Published in final edited form as:
Analyst. 2012 July 7; 137(13): 3028–3038. doi:10.1039/c2an16162a.

Metabolism of Peptide Reporters in Cell Lysates and Single Cells

Angela Proctor[a], **Qunzhao Wang**[a], **David S. Lawrence**[a,b], and **Nancy L. Allbritton**[a,c]

[a]Department of Chemistry, University of North Carolina, Chapel Hill, NC 27599, USA

[b]Division of Chemical Biology and Medicinal Chemistry, School of Pharmacy, University of North Carolina, Chapel Hill, NC 27599, USA

[c]Department of Biomedical Engineering, University of North Carolina, Chapel Hill, NC 27599, USA and North Carolina State University, Raleigh, NC 27695, USA

Abstract

The stability of an Abl kinase substrate peptide in a cytosolic lysate and in single cells was characterized. In the cytosolic lysate, the starting peptide was metabolized at an average initial rate of 1.7 ± 0.3 zmol $pg^{-;1}$ $s^{-;1}$ with a $t_{1/2}$ of 1.3 min. Five different fragments formed over time; however, a dominant cleavage site was identified. Multiple rational design cycles were utilized to develop a lead peptide with a phenylalanine and alanine replaced by an (N-methyl)phenylalanine and isoleucine, respectively, to attain cytosolic peptidase resistance while maintaining Abl substrate efficacy. This lead peptide possessed a 15-fold greater lifetime in the cytosolic lysate while attaining a 7-fold improvement in k_{cat} as an Abl kinase substrate compared to the starting peptide. However, when loaded into single cells, the starting peptide and lead peptide possessed nearly identical degradation rates and an altered pattern of fragmentation relative to that in cell lysates. Preferential accumulation of a fragment with cleavage at an Ala-Ala bond in single cells suggested that dissimilar peptidases act on the peptides in the lysate versus single cells. A design strategy for peptide stabilization, analogous to that demonstrated for the lysate, should be effective for stabilization in single cells.

Introduction

Synthetic peptides have found widespread use in biomedical research particularly as substrates and inhibitors of kinases and peptidases. The chemistry of solid-phase peptide synthesis is well-defined, permitting relatively straightforward construction of large quantities of peptides comprised of native and non-native amino acids. Peptides have a long shelf life and are readily derivatized with labels such as fluorophores and targeting elements (e.g. biotin). Peptides are an attractive alternative to proteins in a variety of applications (e.g. enzyme assays) due to their chemical simplicity and ease of handling. Furthermore, the substrate preference of many protein kinases is largely determined by the amino acid sequence surrounding the phosphoryl-accepting residue. Consequently, consensus peptide sequences have been defined for large numbers of these enzymes.[1–4] In many cases, these short peptide sequences mimic the kinetic properties of the native protein substrates for the enzyme. For kinases, replacement of the residue that undergoes phosphorylation (serine, threonine, or tyrosine) with a non-phosphorylatable analogue (alanine or phenylalanine) often yields a peptide with excellent inhibitory properties for the targeted kinase.[5, 6] Modifications of a peptide substrate with non-native residues can lead to effective and stable inhibitors when the inhibitor peptide binds tightly to the substrate binding domain of the kinase and prevents docking of the target substrate.[5, 7] For these reasons, a wide array of substrate and inhibitor peptides are commercially available to manipulate or monitor a plethora of kinase activities in *in vitro* systems.

In addition to their use in assays with purified kinases, peptides are often used to assay kinase activity in cell lysates and also increasingly in intact cells.[8–15] Cell lysates offer a native enzyme in an environment which retains many of the co-factors and other regulatory elements of the cell. Cell lysates can be prepared in large volumes to meet the sensitivity needs of almost any detection strategy. Manipulation of the reaction environment by addition or removal of modifiers or inhibitors is also relatively trivial in a cell lysate compared to intact cells. However, lysates do not recapitulate the environment of an intact cell in a variety of ways. For example, the contents of a cell lysate are diluted and intermixed when intracellular compartments are fragmented during lysis, resulting in a loss of protein compartmentalization as well as normal spatial relationships found in intact cells. Additionally, the heterogeneity of single cell populations has been well documented[16, 17] and cell lysates eliminate this heterogeneity when samples are pooled. For these reasons, intact cells are also used as an assay format in which peptides act as inhibitors or substrates for enzymes.[18–20] The low molecular weight of peptides offers the advantage of efficient loading into cells relative to intact proteins.[21] The loading of substrate peptides into cells followed by lysis and separation of substrate and product by electrophoresis has been performed to track a variety of enzymatic reactions including those of kinases, acyl transferases, and proteases.[22–24] This strategy offers direct quantification of the substrate and product quantities as well as the identification of the products of competing reactions with zeptomole detection limits.

Despite their benefits as substrates and inhibitors in biomedical research, the use of peptides in the presence of cellular constituents is plagued by unwanted and rapid proteolysis. Exo- and endo-peptidases are an integral part of the protein recycling and antigen processing machinery of cells and ensure that most peptides are rapidly hydrolyzed to their amino acid constituents.[25] While peptidase inhibitors can be used to slow these reactions, they are generally insufficient to eliminate peptide hydrolysis, are often not specific to a single protease, and have significant other off-target effects. Furthermore, many peptidase inhibitors are poorly soluble in aqueous solutions necessitating the use of organic solvents that may interfere with cellular reactions. To prevent peptide bond hydrolysis, a number of alternative strategies have been utilized to impart stability to peptides. Peptide cyclization reduces degradation by creating a fixed secondary structure preventing protease access to the peptide bond.[26–29] Although circularized peptides are excellent at resisting peptidase actions, peptide cyclization can be difficult and is often of low yield due to competing intermolecular reactions. More importantly, cyclization often reduces or eliminates the ability of kinases to phosphorylate the peptide. Another method frequently utilized to impart stability to peptides is the addition of a polyethylene glycol moiety (PEGylation); shorter PEG chains (average molecular weight of approximately 6,000 Da) attached to peptides show better affinity for the targeted enzyme yet less ability to resist degradation than PEGylated peptides with average molecular weights near 25,000 Da.[30–32] The addition of a PEG group greatly increases substrate molecular weight and diminishes the chemical differences of the substrate and product, making detection and quantification of the substrate and product challenging. The polydispersity of molecular weights in PEG formulations also yields unacceptably broad electrophoretic peaks. Introduction of non-native residues into peptides, which can be achieved with standard solid phase synthesis strategies, has been shown to block unwanted proteolytic reactions. This strategy has been particularly successful in stabilizing inhibitor peptides.[30, 33–38] Extensive libraries of non-native amino acids provide numerous possibilities for the construction of selective inhibitors or substrates, as demonstrated by Lee *et al.* in 2004.[14] However, design of peptides that act as substrates for specific enzymes yet are peptidase resistant has been challenging.

A strategy was developed to rationally design a kinase-substrate peptide with increased stability in cell lysates and intact cells. A consensus substrate sequence was selected for Abl

kinase and used as the starting point for the design modifications. Abl kinase was chosen because of its role in chronic myelogenous leukemia (CML), where a constitutively active Abl enzyme results from the fusion of the Bcr to Abl protein in a majority of patients with CML.[39] The average rate and locations of lysate-induced cleavage sites in an Abl peptide substrate were identified by capillary electrophoresis. The starting peptide was then modified by replacement of residues adjacent to the identified cleavage site with non-native amino acids and then re-evaluated for peptidase resistance in the cell lysate. The modified peptides were also evaluated for Abl substrate suitability. Multiple cycles of peptide modification and assessment of protease resistance and kinase substrate efficacy generated a final modified peptide that was characterized in a cell lysate. The substrate was also assessed for phosphorylation by Abl relative to the starting sequence. Proteolytic sensitivity of both the starting peptide sequence as well as the final modified peptide was also assessed in intact single cells.

