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N-Gemini peptides: cytosolic protease resistance via N-terminal dimerization of unstructured peptides

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Abstract

Herein we describe a synthetically simple strategy for increasing the lifetime of unstructured peptides in cytosolic environment via dimerization at the N-terminus to block threading into the catalytic cleft of cytosolic proteases. We establish this approach with kinase substrates, allowing for phosphorylation in cells as a demonstration of protease resistance.

There is great interest in developing bioactive peptides that function within cells. However, a primary limitation of peptides is that they are rapidly degraded in cells. A number of aminopeptidases and endopeptidases are known to exist free in the cytosol of cells, including tripeptidylpeptidase II (TPPII),^{1–3} thimet oligopeptidase (TOP),⁴ prolyl oligopeptidase (POP),⁵ and leucine aminopeptidase (LAP).^{6,7} A common feature of these peptidases is a narrow cleft that contains the catalytic site, and evidence suggests that the N-terminus of peptides must thread into the cleft.^{5,8–12} Several methods have been reported in the literature that take advantage of these features to create more protease-resistant peptides. While N-terminal acetylation of peptides modestly extends their lifetime by about a factor of 2 in serum,¹³ this approach has little effect on peptide lifetimes in cell lysates.^{14,15} A common approach involves incorporation of unnatural amino acids into the sequence.^{16,17} However, this approach often requires an iterative design process that can be time consuming and costly, as a delicate balance between resistance and peptide efficacy must be maintained. Furthermore, while a wide range of peptide stapling methodologies,^{18,19} hydrogen bond

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Conflicts of interest

There are no conflicts to declare.

surrogates,^{20,21} and mixed α/β -peptides^{22–24} have been developed, these are limited to α -helical peptides. PEGylation of peptide substrates has also been used to increase resistance, though this often requires 10–40 kDa branched PEG units, and can result in changes in the properties of the peptide and sterics can interfere with binding.²⁵ Previous work in our lab has shown that appending small cyclic peptides to the N-terminus of a linear peptide can extend its lifetime in cytosolic environment, taking advantage of the size of the catalytic cleft as well as the N-terminus recognition.¹⁴ However, cyclization can be synthetically challenging or low yielding, diminishing broad utility. There is one example demonstrating that non-covalent binding to the N-terminus of an unstructured peptide can increase its proteolytic stability in vitro, though it has only been demonstrated with aminopeptidase N (APN).²⁶ Additionally, disulphide linkage of an aromatic moiety that can form π – π stacking interactions with sidechains was reported to increase proteolytic stability toward chymotrypsin.²⁷ Finally, we have investigated appending short PEG chains as well as bulky groups to the N-terminus of unstructured peptides with only limited success.^{15b,c}

In an ongoing effort in our labs, we aimed to increase protease resistance of a number unstructured peptides that are substrates for kinases in order to develop single-cell kinase assays via capillary electrophoresis-laser induced fluorescence (CE-LIF) analysis of peptide phosphorylation.^{14,16} However, peptide degradation is competitive with phosphorylation,^{14,16,28} making such an assay impossible without some method for increasing protease resistance (Scheme 1).

In considering straightforward synthetic approaches to masking the N-terminus to prevent threading into the cytosolic peptidases, we hypothesized that N-terminal dimerization of a peptide could mask its N-terminus in the same way that cyclization would, but without the added conformational constraints that can often decrease substrate efficacy (Scheme 1). We call these head-to-head dimeric peptides N-gemini peptides, similar to gemini surfactants.²⁹ Such dimerization is synthetically easier than peptide cyclization, and faster and cheaper than iterative design with unnatural amino acids, without risking the loss of substrate activity. Herein we report the validation of this protection approach in HeLa cell lysates, in which we applied N-terminal dimerization to three different kinase substrates. We then further demonstrated the use of one of those substrates in an intracellular kinase assay (Scheme 1). We demonstrate that N-terminal dimerization improves the half-lives of unstructured peptides by up to 28-fold, which is on par with protection of an unstructured peptide by capping with N-terminal cyclic β -turn^{14,15} or by incorporation and optimization of unnatural amino acids into the substrate sequence.^{16,30} Importantly, the approach reported here is far more synthetically accessible, thus providing a significant advancement over previous methods and a new tool for achieving protease-resistant unstructured peptides.

