Proteolytic excision and in situ cyclization of a bioactive loop from an REI-V_L presentation scaffold

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Abstract

Covalent cyclization of peptides is an important tool in structure–function analysis of bioactive peptides, because it constrains the molecule to enrich or exclude the receptor-bound conformation. Previously we described a 2-step procedure for cyclizing purified, native peptides in aqueous solution by reacting a Met or Lys side chain with an iodoacetylated N-terminus (Wood SJ, Wetzel R, 1992a, *Int J Pept Protein Res* 39:533–539). We show here that the cyclization reaction scheme can be extended to peptides excised from proteins by endo-LysC proteolysis, which generates fragments terminating with Lys. To illustrate the method, we used an immunoglobulin V_L domain (REI-V_L) with an RGD-containing sequence engineered into its CDR3 and flanked by Lys residues. This REI-V_L/RGD hybrid displayed an IC₅₀ of 24 nM for ligand competition at the platelet fibrinogen receptor $\alpha_{IIb}\beta_3$. The RGD-containing peptide excised by endo-LysC from the REI-V_L presentation scaffold exhibited an IC₅₀ of about 50 nM, and the corresponding cyclized peptide, an IC₅₀ of about 10 nM. Significantly, both the N^α-acylation and the cyclization reactions occur efficiently even in the context of the other endo-LysC fragments of REI-V_L, which suggests that the reaction may prove useful in converting mixtures of endo-LysC products of many proteins into the corresponding cyclic peptides in situ.

Keywords: cyclization; iodoacetylation; mutagenesis; presentation scaffold; REI-V_L; structure–function analysis

Most peptide synthesis methods are based in a traditional organic chemistry approach, relying on the use of nonaqueous solvents and on strategies that include protection and deprotection of sensitive side chains. With the advent of methods for the production of polypeptides by recombinant DNA methods, however, it has become important to identify mild, highly selective chemical modification methods that can be carried out efficiently in aqueous media without previously applied protecting groups. One classic method is the acylation of peptides at acid pH, which gives strong reaction preference to an N-terminal α -group in the presence of 1 or more Lys ϵ -amino groups (Means & Feeney, 1971). More recently, this chemistry was extended to *iodoacetylation* of peptides (Wetzel et al., 1990) for crosslinking to proteins (Wetzel et al., 1990), carbohydrates (Wood & Wetzel, 1992b), and other functionalities (Stults et al., 1993). In addition, N^α-iodoacetyl groups introduced by such procedures have been used for efficient aqueous phase cyclization of purified unprotected peptides via intramolecular crosslinking to nucleophilic amino acid side chains (Wood & Wetzel, 1992a; Lee et al., 1993).

Presentation scaffolds are small protein domains for displaying installed peptide ligands, which may prove useful in elucidating the receptor-bound conformation of a peptide (Wetzel, 1991; Lee et al., 1993; Pessi et al., 1993; Yamada et al., 1993), in therapeutic applications (White et al., 1961; Maeda et al., 1989; Sollazzo et al., 1990; Wolfson et al., 1991; Zanetti et al., 1993), and in mapping ligand binding sites of receptors (Freimuth et al., 1990; Langen & Taylor, 1992). While conceptually attractive, however, presentation scaffold experiments may sometimes prove difficult to interpret. One way to initially assess the contribution of the scaffold to the activity of an installed peptide is to compare the activity of the scaffold chimera to the activities of the corresponding acyclic and cyclic peptides. For use in coordination with random mutagenesis approaches to optimizing the binding of scaffold–ligand hybrids, we sought an efficient means of preparing each acyclic and cyclic peptide directly from the corresponding hybrid protein.

In this paper we describe the introduction of an RGD-containing peptide cassette into a loop of the presentation scaffold REI-V_L in such a way that it can be (1) excised as an intact peptide by a lysine-specific protease and (2) cyclized in high yield via the C-terminal Lys of the cassette. Significantly, despite the potential for side reactions such as heteropolymer formation (Robey & Fields, 1989), this reaction sequence can be carried out at equivalent, good overall yields of cyclic peptide even when

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conducted on the crude proteolysis digest. The sequence RIPRGDMPK, in the context of CDR3 of REI-V_L, was in this manner shown to possess binding activity to the integrin $\alpha_{IIb}\beta_3$ at a level intermediate between its activities as the corresponding cyclic and acyclic peptides; equivalent activities were obtained whether the reaction was conducted on pure peptide or endo-LysC digest.

Results

A mutant of the V_L domain of the Bence-Jones light chain REI containing the sequence KRIPRGDMPK in CDR3 was constructed in the synthetic DNA for REI-V_L (Lee et al., 1993) by cassette mutagenesis (Wells et al., 1985). The protein was expressed by secretion into the periplasm of *Escherichia coli* (Chan et al., manuscript in prep.) and purified. Like other mutants of REI-V_L containing RGD sequences in CDR3 (Lee et al., 1993), this new mutant, REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK)¹, exhibits strong competitive inhibition of fibrinogen binding to the platelet receptor $\alpha_{IIb}\beta_3$ (Table 1).