Materials and Methods

Chemicals

Peptide synthesis reagents were purchased from Aldrich or Fisher except for the following: 2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU), 1-(Mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole (MSNT), 9-Fluorenylmethoxycarbonyl (Fmoc) amino acids, 5-carboxyfluorescien (5-FAM) and resins were received from ChemPep or NovaBiochem; N-Hydroxybenzotriazole (HOBt) was obtained from AnaSpec; and Fmoc-3-(2-Naphthyl)-L-Alanine was purchased from Peptides International. All other chemicals used in the assays were procured from Fisher or Sigma except for the following: Active Abl-1 enzyme was purchased from Invitrogen; bovine serum albumin (BSA) was received from Calbiochem; Roswell Park Memorial Institute Media (RPMI-1640) and Dulbecco's Modified Eagle's Medium (DMEM) were obtained from Cellgro; Penicillin/streptomycin was procured from Gibco; and fetal bovine serum (FBS) was purchased from Atlanta Biologicals.

Peptide synthesis and preparation

Full-length substrate peptides with an amidated C terminus were synthesized via standard Fmoc peptide synthesis (Prelude Peptide Synthesizer, Protein Technologies, Tucson, AZ) utilizing TGR resin. Coupling was performed with two 5 min incubations in dimethylformamide (DMF) with 5 equivalents (eq) amino acid, 5 eq HCTU, and 10 eq N,N-Diisopropylethylamine (DIPEA). Fmoc deprotection was achieved with two 2.5 min incubations with 20% piperidine in DMF. The free N-terminus was reacted with 5 eq 5-FAM, 5 eq diisopropylcarbodiimide (DIC), and 5 eq HOBt in DMF overnight, then treated with 30% piperidine in DMF for 30 min. The peptide was cleaved with trifluoroacetic acid:water:triisopropylsilane (TFA:H_2O:TIS) in a ratio of 95:2.5:2.5, precipitated with ether, and dried in air. Peptide purity was assessed with HPLC-MS and further purification via HPLC was performed if needed. Peptides were dissolved in Tris buffer, pH 7.5, aliquoted, and stored at −80 °C.

In some instances, amino acids were coupled overnight in N-Methyl-2-pyrrolidone (NMP) with 5 eq amino acid, 5 eq bromo-tris-pyrrolidino phosphonium hexafluorophosphate (PyBrop), and 10 eq DIPEA. Capping with acetic anhydride was performed if necessary and product formation was assessed using HPLC-MS.

Peptide fragment standards with a free carboxylic acid at the peptide C terminus were synthesized using either a Wang or 2-Chlorotrityl resin. When using the Wang resin, 10 eq of the first Fmoc amino acid in CH_2Cl_2 was reacted with 7.5 eq 1-methylimidazole and 10

eq MSNT for 15 minutes in a dry glass vial on a shaker. The reaction mixture was transferred to another dry glass vial containing the Wang resin and incubated for 1 h. For the 2-Chlorotrityl resin, 1 eq of the first Fmoc amino acid and 4 eq DIPEA in dry CH_2Cl_2 was reacted with the 2-Chlorotrityl resin for 2 h in a dry glass vial on a shaker. The remaining amino acids were attached as described for the amidated peptides above.

Cell culture

Baf/BCR-ABL cells are a mouse B-cell lymphoma line that was stably transfected with and overexpress Bcr-Abl.[43] HeLa cells were obtained from the American Type Culture Collection. Baf/BCR-ABL cells were cultured in RPMI-1640 medium supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL). HeLa cells were cultured in DMEM medium supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL). All cells were maintained in a humidified atmosphere of 37 °C in 5% CO_2. HeLa cells used for single cell-CE experiments were plated the day before onto custom chambers, prepared by using poly(dimethyl siloxane) (PDMS, Sylgard 184) to glue a silicon O-ring (McMaster-Carr) to a #1 glass coverslip (Fisher). A dilute cell suspension was added to 500 μL of DMEM media in the chamber and the chambers were placed in the humidified incubator until use in the experiments.

Measurement of peptide degradation in a cell lysate

A Baf/BCR-ABL cell pellet was washed with and resuspended in phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Na_2HPO_4, 27 mM KCl, 1.75 mM KH_2PO_4, pH 7.4). The cells were submerged in liquid nitrogen for 1 min and rapidly thawed at 37 °C for a total of three cycles. The mixture was centrifuged at 14,000 x g for 5 min at 4 °C. The supernatant was transferred to a clean centrifuge tube and maintained on ice until use in the assay. Total protein concentration in the supernatant was measured using fluorescamine.[42] Briefly, fluorescamine (10 μL, 3 mg/mL in acetone) was added to a cell lysate (30 μL) and incubated for 5 min at 25 °C. Fluorescence was measured with a fluorescence plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) with an excitation of 390 nm (bandwidth of 9 nm) and emission of 475 nm (bandwidth of 15 nm).

Assay of peptide degradation was performed by mixing peptide (1 μM) with the Baf/BCR-ABL cell lysate (3 mg/mL total cell protein) and incubating at 37 °C. Aliquots were removed from the reaction mixture at various time intervals. The reactions were 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 addition of the substrate peptide. Reaction mixtures were then separated by capillary electrophoresis and detected by 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. The average initial degradation and fragmentation rates were calculated using the first two time points by monitoring the change in peptide amount divided by the change in time per amount cytosolic protein. The units are defined as zmol of peptide per pg of cytosolic protein per s, or zmol pg^{-1} s^{-1}.

in vitro kinase assay

Protein kinase assays were performed at 30 °C in assay buffer [50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, 1 mM ATP] with Abl-1 kinase (12 nM) and substrate (29 μM). Aliquots were then removed from the reaction mixture at varying times. The reactions were stopped by heating at 90 °C for 4 min. Additionally, a negative control with no ATP was simultaneously assayed and sampled. The amount of peptide phosphorylation was measured using capillary electrophoresis coupled with laser-induced fluorescence (LIF) to separate the substrates and products and quantify peak areas. The samples with and without

ATP were compared to identify the phosphorylated product and the non-phosphorylated precursor (Supplementary Figure S1).

Measurement of kinetic parameters

Protein kinase assays were performed as described in the *in vitro* kinase assay section with the following exceptions: substrate concentration ranged from 10 to 100 μM; Abl-1 (Invitrogen, Carlsbad, CA) enzyme concentration was 12 nM for QW-III-67B and 6 nM for QW-V-48B. The immobilized metal ion affinity-based fluorescent polarization (IMAP) assay (Molecular Devices Corp., Sunnyvale, CA) was used to measure the amount of phosphorylated peptide in reaction mixtures (http://www.moleculardevices.com/pages/reagents/imap.html). A calibration curve was constructed by measuring the anisotropy of solutions with known ratios of phosphorylated to non-phosphorylated peptide. The standard with 100% phosphorylated peptide was prepared using Abl-1 kinase and the percentage phosphorylation was verified with capillary electrophoresis. Anisotropy was measured using a fluorescence plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA) with an excitation of 485 nm (bandwidth of 9 nm) and emission of 525 nm (bandwidth of 15 nm). Samples were diluted to the working concentration of 100 nM for the IMAP assay with a buffer containing 10 mM Tris-HCl (pH 7.2), 10 mM $MgCl_2$, and 0.01% Tween-20.

Capillary electrophoresis

Laser-induced fluorescence (LIF, 488 nm) was used for peptide detection during capillary electrophoresis (ProteomeLab PA800, Beckman Coulter, Fullerton, CA). Fused-silica capillaries [50 μm inner diameter, 360 μm outer diameter, (Polymicro Technologies, Phoenix, AZ)] had a total length of 30 cm with an effective length of 20 cm. Capillaries were conditioned prior to use with 0.1 M NaOH for 12 h, H_2O for 1 h, 0.1 M HCl for 6 h, and H_2O again for 12 h. After each sample, the capillary was sequentially rinsed with 1 M NaOH, H_2O, and buffer for 2 min by applying a pressure of 20 psi to the capillary inlet. A sample plug was hydrodynamically loaded into the capillary by applying 0.5 psi to the inlet for 5 s. Electrophoresis was initiated by application of a negative voltage to the outlet. For the assays employing a cell lysate, the electrophoretic buffer was 100 mM Tris and 100 mM Tricine, pH 8.1 and the field strength was 500 V/cm. For the *in vitro* kinase assay samples, the electrophoretic buffer was 100 mM Tris, 100 mM Tricine and 5 mM SDS, pH 8.1 and field strength was 600 V/cm. The data was analyzed using commercial software (32 Karat, version 8.0, Beckman Coulter, Fullerton, CA).

