinjected in single cells.

Table 1

Peptide Properties.

Peptide	Sequence	$t_{50\%P}$ (min)	$t_{1/2}$ (min)	Initial Dominant Fragment
I	6FAM-GRPRAATFAEG	17	20	5-mer
III-A	6FAM-GRP-βArg-AATFAEG	NP	9	ND
III-B	**6FAM-GRP-MeArg-AATFAEG**	**1013**	**2**	**6-mer**
III-C	6FAM-GRP-Arg(Me2)-AATFAEG	NP	8	ND
III-D	6FAM-GRP-DArg-AATFAEG	NP	17	ND
IV-A	6FAM-GRP-MeArg-A-βAla-TFAEG	$>10^5$	14	ND
IV-B	6FAM-GRP-MeArg-A-DAla-TFAEG	$>10^5$	32	ND
IV-C	6FAM-GRP-MeArg-A-Glu-TFAEG	679	37	ND
IV-D	**6FAM-GRP-MeArg-A-FTFAEG**	**30**	**14**	**8-mer**
IV-E	6FAM-GRP-MeArg-A-MePhe-TFAEG	125	2	ND
V-A	6FAM-GRP-MeArg-AFT-Phe(F5)-AEG	174	88	ND
V-B	**6FAM-GRP-MeArg-AFTF-DAla-EG**	**306**	**895**	**9-mer**
V-C	**6FAM-GRP-MeArg-AFTF-Sarc-EG**	**21**	**84**	**9-mer**
VI-A	6FAM-GRP-MeArg-AFTF-Sarc-NH_2	36	13	ND
VI-B	6FAM-GRP-MeArg-AFTF-MeAla-NH_2	34	92	5-mer

[a] $t_{50\%\ P}$ is the time required for 50% of the peptide to be phosphorylated and [b] $t_{1/2}$ is the half-life of the intact peptide in a HeLa cytosolic lysate. NP = no phosphorylation. ND = not determined. Peptides shown in bold were used as base peptides for further modification in the next iteration. The amino acids are represented by their standard one letter code except for the following, which are defined as: βAla (Beta-alanine), MeAla (N-methylated alanine), DAla (D-alanine), βArg (Beta-arginine), MeArg (N-methylated arginine), Arg(Me2) (N-ω,ω-dimethyl-L-arginine(symmetrical)), MePhe (N-methylated phenylalanine), Phe(F5) (pentafluorophenylalanine), and Sarc (sarcosine, or N-methyl glycine).