The N-gemini peptides consisted of the substrate sequence on the C-terminus, a spacer consisting of a polyethylene glycol (PEG) chain (PEG³ or PEG⁴), 5(6)-FAM or (5)-FAM conjugated to a lysine side chain, and a glycine (Figure 1B).³¹ Succinic acid was selected as the dicarboxylate linker for initial investigations to give the peptide dimer. Three peptide sequences known to function as kinase substrates were chosen, a PKB substrate (GRPRAATFAEG-NH₂),³² a PKC substrate (QKRPSQRSKYL-NH₂),³³ and an Abl substrate (EAIYAAPFAKKK-NH₂).³⁴ The N-gemini peptides were dimerized on resin

using conditions adapted from Kier and Andersen.³⁵ Monomeric peptides, 1–3, consisting of the substrate sequence capped with 5(6)-FAM (mixture of isomers) at the N-terminus (Figure 1A) were used as controls.

The protease resistance of the peptide dimers was determined by incubating them in HeLa cell lysates at 37 °C as a model for the cytosol. These conditions are likely harsher than cytosol, as lysis releases additional proteases from organelles. Aliquots were removed and quenched at various time points and the amount of intact peptide left was determined by capillary electrophoresis paired with laser induced fluorescence (CE-LIF) (Figure S3). The control peptides all degraded rather quickly, with half-lives of 2–15 minutes, depending on the sequence (Figure S4 and Table 1). The N-gemini peptides 4–6, in contrast, exhibit a 24-, 28-, and 7.5-fold increase in protease resistance of the peptide dimers over their un-dimerized counterparts, despite the fact that they are still unstructured (Figure S4 and Table 1). This increase in protease resistance is on the same order as the best methods reported to date, including appending a cyclic β -hairpin,¹⁴ which is synthetically more challenging, or incorporation of unnatural amino acids within the substrate itself,^{16,17} which requires significantly more optimization of both degradation resistance and substrate activity. The increased resistance is presumably due to the fact that the peptide dimers can only enter the catalytic cleft of peptidases starting with the C-terminus, hindering their recognition by the peptidases. To confirm that these results do not reflect co-elution of the parent peptide and fragments, CE-MS was used to analyze the samples from the longest-lived peptide, 5, and its control peptide, 2 (Figure S6). Based on integration of the peaks relative to an internal standard, the half-life of peptide 2 was 5 minutes, while the half-life of peptide 5 was 2 hours, representing the same fold-increase seen by CE-LIF, and verifying that co-elution of peptide fragments was not an issue. Note that the difference in absolute lifetimes arise from batch-to-batch variability in the cell lysates and/or differences in the detection techniques.

To evaluate the role of the linker, two additional versions of peptide 5 were synthesized, one using isophthalic acid (peptide 7) and one using terephthalic acid (peptide 8) as the linker (Figure 1B). The half-lives of peptides 7 and 8 were close to that of peptide 5 at approximately 7 hours (Figure S5), indicating the linker used does not significantly affect the degradation resistance, suggesting that the masking of the N-termini, rather than the particular structure of the linker, influences degradation rates.

Enzymatic assays were performed to assess the ability of peptides 1–6 to act as substrates for their respective enzymes. All peptides were synthesized using only one isomer of FAM ((5)-FAM) and a PEG⁴ chain (herein referred to as peptides 1*–6*), since dimers with differing lengths of PEG chains and even the 5- and 6- isomers of FAM can be separated by CE, complicating the analysis. The peptides were incubated with the appropriate purified kinase at 30 °C and phosphorylation was monitored over time using CE-LIF (Figure S8). The identification of phosphorylated peaks was confirmed by HR ESI-MS (Figures S9 & S10). All N-gemini peptides were phosphorylated by their respective kinase, on one or both of the phosphorylation sites, indicating that the dimerization did not hinder the peptide's ability to act as a substrate. Dimers 4* and 5* were phosphorylated to a similar extent as their un-dimerized counterpart, demonstrating that the rate of phosphorylation was not significantly perturbed (Table 2). Importantly, this means that re-optimization of the

substrate is not necessary to achieve degradation resistance, as is often needed with other approaches.^{16,17} Separation could not be achieved with peptide **6***, so the degree of phosphorylation could not be quantified (Figure S8).