Single cell capillary electrophoresis

Single cell capillary electrophoresis was performed using a custom-made CE system with LIF detection, as described previously.[40] Fused-silica capillaries [30 μm inner diameter, 360 μm outer diameter, (Polymicro Technologies, Phoenix, AZ)] with a total length of 38 cm and an effective length of 21.5 cm were conditioned as described above. A negative voltage of 14 kV was applied to the outlet reservoir while the inlet reservoir was held at ground. The electrophoretic buffer was 100 mM Tris and 100 mM Tricine, pH 8.1. 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, 25 °C) during experiments, with the flow turned off immediately prior to cell lysis and loading into the capillary. Laser-based cell lysis was achieved with a focused Nd:YAG laser as previously described.[41] To identify peptide fragments formed in cells, standards of peptide fragments (100 nM) were hydrodynamically loaded into the capillary immediately following loading of a single HeLa cell into the capillary. The HeLa cell was not loaded with peptide prior to loading into the capillary. The lysed HeLa cell provided a milieu for the subsequently loaded standards similar to that for peptide obtained from a lysed cell. To calibrate the amount of peptide on the electropherograms, a known concentration of a 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.[22] Data was collected with custom software (LabVIEW 9.0.1, National Instruments, Austin, TX) and analyzed utilizing Origin software (version 7.5, OriginLab Corporation, Northampton, MA).

Results and Discussion

Selection of the starting peptide

The starting peptide QW-III-67B (5FAM-GGAYAAPFKKKA) is based on a related sequence obtained from an oriented peptide library by Songyang *et al.* directed at determining tyrosine kinase specificity.[44] The Songyang substrate was phosphorylated with good efficiency by Abl and was minimally phosphorylated by Src, a close relative of Abl that shares a similar consensus sequence. Another similar sequence has been shown to have good efficiency for both Abl and Bcr-Abl.[45] Based on these favorable properties and the good specificity for Abl kinase, peptide QW-III-67B was chosen as the starting peptide for this work.

Characterization of peptide QW-III-67B degradation in cytosolic lysates

To determine whether the peptide QW-III-67B is degraded by cytosolic peptidases, the peptide was incubated in a Baf/BCR-ABL cell lysate. After varying times, aliquots were removed and separated by electrophoresis followed by detection of all fluorescent peptides (Figure 1A–1C). At time zero, a single peak with a migration time identical to that of the standard intact peptide QW-III-67B was present. Within one minute, three peaks were seen, the intact peptide and two peaks with longer migration times. Under these conditions, the starting peptide possessed a half-life of 1.3 ± 0.4 min in the cytosolic lysate. The average rate of peptide breakdown in the cell lysate over 5 min was 1.7 ± 0.3 zmol pg^{-1} s^{-1} and approximately 6.2 ± 2.7 % of the intact peptide remained after 5 min. After 30 min, the initial peak was no longer identified and five additional peaks had formed, all with migration times slower than the intact peptide. Given the presence of peptidases in a cytosolic lysate, it was likely that these peaks were proteolytic fragments of the intact starting substrate. In order to determine the temporal pattern of fragment formation, the percentage of each fragment with respect to total peptide was plotted as a function of time (Figure 1D). The initial fragment was generated nearly as fast as the intact peptide was degraded, with an average initial rate of 1.2 ± 0.1 zmol pg^{-1} s^{-1}. It is possible that the later appearing fragments were formed as a consequence of further peptidase action on the first fragment. Phosphorylated product was not observed since neither ATP nor phosphatase inhibitors were present in the assay.

To compare the breakdown of QW-III-67B in lysates generated with different cells, the peptide was incubated in a HeLa cell lysate. During various times, aliquots were removed and separated by capillary electrophoresis. A peptide-fragment pattern similar to that observed in the Baf/BCR-ABL lysate was present (Figure 1E). The initial rate of breakdown in the HeLa cell lysate was 1.3 zmol pg^{-1} s^{-1} similar to that in the Baf/BCR-ABL lysate. Additionally the major fragment that formed over time possessed a migration time identical to the major peak formed in the Baf/BCR-ABL lysate. This product formed at a rate of 1 zmol pg^{-1} s^{-1} nearly identical to result in the Baf/BCR-ABL lysate. Two additional peaks formed on slower time scales and the migration times of these peaks matched the two secondary peaks formed in the Baf/BCR-ABL lysate. The pattern and rate of breakdown in HeLa and Baf/BCR-ABL lysates was very similar despite the difference in species and tumor type. For this reason, subsequent lysate experiments utilized only the Baf/BCR-ABL cells.

Characterization of peptides following lysine replacement

Since trypsin-like proteases are common in cells and favor positively charged residues, the initial cleavage site was thought to be at one of the lysine residues near the carboxy terminus of the peptide.[46] Ornithine, a lysine analog, possesses a positively charged side chain but with one less carbon (Figure 2). Replacement of lysine with ornithine has been shown to limit the ability of trypsin to act on lysine-containing substrates.[47] Peptides with ornithine inserted into the peptide in place of one, two, or all three lysine residues (peptides QW-III-90A through QW-III-90G in Table 1) were synthesized, purified, and then incubated in the Baf/BCR-ABL cytosolic lysate for varying times. Aliquots were removed and electrophoresed to assess the formation of fluorescent fragments. The resulting traces were very similar to that of the control peptide, with the initial timepoint for each peptide showing a single peak that migrated at the same time as the intact parent. Subsequent time points demonstrated the disappearance of the initial peak and the appearance of multiple peaks with slower migration times, presumably fragment peptides of the lysine-substituted peptides. To determine the pattern of fragment formation, the fraction of peptide present as each fragment was plotted as a function of time, with results for each of the seven modified peptides appearing very similar to that of the starting peptide QW-III-67B (Supplementary Figure S2). Irrespective of the number or location, ornithine-for-lysine substitution provided only a modest enhancement in stability relative to that of the starting peptide, with the peptide half-lives ($t_{1/2}$) in the lysate ranging from 4.3 to 6.0 min compared to the 1.3 min for the starting peptide (Table 1).

To determine whether the ornithine-for-lysine-substituted peptides retain the ability to be phosphorylated by Abl kinase, each modified peptide was incubated with Abl kinase *in vitro* and aliquots were removed over time and separated. The relative peak areas of the phosphorylated and non-phosphorylated peptides were used as a measure of the Abl substrate efficacy. Half of the ornithine-containing peptides were phosphorylated at a faster rate than that of the QW-III-67B peptide. When the time for 50% peptide phosphorylation ($t_{50\%P}$) was measured, three of the double-ornithine peptides and one of the single-ornithine peptides achieved a 1.7-6X shorter $t_{50\%P}$ than that of QW-III-67B. The remaining single and triple-ornithine peptides were phosphorylated more slowly than QW-III-67B with a 3-4X greater $t_{50\%P}$. While peptidase resistance was slightly improved and kinase substrate suitability was acceptable, the ornithine-containing peptides did not possess dramatically improved proteolytic resistance relative to the starting peptide, suggesting that the lysine residues might not be the initial site of cleavage.