Figure 1A shows the HPLC profile of peptides generated by digestion of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK) with endo-LysC. The digest goes to 50–70% completion based on peak areas from analytical reversed-phase HPLC (RP-HPLC) analysis. Reduction and alkylation of the protein prior to digestion resulted in no significant increase in the extent of completion of the digest (data not shown) and were therefore not routinely done. The fragments expected from the proteolysis of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK) with endo-LysC are shown in Table 2. Fragments 1 and 4 are expected to be disulfide linked in the digest, as they are in the protein.

Analysis of fractions from an HPLC separation of the endo-LysC digest of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK) (Fig. 1A) revealed that only 1 fraction contained material with an affin-

¹ The nomenclature for a mutant protein containing an inserted sequence is as described. Following the name of the wild-type protein is the designation for the wild-type residues deleted ($\Delta 90-97$), and after the slash is the designation of the sequence number of the first inserted residue followed by the inserted sequence in single-letter code.

Table 1. IC₅₀ values for inhibitors of fibrinogen binding to $\alpha_{IIb}\beta_3$ ^a

Preparation	IC ₅₀ (nM)
REI-V _L ($\Delta 90-97/+90$ KRIPRGDMPK)	24 ± 3 (6)
RIPRGDMPK	50 ± 6 (5)
RIPRGDMPK, cyclic	11 ± 3 (5)
REI-V _L ($\Delta 90-97/+90$ KRIPRGDMPK) Lys-C digest	40 ± 7 (6)
REI-V _L ($\Delta 90-97/+90$ KRIPRGDMPK) Lys-C digest cyclized	8 ± 2 (4)

^a Assayed on microtiter plates by the method of Smith et al. (1990). Estimates in the error are standard deviations. The number in parentheses represents the number of independent determinations. The amounts of isolated peptides were calculated from peak areas on analytical HPLC. The amounts of available peptide in crude protease digests were calculated from total protein after estimating the extent of completion of protease digestion and/or recovery from batchwise HPLC resin partial purification from HPLC analysis.

Fig. 1. Reversed-phase HPLC chromatograms. **A:** Endo-LysC digest of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK). **B:** Reaction mixture of iodoacetylation of A. **C:** Reaction mixture from the cyclization of the products generated in B at pH 10. Peptide elution was monitored by absorbance at 215 nm. Arrows indicate the elution positions of (a) undigested REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK), (b) RIPRGDMPK, (c) N^α-iodoacetyl-RIPRGDMPK, and (d) Lys-cyclized N^α-iodoacetyl-RIPRGDMPK.

ity for the $\alpha_{IIb}\beta_3$ receptor: the peak eluting at 14 min (data not shown). Amino acid sequencing of this peak (Fig. 2A) demonstrated that it contained approximately equimolar amounts of 2 peptides. One peptide was the expected RGD-containing peptide, RIPRGDMPK, and the other contained the carboxyl-terminal peptide from REI-V_L, LQITR (Table 1) (data not shown). Iodoacetylation of this 14-min HPLC peak fraction yielded only 2 detectable products, one derived from each of the starting peptides (Fig. 2B). Previous experience (Wetzel et al., 1990) and the behavior of the products (see below) suggest these to be the N^α-iodoacetyl derivatives. Amino acid composition

Table 2. Sequences expected from an endo-LysC digest of the mutant REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK)

Sequence	Fragment number (from N-terminus)
DIQMTQSPSSLSASVGDRTITCQASQDIK	1
YLNWYQQTPGK	2
APK	3
LLIYEASNLQAGVPSRFSGSGSGTDYFTFTISLQPEDATYYCQK	4
RIPRGDMPK	5
FGQGTK	6
LQITR	7

Fig. 2. Reversed-phase HPLC chromatograms. **A:** Purified peak exhibiting $\alpha_{11b}\beta_3$ affinity. **B:** Reaction mixture from iodoacetylation of **A** showing elution positions of N^α -iodoacetyl-RIPRGDMPK (17 min) and N^α -iodoacetyl-LQITR (18.5 min). **C:** Reaction mixture generated by incubation of pure N^α -iodoacetyl-RIPRGDMPK at pH 2, indicating the Met-cyclized diastereomeric products (14.5, 15 min) and unreacted N^α -iodoacetyl-RIPRGDMPK (17 min). **D:** Reaction mixture generated by incubation of pure N^α -iodoacetyl-RIPRGDMPK at pH 10, showing the predominant Lys-cyclized peptide (16 min) and a small amount of Met-cyclized material (14.5, 15 min). Peptide elution was monitored by absorbance at 215 nm.

showed that the product peak eluting at 18 min corresponds to a modified LQITR peptide, whereas the 16-min peak is the derivatized RIPRGDMPK (data not shown). The 2 N^α -iodoacetylated peptides were each obtained at 85% yield.