Since previous work in our lab has been done on developing a kinase reporter based on peptide **1**,¹⁹ we evaluated phosphorylation of peptides **1*** & **4*** in cytosolic lysates for comparison. Peptides **1*** & **4*** were incubated at 30 °C in Baf/BCR-Abl cytosolic lysate containing ATP and a cocktail of phosphatase and protease inhibitors, and phosphorylation was monitored using CE-LIF (Figure 2). Both peptides **1*** & **4*** were phosphorylated at comparable rates: at 2 hours, peptide **1*** was 87±2% phosphorylated and peptide **4*** was 86±0.1% phosphorylated relative to non-degraded peptide (due to the overlay, peaks for peptide **4*** were deconvoluted as described previously³⁶ using Origin 9.0. The error reported for calculated peak area was 3–9%).

The intracellular phosphorylation of peptide **4*** was then assessed. Peptide **1*** or **4*** was loaded via induced pinocytosis, along with sodium pervanadate a phosphatase inhibitor, into live Baf/BCR-Abl cells and incubated at 37 °C for 25 minutes. The cells were then lysed and the cellular contents analyzed by CE-LIF (Figure 3). In that timeframe, peptide **1*** appeared to have completely degraded, as no intact phosphorylated or un-phosphorylated peptide was seen. Peptide **4***, however, exhibited only partial degradation, and both un-phosphorylated and di-phosphorylated peptide were observed. The peptide dimer was 54±8% phosphorylated relative to intact peptide remaining, and together, those constituted 19±2% of all species present. These results demonstrate that even within cells, the N-gemini peptide is long-lived enough, and reactive enough, to get phosphorylated faster than it is degraded, unlike the control peptide.

In summary, we developed a synthetically simple method to impart protease resistance onto peptides via dimerization at the N-terminus. Three different kinase substrates were protected using this strategy, leading up to a 28-fold increase in half-lives in cell lysate, the longest lived with a half-life of 7 hours. The same Abl substrate, which we had previously protected using a cyclized β -turn at the N-terminus,¹⁶ exhibited a similar level of degradation resistance as the N-gemini peptide, but with a difficult, low-yielding synthesis. Importantly, the N-gemini peptides were still recognized by their respective kinases and maintained the ability to act as efficient substrates. The increased lifetime of a dimerized Abl-1 substrate peptide (peptide **4***) in living Baf/BCR-Abl cells was demonstrated.

While the degree of protease resistance was sequence-dependent, even the smallest enhancement in resistance (7-fold) is significant, suggesting that this is a promising “first line of defence” against proteases, prior to exploring more challenging approaches. This strategy is expected to further promote the use of peptide reporters for the study of intracellular enzymatic activity. Furthermore, this approach may be applicable more widely to other classes of bioactive peptides as a general approach to increasing protease resistance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(a) Control peptides. All peptides are amidated at the C-terminus. (b) General construct of substrate dimers. The linker (red) is connected by amide bonds to the spacer (blue), and the substrate (black) is amidated at the C-terminus.

Figure 2.

Electropherograms monitoring the phosphorylation of peptides 1* (a & b) and peptide 4* (c & d) in Baf/BCR-Abl cytosolic lysates. Time points were taken at (top to bottom) $t=1$, 1.5, & 2 hours. (a) & (c) are the negative controls in which no ATP was added. Time points were taken at (top to bottom) $t=1$, 1.5, & 2 hours. U = unphosphorylated; P = phosphorylated; Mono-P = mono-phosphorylated; Di-P = dephosphorylated.

Figure 3.

Electropherograms measuring the amount of peptide **1*** (b) and **4*** (d) phosphorylated in Baf/BCR-Abl cells after pinocytosis and 25 minutes of incubation. (a) and (c) show time points from in vitro assays of peptide **1*** and **4***, respectively, for comparison of migration times. U = unphosphorylated; P = phosphorylated; Mono-P = mono-phosphorylated; Di-P = dephosphorylated.

Scheme 1.

(a) Intracellular phosphorylation assay analyzed by CE-LIF using a fluorescent peptide substrate; green star represents the fluorophore. (b) N-gemini peptide where red octagon represents the linker and the green star represents the fluorophore.

Table 1Half-lives of peptides **1–6** in HeLa cytosolic lysates.^a

Peptide	t1/2 (minutes)	Fold Increase
1	5±1	----
2	15±1	----
3	2±0.2	----
4	120±5	24
5	420±24	28
6	15±3	7.5

^aError was determined based on the standard deviation from three runs.

Table 2Extent of in vitro phosphorylation of peptides **1***–**6*** after 20 minutes.^a

Peptide	% Phosphorylated
1*	17±2
2*	17±2
3*	42±6
4*	16±1
5*	30±2
6* b	N.D. ^b

^aError represents the standard deviation of three runs.^bNot determined.