Identification of the peptide fragments

To identify the primary site of peptide-bond hydrolysis, standards consisting of all possible fluorescent peptide fragments formed from QW-III-67B were synthesized and characterized by electrophoresis. Under the electrophoretic conditions used, each peptide fragment migrated at a unique time and was present as a single major peak. Thus, the fragments formed and peptide cleavage sites targeted by the cytosolic lysate should be readily identifiable. The starting peptide, QW-III-67B, was incubated in a cell lysate and after the reaction was stopped, each of the possible fluorescent peptide standards was sequentially added to the lysate mixture to identify the lysate peaks. Each peak present in the cytosolic lysate matched to a fragment peak suggesting that the additional peaks on the electropherogram were indeed fragments of the original peptide (Figure 1). A 7-residue fragment (peptide f) generated by cleavage at the proline-phenylalanine bond formed initially and in the greatest quantity. 3, 4, and 5-residue fragments also formed over time but with a delay relative to that of the 7-mer. Since the proline-phenylalanine bond appeared to be the site of initial peptide cleavage, these residues were targeted for modification to enhance stability.

Characterization of peptides with proline replacement

The proline and phenylalanine in QW-III-67B were replaced with native and non-native residues (Figure 2) and peptide stability assessed. Proline has a structure unique to the native residues in that the backbone nitrogen is linked via a ring to the side chain, creating a bend in the peptide backbone. Three strategies were attempted to replace the proline with a goal of diminishing peptidase rather than kinase action: *i)* an alanine was inserted in place of the proline to eliminate the bend in the peptide backbone; *ii)* native residues with a side chain ring were substituted to maintain a similar side chain character while still eliminating the backbone bend; and *iii)* the non-native residue sarcosine (N-methylglycine) was used to maintain the backbone kink yet eliminate the closed-ring structure.[48] Peptides QW-IV-74A to QW-V-74E (Table 1) were synthesized, purified, incubated in a cytosolic lysate and then separated by CE to determine whether fluorescent fragments were formed. In each case, approximately eight fluorescent fragments were seen within 5 min of incubation in the lysate. Although these fragment peaks grew larger over time, additional peaks were not observed. To compare the peptidase resistance of these peptides to that of the prior peptides, the percentage of peptide fragment over time was measured and the $t_{1/2}$ calculated. The $t_{1/2}$ of all of the proline substituted peptides was improved relative to that of the starting peptide QW-III-67B and the ornithine-containing peptides (Table 1). The peptide possessing a sarcosine displayed the most dramatic increase in stability with a $t_{1/2}$ of 24.0 min compared to the 1.3 min of the starting peptide (Figure 3A).

To determine whether the proline-substituted peptides also retained the ability to be phosphorylated by Abl kinase, each modified peptide was incubated with Abl kinase *in vitro* and phosphorylation measured over time. Minimal phosphorylation was observed for each of the five peptides over the assay period. Based on the measured phosphorylation rate, $t_{50\%P}$ for all of these peptides was estimated to be $>10^5$ min or >650-times longer than the 150 min of the starting peptide (Figure 3B). Despite the increased peptidase resistance, the poor substrate efficacy of the proline-substituted peptides rendered these modifications unsuitable for the creation of protease-resistant Abl kinase substrates.

Characterization of peptides with phenylalanine replacement

Phenylalanine possesses a bulky hydrophobic side group, and therefore non-native residues with similar features, including β-(2-naphthyl)-L-alanine and 3-nitrotyrosine, were substituted in place of phenylalanine (Figure 2). Since both N-methylated and D-amino acids have been shown to impart stability to inhibitor peptides, D-phenylalanine and (N-methyl)phenylalanine were also incorporated as replacements for phenylalanine.[27, 35, 36, 38] Peptides QW-IV-85B through QW-V-23E (Table 1) were synthesized, purified, and then incubated in the Baf/BCR-ABL cytosolic lysate for varying times. Aliquots were removed and electrophoresed to assess the formation of fluorescent fragments. Within 1 min, the β-(2-naphthyl)-L-alanine-substituted peptide showed the appearance of a second peak in the electropherogram with a migration time slower than the intact peptide. This second peak grew larger over the course of the assay, and a much smaller, slower migrating peak first appeared after 30 min incubation. The intact peptide disappeared between 15 and 30 min. A similar pattern and time course was also observed for the 3-nitrotyrosine-substituted peptide. The electropherogram of the lysate-incubated, D-phenylalanine peptide did not show additional peaks until after 5 min of incubation. Unlike the prior two peptides, 30% of the D-phenylalanine peptide remained intact at 60 min. The (N-methyl)phenylalanine-substituted peptide showed results similar to that of the D-phenylalanine-substituted peptide but had an even slower rate of fragment formation with 70% of the peptide co-migrating with that of the starting peptide after 60 min (Supplementary Figure S3). At this time, 5 additional peaks were present on the electropherogram of the (N-methyl)phenylalanine-substituted peptide. To compare cytosolic peptidase resistance among these peptides, the

amount of intact peptide was plotted as a function of time and $t_{1/2}$ was calculated. As expected, the (N-methyl)phenylalanine-substituted peptide showed the most dramatic increase in lifetime, with a 40-fold increase relative to that of the starting peptide QW-III-67B (Figure 3A). The D-phenylalanine-substituted peptide was also longer lived with a 30-fold increase in t1/2. The remaining two peptides showed more modest results, with a 4 to 7-fold increase in peptide lifetime (Figure 3A).

The ability of each phenylalanine-modified peptide to be phosphorylated *in vitro* by Abl kinase was also assessed as described above. Two of the peptides, the (N-methyl)phenylalanine- and the 3-nitrotyrosine-substituted peptides, showed minimal phosphorylation, with $t_{50\%P} > 10^5$ min (Figure 3B). The β-(2-naphthyl)-L-alanine- and the D-phenylalanine-substituted peptides were phosphorylated at similar rates to the parent peptide with a $t_{50\%P}$ of 190 and 82 min, respectively (Figure 3B).

Design and characterization of an (N-methyl)phenylalanine-substituted lead peptide

The (N-methyl)phenylalanine-substituted peptide QW-IV-85B was the most promising peptide with respect to peptidase resistance but performed poorly as an Abl substrate. Placement of an isoleucine in the position immediately upstream of the phosphorylated tyrosine has been shown to improve Abl phosphorylation efficiency of some substrates, but can make the peptide more suitable for phosphorylation by Src.[4, 44] To improve the substrate behavior of the (N-methyl)phenylalanine-substituted peptide, the alanine adjacent to the tyrosine was replaced with an isoleucine. This new peptide (QW-V-48B in Table 1) was characterized for peptidase resistance as well as Abl- and Src-substrate performance. After a 3 min incubation in the cytosolic lysate, a small peak in addition to QW-V-48B appeared. Over a 5 min time, the average rate of breakdown was 0.20 ± 0.03 zmol pg^{-1} s^{-1} and 79.6 ± 2.5% remained intact in the lysate. By 10 min, the second peak had increased and was accompanied by two additional peaks. The $t_{1/2}$ of this peptide was 19.1 ± 3.3 min or 15-fold longer than that of the starting peptide yet >2 fold less than that of the peptide without isoleucine (Table 1, Figure 3A). To determine whether this peptide was a substrate for Src, the peptide was incubated with various concentrations of Src kinase and the amount of phosphorylation measured. At all concentrations, no phosphorylation by Src was observed (Supplementary Figure S4). To determine whether the isoleucine improved Abl-substrate performance, the QW-V-48B peptide was incubated with Abl kinase and the amount of phosphorylation measured. Incorporation of the isoleucine decreased the $t_{50\%P}$ by >2000-fold relative to that of the peptide with only the (N-methyl)phenylalanine-substitution (Figure 3B). The isoleucine peptide also possessed a 3.5X lower $t_{50\%P}$ than the original starting peptide. Based on the combined ability of this peptide to resist degradation, resist Src phosphorylation yet remain a substrate for Abl, peptide QW-V-48B was chosen for further characterization and evaluation.

Characterization of the lead peptide QW-V-48B in a cytosolic lysate

Although the lead peptide QW-V-48B possessed a substantially increased lifetime relative to that of the starting peptide QW-III-67B, QW-V-48B was still degraded into multiple different fragments over time. In order to understand which residues were the preferred sites of cleavage, all possible fluorescent fragments were synthesized and characterized by electrophoresis. Under the electrophoretic conditions used, all of these fragment peptides were resolved from each other and from the parent peptide. The lead peptide, QW-V-48B, was incubated in a Baf/BCR-ABL cell lysate and each of the possible fluorescent peptide standards were sequentially added to the mixture to identify the unknown peaks in the electropherogram. Each of the peaks present in the cytosolic lysate was matched to a fragment peak (Figure 4). The first fragment formed was a 5-residue peptide cleaved at the alanine-alanine bond (peptide t) (Figure 4B,E). The amount of each fragment graphed as a

function of time (Figure 4D) demonstrated that the 5-mer formed at an initial average rate of 0.11 ± 0.02 zmol pg^{-1} s^{-1} and accounted for approximately 50% of all peptide present by 60 min (Figure 4D). Other peptides that formed over time were the 3, 4, and 11-residue fragments, but they formed on longer time scales relative to that of the 5-mer (Figure 4C). Significantly, there was no evidence of the 7-mer fragment that would be generated from cleavage between the proline and (N-methyl)phenylalanine. Thus the substitution of (N-methyl)phenylalanine for phenylalanine greatly enhanced resistance to proteolysis.

The kinetic parameters of K_M and k_{cat} were determined for both peptides in order to quantitatively compare the ability of the starting peptide and lead peptide to act as substrates for Abl. Varying concentrations of each peptide were incubated with Abl kinase and the initial reaction velocity calculated. Peptide QW-V-48B had a lower K_M than QW-III-67B (20 μM compared to 58 μM) and also a higher turnover number, (k_{cat} = 3,000 min^{-1} compared to 2,100 min^{-1}). The lead peptide QW-V-48B was both a better Abl substrate and possessed greater peptidase resistance than the starting peptide QW-III-67B.

Characterization of peptides in single HeLa cells

Although use of cytosolic lysates enables facile optimization of substrate peptide properties, most cellular processes are dramatically altered in this homogenous, diluted environment. In particular, peptidases located within organelles are expected to be released upon cell lysis. Thus a peptide in a cell lysate may encounter a different repertoire of peptidases relative to a peptide in the cytosol of an intact cell and it is important to understand whether the peptide fragments and fragmentation rates from a lysate faithfully mimic that formed in intact cells. In order to assess and quantify the peptide fragments generated in single cells, peptide QW-III-67B was microinjected into a single HeLa cell (24 ± 8 amol, n = 4 cells). HeLa cells were utilized for single cell studies since they were more easily microinjected than non-adherent cells and possessed a similar fragmentation profile to that of Baf/BCR-ABL cell lysates (Figure 1E). After a 5 min incubation, cells were lysed and loaded into an overlying capillary and the different fluorescent peptides detected and quantified.[41] The peptide was nearly completely metabolized, with only 10 ± 2 % remaining intact (Table 2). Under the conditions used, the average rate of QW-III-67B degradation was calculated to be 0.7 ± 0.2 zmol pg^{-1} s^{-1} assuming a cellular protein concentration of 100 mg/mL and a cell volume of 1 pL.[49] Nine different peaks were observed on the electropherogram (Figure 5A), compared to the six peaks seen in the lysate samples (Figure 1C). The peptide fragment standards were utilized to identify the source of the peaks obtained from the single cells and each fragment generated in the single cells co-migrated with a standard fragment (Figure 5A). The most abundant fragment generated was the 5-mer (peptide h) that amounted to 36 ± 6% of all peptides. This same 5-mer was also generated in the lysate studies but at approximately half the amount observed in single cells at 5 min. The 7-mer fragment (peptide f) that was generated the fastest in the lysate studies was present, but to a lesser extent (5 ± 2 %) in the intact cells. In single cells, the 4, 6, and 11-residue fragments were also seen at amounts greater than 10 % (Table 2) while of these, only the 4-mer was seen in the lysate studies (Figure 1). The 2-mer seen in small amounts in the lysate studies was not seen at all in the intact cell studies. Although the pattern of peptide fragmentation was similar in single cells compared to lysates, there were important differences. While the peptide concentration incubated in the cell lysate was roughly 25-times lower than that loaded into single cells and the lysate protein was 30-times more dilute than a single cell, the rate of peptide metabolism in the cell lysate still remained significantly greater than that of peptide in an intact cell over the same time period. Thus the peptidase activity in cell lysates was considerably greater than that in the cytosol of an intact cell most likely due to the release of sequestered peptidases from ruptured organelles in the lysate. A second significant difference is the predominance of peptide h (representing Ala-Ala bond cleavage) in the single cells relative

to other peptide fragments suggesting that the Ala-Ala bond may be the dominant cleavage site in single cells and not the Pro-Phe which dominated in the lysate. No phosphorylated product was detected or expected since HeLa cells do not express Bcr-Abl.

The lead peptide QW-V-48B was microinjected into a single HeLa cell, incubated for 5 min, and then the cellular contents were electrophoretically separated. On average, 28 ± 6 amol (n = 4 cells) of QW-V-48B was loaded into the cells. The peptide was degraded at an average rate of 0.6 ± 0.1 zmol pg^{-1} s^{-1} with 33 ± 14% remaining intact after 5 min (Table 2). Thus the average rate of metabolism in single cells was similar for the starting and lead peptides. Five peaks were observed on the electropherogram (Figure 5B) and the migration time of each of these peaks matched to that of a QW-V-48B fragment standard (Figure 5B, D). The 7-residue fragment representing cleavage between the proline and (N-methyl)phenylalanine was not present in the single cells. This bond was effectively stabilized by replacement with a non-native amino acid. However, 4, 5, and 11-residue peptide fragments were present with the 5-mer (peptide t), representing the Ala-Ala bond cleavage, being the most abundant with 22 ± 10 % present after 5 min. The 2-mer observed in cell lysates (Figure 4C) was absent from the single cells; however, the single cells possessed a 6-amino acid fragment not observed in the lysates. As with QW-III-67B, peptide metabolism in the single cell was similar to that in a lysate but with some key differences. Since the rate of metabolism of QW-V-48B and QW-III-67B in single cells was nearly identical, replacement of the Pro-Phe bond did not stabilize the peptide in the single cells. The predominance of peptide h and t for both QW-III-67B and QW-V-48B in single cells suggests that the Ala-Ala bond is the preferred location for bond cleavage in the intact cytosol where disruption of the organelles has not occurred. No phosphorylated product was detected in any single cells.

Conclusions

Identification of the exact cleavage locations within a peptide permitted rational peptide redesign by placement of non-native residues at hydrolysis sites, resulting in increased peptide lifetimes in a cytosolic lysate. By contrast, both the starting and lead peptides showed similar degradation rates in intact cells suggesting that the cell lysate system may not be a good model for estimating intracellular rates and patterns of peptide proteolysis. It is likely that different peptidases acted on the substrates in single cells compared to that in lysates, leading to differences in primary cleavage sites and rates of proteolysis. One caveat is that only a single time point was sampled for the single cells and formation of the fragments over time was not determined. Assay of single cells loaded with peptide and incubated for other times would lead to a more complete picture of peptide fragmentation and permit further analysis of substrate lifetime in intact cells. A key goal for future work is the creation of proteolytic resistant intracellular substrates of the Abl kinase.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding for this work was provided by the NIH (R01CA139599 and R01CA140173) and the University Cancer Research Fund of North Carolina.

References

1. Pungaliya PP, Bai Y, Lipinski K, Anand VS, Sen S, Brown EL, Bates B, Reinhart PH, West AB, Hirst WD, Braithwaite SP. PLoS ONE. 2010; 5:1–13.

2. Alessi DR, Caudwell FB, Andjelkovic M, Hemmings BA, Cohen P. FEBS Lett. 1996; 399:333–338. [PubMed: 8985174]
3. Nishikawa K, Toker A, Johannes F, Songyang Z, Cantley LC. J Biol Chem. 1997; 272:952–960. [PubMed: 8995387]
4. Wu J, Afar DEH, Phan H, Witte ON, Lam KS. Com Chem High T Scr. 2002; 5:83–91.
5. Lawrence DS. Biochim Biophys Acta. 2005; 1754:50–57. [PubMed: 16172031]
6. Litman P, Ohne O, Ben-Yaakov S, Shemesh-Darvish L, Yechezkel T, Salitra Y, Rubnov S, Cohen I, Senderowitz H, Kidron D, Livnah O, Levitzki A, Livnah N. Biochemistry. 2007; 46:4716–4724. [PubMed: 17397140]
7. Ngoei KRW, Catimel B, Church N, Lio DS, Dogovski C, Perugini MA, Watt PM, Cheng H, Ng DCH, Bogoyevitch MA. Biochem J. 2011; 434:399–413. [PubMed: 21162712]
8. Wu D, Sylvester JE, Parker LJ, Zhou G, Kron SJ. Biopolymers. 2010; 94:475–486. [PubMed: 20593469]
9. Xu X, Liu X, Nie Z, Pan Y, Guo M, Yao S. Anal Chem. 2011; 83:52–59. [PubMed: 21128608]
10. Wu D, Mand MR, Veach DR, Parker LL, Clarkson B, Kron SJ. Anal Biochem. 2008; 375:18–26. [PubMed: 18194660]
11. Han X, Yamanouchi G, Mori T, Kang J, Niidome T, Katayama Y. J Biomol Screen. 2009; 14:262.
12. Shults MD, Janes KA, Lauffenburger DA, Imperiali B. Nat Methods. 2005; 2:277–283. [PubMed: 15782220]
13. Wang Q, Zimmerman EI, Toutchkine A, Martin TD, Graves LM, Lawrence DS. ACS Chem Biol. 2010; 5:887–895. [PubMed: 20583816]
14. Lee JH, Nandy SK, Lawrence DS. J Am Chem Soc. 2004; 126:3395.
15. Stawikowski M, Stowikowska R, Jaśkiewicz A, Zablotna E, Rolka K. ChemBioChem. 2005; 6:1057–1061. [PubMed: 15883970]
16. Sims CE, Allbritton NL. Curr Opin Biotech. 2003; 14:23–28. [PubMed: 12565998]
17. Kovarik ML, Allbritton NL. Trend Biotechnol. 2011; 29:222–230.
18. Wang Q, Dai Z, Cahill SM, Blumenstein M, Lawrence DS. J Am Chem Soc. 2006; 128:14016–14017. [PubMed: 17061870]
19. Lawrence DS, Wang Q. ChemBioChem. 2007; 8:373–378. [PubMed: 17243187]
20. Priestman MA, Lawrence DS. Biochim Biophys Acta. 2010; 1804:547–558. [PubMed: 19765679]
21. Lee S, Kim E, Suk K, Lee W. Immunology. 2011; 134:224–233. [PubMed: 21896016]
22. Meredith GD, Sims CE, Soughayer JS, Allbritton NL. Nat Biotechnol. 2000; 18:309–312. [PubMed: 10700147]
23. Arkhipov SN, Berezovski M, Jitkova J, Krylov SN. Cytom Part A. 2005; 63A:41–47.
24. Brown RB, Hewel JA, Emili A, Audet J. Cytom Part A. 2010; 77A:347–355.
25. Reits E, Griekspoor A, Neijssen J, Groothuis T, Jalink K, van Veelen P, Janssen H, Calafat J, Drijfhout JW, Neefjes J. Immunity. 2003; 18:97–108. [PubMed: 12530979]
26. Tal-Gan Y, Hurevich M, Klein S, Ben-Shimon A, Rosenthal D, Hazan C, Shalev DE, Niv MY, Levitzki A, Gilon C. J Med Chem. 2011; 54:5154–5164. [PubMed: 21650457]
27. Molhoek EM, van Dijk A, Veldhuizen EJA, Haagsman HP, Bikker FJ. Peptides. 2011; 32:875–880. [PubMed: 21376095]
28. Ovadia O, Linde Y, Haskell-Leuvano C, Dirain ML, Sheynis T, Jelinek R, Gilon C, Hoffman A. Bioorgan Med Chem. 2010; 18:580–589.
29. Nguyen LT, Chau JK, Perry NA, de Boer L, Zaat SAJ, Vogel HJ. PLoS ONE. 2010; 5:1–8.
30. Brinckerhoff LH, Kalashnikov VV, Thompson LW, Yamshchikov G, Pierce RA, Galavotti HS, Engelhard VH, Slingluff CL Jr. Int J Cancer. 1999; 83:326–334. [PubMed: 10495424]
31. Bellmann-Sickert K, Elling CE, Madsen AN, Little PB, Lundgren K, Gerlach L, Bergmann R, Holst B, Schwartz TW, Beck-Sickinger AG. J Med Chem. 2011; 54:2658–2667. [PubMed: 21410292]
32. Pasut G, Veronese FM. Adv Drug Deliver Rev. 2009; 61:1177–1188.
33. Koda Y, Liang MT, Blanchfield JT, Toth I. Int J Pharm. 2008; 356:37–43. [PubMed: 18272306]
34. Cros CD, Toth I, Blanchfield JT. Bioorgan Med Chem. 2011; 19:1528–1534.

35. Neundorf I, Rennert R, Franke J, Közle I, Bergmann R. Bioconjugate Chem. 2008; 19:1596–1603.
36. Tugyi R, Uray K, Iván D, Fellinger E, Perkins A, Hudecz F. PNAS. 2005; 102:413–418. [PubMed: 15630090]
37. Gentilucci L, De Marco R, Cerisoli L. Curr Pharm Design. 2010; 16:3185–3203.
38. Pappa EV, Zompra AA, Spyranti Z, Diamantopoulou Z, Pairas G, Lamari FN, Katsoris P, Spyroulias GA, Cordopatis P. Biopolymers. 96:260–272. [PubMed: 20632397]
39. Bartram CR, de Klein A, Hagemeijer A, van Agthoven T, van Kessel AG, Bootsma D, Grosveld G, Ferguson-Smith MA, Davies T, Stone M, Heisterkamp N, Stephenson JR, Groffen J. Nature. 1983; 306:277–280. [PubMed: 6580527]
40. Kottegoda S, Aoto PC, Sims CE, Allbritton NL. Anal Chem. 2008; 80:5358–5366. [PubMed: 18522433]
41. Sims CE, Meredith GD, Krasieva TB, Berns MW, Tromberg BJ, Allbritton NL. Anal Chem. 1998; 70:4570–4577. [PubMed: 9823716]
42. Funk GM, Hunt CE, Epps DE, Brown PK. J Lipid Res. 1986; 27:792–795. [PubMed: 3760715]
43. Mahon FX, Deininger MWN, Schultheis B, Chabrol J, Reiffers J, Goldman JM, Melo JV. Blood. 2000; 96:1070–1079. [PubMed: 10910924]
44. Songyang Z, Carraway KL III, Eck MJ, Harrison SC, Feldman RA, Mohammadi M, Schlessinger J, Hubbard SR, Smith DP, Eng C, Lorenzo MJ, Ponder BAJ, Mayer BJ, Cantley LC. Nature. 1995; 373:536–539. [PubMed: 7845468]
45. Griswold IJ, MacPartlin M, Bumm T, Goss VL, O'Hare T, Lee KA, Corbin AS, Stoffregen EP, Smith C, Johnson K, Moseson EM, Wood LJ, Polakiewicz RD, Druker BJ, Deininger MW. Mol Cell Biol. 2006; 26:6082–6093. [PubMed: 16880519]
46. Stroud RM. Sci Am. 1974; 231:74–88. [PubMed: 4846962]
47. Seely JH, Benoiton NL. Can J Biochem Cell B. 1970; 48:1122–1131.
48. Tran TT, Treutlein H, Burgess AW. Protein Eng Des Sel. 2006; 19:401–408. [PubMed: 16799150]
49. Dovichi NJ, Hu S. Curr Opin Chem Biol. 2003; 7:603–608. [PubMed: 14580565]

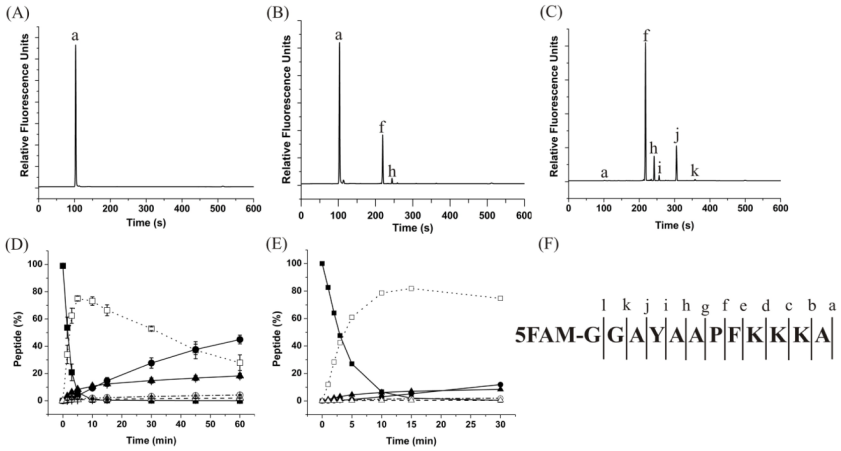

Figure 1.

Degradation profile of the starting peptide (QW-III-67B) in a cell lysate. (A) –(C) Electropherograms of the peptide after incubation in the Baf/BCR-ABL lysate for 0 (A), 1 (B), or 30 (C) min. (D) Formation of peptide fragments over time in the Baf/BCR-ABL lysate. (E) Formation of peptide fragments over time in a HeLa–cell lysate. Shown on the y-axis is the percentage of peptide present as a fragment. The symbols are defined as: filled square (peptide a or starting peptide), open square (peptide f), closed triangle (peptide h), open circle (peptide i), closed circle (peptide j) and open triangle (peptide k). The symbols and error bars represent the average and standard deviation of the data points. (F) The uppercase letters are the single amino acid abbreviations for the starting peptide sequence. The lowercase letters indicate the cleavage locations that generate the indicated peptide fragments.

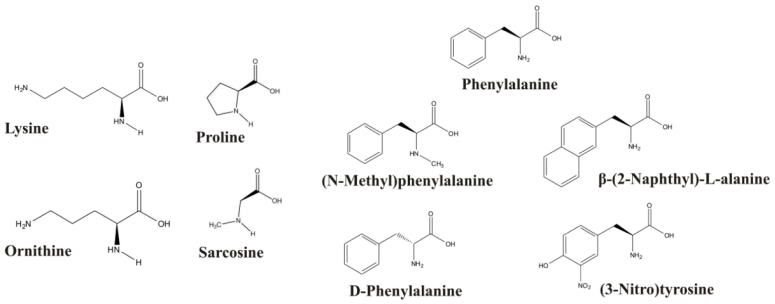

Figure 2.
The native residues and the non-native residues that were inserted into the peptide to stabilize the peptide against hydrolysis.

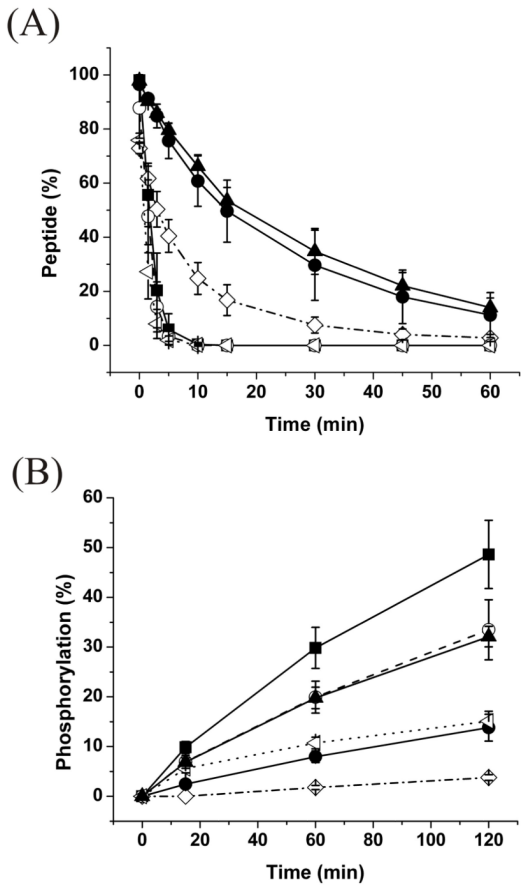

Figure 3.

Baf/BCR-ABL cytosolic lysate degradation (A) and *in vitro* phosphorylation (B) of modified peptides. Filled square (QW-III-67B, starting peptide); open diamond (QW-IV-74D, sarcosine-substituted peptide); closed circle (QW-IV-85B, (N-methyl)phenylalanine-substituted peptide); open circle (QW-V-23C, β-(2-naphthyl)-L-alanine-substituted peptide); open triangle (QW-V-23E, 3-nitro tyrosine-substituted peptide); and closed triangle (QW-V-48B, isoleucine and N-methylated phenylalanine-substituted peptide). The symbols and error bars represent the average and standard deviation of the data points.

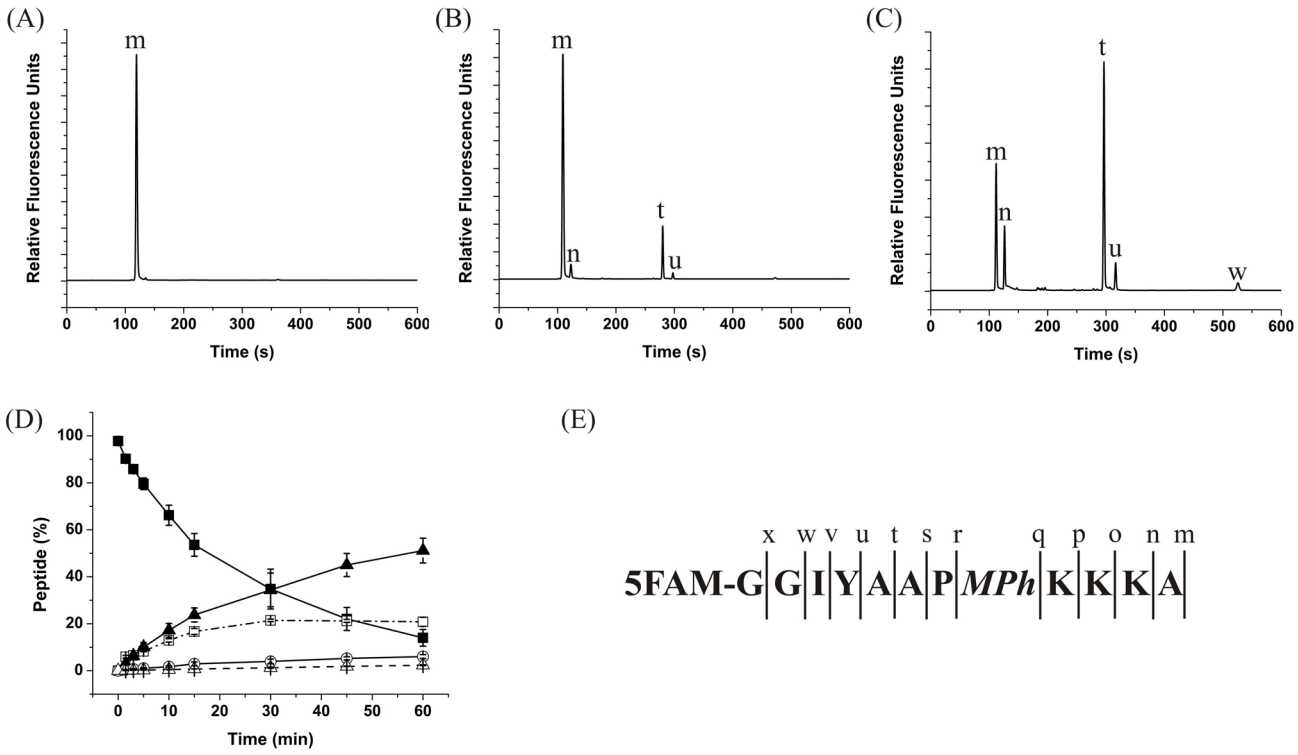

Figure 4.

Degradation profile of the lead peptide (QW-V-48B) in a Baf/BCR-ABL cell lysate. (A) – (C) Electropherograms of the peptide after incubation in the lysate for 0 (A), 1 (B), or 30 (C) min. (D) Formation of peptide fragments over time. Shown on the y-axis is the percentage of peptide present as a fragment. The symbols are defined as: filled square (peptide m), open square (peptide n), closed triangle (peptide t), open circle (peptide u), open triangle (peptide w). The symbols and error bars represent the average and standard deviation of the data points. (E) The uppercase letters are the single amino acid abbreviations for the lead peptide sequence and *MPh* represents N-methylated phenylalanine. The lowercase letters indicate the cleavage locations that generate the indicated peptide fragments.

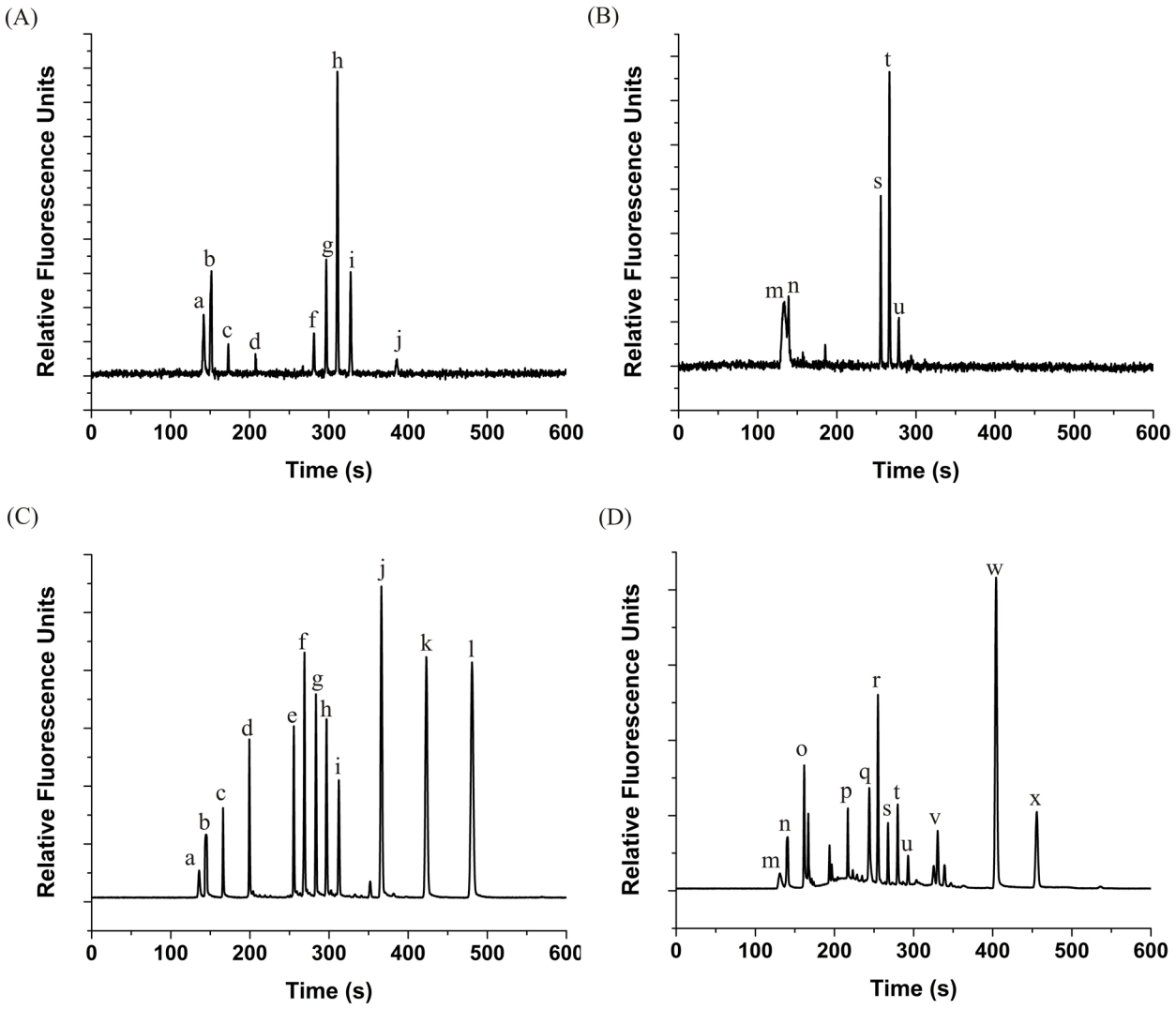

Figure 5.

Degradation of peptides in single HeLa cells. Cells were microinjected with QW-III-67B (A,C) or the lead peptide QW-V-48B (B,D). (A,B) Electropherogram of a single cell 5 min after microinjection. The lowercase letters label the peptide fragments as defined in Figures 1F and 3F. (C,D) Identification of fragments formed in single cells. Single cell contents were loaded into a capillary 5 min after starting or lead peptide microinjection into the cell. Immediately after loading the cell contents into the capillary, the fragments of the starting peptide QW-III-67B (C) or lead peptide QW-V-48B (D) were loaded into the capillary. A voltage was then applied to the capillary to initiate simultaneous electrophoresis of the cellular contents and fragment standards.

Table 1

Peptide Name	Sequence	$t_{1/2}$[a] (min)	$t_{50\%\ p}$[b](min)
QW-III-67B	FAM-GGAYAAPFKKKA	1.3	150
QW-III-90A	FAM-GGAYAAPFKKOA	4.6	580
QW-m-90B	FAM-GGAYAAPFKOKA	5.3	500
QW-III-90C	FAM-GGAYAAPFOKKA	4.3	86
QW-III-90D	FAM-GGAYAAPFKOOA	4.9	44
QW-III-90E	FAM-GGAYAAPFOKOA	5.0	25
QW-III-90F	FAM-GGAYAAPFOOKA	6.0	90
QW-III-90G	FAM-GGAYAAPFOOOA	5.7	650
QW-IV-74A	FAM-GGAYAAAFKKKA	15.1	$>10^5$
QW-IV-74B	FAM-GGAYAAWFKKKA	8.9	$>10^5$
QW-IV-74C	FAM-GGAYAATKKKKA	10.6	$>10^5$
QW-IV-74D	FAM-GGAYAA-Sarc-FKKKA	24.0	$>10^5$
QW-IV-74E	FAM-GGAYAAFFKKKA	9.5	$>10^5$
QW-IV-85B	FAM-GGAYAAP-MPh-KKKA	52.6	$>10^5$
QW-V-23B	FAM-GGAYAAP-DPh-KKKA	36.3	82
QW-V-23C	FAM-GGAYAAP-Nal-KKKA	5.7	190
QW-V-23E	FAM-GGAYAAP-Tyr(3-$N0_2$)-KKKA	4.6	$>10^5$
QW-V-48B	FAM-GGIYAAP-MPh-KKKA	19.4	42

Table 2

Starting Peptide QW-III-67B	Peptide (%)	Lead Peptide QW-V-48B	Peptide (%)
a	10 ± 2	m	33 ± 14
b	15 ± 8	n	15 ± 9
c	2 ± 0.6	s	13 ± 4
d	0.7 ± 0.5	t	22 ± 10
f	5 ± 2	u	17 ± 15
g	13 ± 4		
h	36 ± 6		
i	15 ± 5		
j	3 ± 2		