The sequence RIPRGDMPK contains 2 nucleophilic amino acids, Met and Lys, capable of undergoing the cyclization reaction with an iodoacetylated N-terminus (Wood & Wetzel, 1992a). The product of cyclization via Met can be cleanly produced by incubating N^α -iodoacetyl-RIPRGDMPK at pH 2, a pH at which the lysine ϵ -amino group, being protonated, cannot react (Wood & Wetzel, 1992a). Figure 2C shows the results of an incomplete methionine cyclization reaction, which proceeds with a $t_{1/2}$ of about 15 h and generates a product mixture eluting at 14.5 and 15 min. (Methionine-cyclized peptides generally exhibit a characteristic set of diastereomers that migrate slightly differently in RP-HPLC [Wood & Wetzel, 1992a].) Figure 2D shows that reaction of N^α -iodoacetyl-RIPRGDMPK at pH 10 (a pH at which either Met or Lys can react) gives a small amount of the Met-cyclized material but a much higher yield of a different product eluting at 15.7 min, with a $t_{1/2}$ of about 5 h. The elution position of this product—earlier than the acetylated peptide but later than the Met-cyclized peptides, is consistent with a Lys-cyclized peptide based on reactions of similar peptides (Table 3). Further, composition analysis indicates the disappearance of Lys, and mass spectrometric analysis gives a parent ion consistent with cyclized peptide. The yield of this Lys-cyclized peptide, after RP-HPLC purification, is 77%, based on iodoacetyl-peptide. The excision/cyclization reaction sequence is summarized in Figure 3.

In previous studies it was demonstrated that, for homologous peptides of the same length, the rates of cyclization via a C-terminal Met and via a C-terminal Lys are essentially identical at pH 10 (Wood & Wetzel, 1992a). It might thus seem surprising that in a direct competition in RIPRGDMPK, cyclization via Lys, which requires cyclization of more residues, so predominates over Met-cyclization. The answer probably lies in the first Pro residue in the sequence. Previous work with a pentapeptide series also indicated that, all other things being equal, a Pro res-

Table 3. Cyclization product distribution in a series of sequence-related peptides^a

Sequence	Product distribution of pH 10 reaction	
	Met-cyclic	Lys-cyclic
RGDMPDDK	100	0
IPRGDMPDK	63	37
RIPRGDMPDDK	41	59
SRIPRGDMPDDK	39	61
ISRIPRGDMPDDK	49	51
RIPRGDMPK	17	83

^a Listed are the percentages of total product mixture by HPLC analysis after incubation of the corresponding iodoacetyl-peptides at pH 10 as described in the Materials and methods. Each product was confirmed as a cyclic monomer by loss of iodide as shown by the laser desorption mass spectrometry parent ion. Amino acid composition analysis confirmed the involvement of Met or Lys in the cyclization by loss of the residue. In general, Met-cyclic product appears as 2 peaks, or a single poorly resolved peak, in HPLC analysis, eluting earlier than the single peak of the Lys-cyclic product, which in turn elutes earlier than the iodoacetyl peptide.

Fig. 3. **A:** Amino acid sequence for the framework and hypervariable loop residues (residues 85–102) of the β -hairpin containing CDR3 in wild-type REI (Palm, 1970). **B:** The corresponding loop from the mutant REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK). The RGD-containing sequence from kistrin (Dennis et al., 1989) was installed into CDR3 by replacing the YQSLPYT sequence at positions 90–97 of the wild type with the sequence KRIPRGDMPK, which adds flanking Lys residues to the kistrin-derived sequence. Treatment with endo-LysC generates the peptide RIPRGDMPK, which can then be acylated with iodoacetic anhydride and subsequently cyclized via Lys by increasing the pH to 10. The pH 10 side product obtained by cyclization via the Met side chain is not shown.

idue inserted between the N-terminus and the nucleophilic side chain slows the cyclization reaction rate by about an order of magnitude (Wood & Wetzel, 1992a). Table 3 shows a series of N^{α} -iodoacetylpeptides, related in sequence to the guest peptide in REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK), and the product distribution of their cyclization at pH 10. It can be seen that in the peptide RGDMPDDK, cyclization of the Met residue is strongly favored in comparison to cyclization via the C-terminal Lys. All other peptides in Table 3, however, exhibit significant amounts of Lys-cyclized product in addition to the Met-cyclized peptide. We hypothesize that the peptidyl-prolyl bond, found between the N-terminus and Met in all of the peptides except RGDMPDDK, preferentially slows the cyclization via Met relative to Lys, due to the smaller loop size of the Met-cyclized product in this sequence. Table 3 shows that the bias in favor of cyclization to the C-terminal Lys is even more dramatic in RIPRGDMPK, the peptide excised from the scaffold chimera (Fig. 2D).

In principle, these iodoacetylation and cyclization reactions should proceed even in a mixture of peptides. The iodoacetylation reaction is highly selective for any α -amino group (Wetzel et al., 1990). Furthermore, at peptide concentrations in the 1-mg/mL range or below, intermolecular alkylation and polymerization are not observed as a side reaction during cyclization (S.J. Wood & R. Wetzel, unpubl.; Robey & Fields, 1989). The chemistry was therefore attempted on the endo-LysC reaction mixture. The RP-HPLC chromatogram of the endo-LysC digestion of REI ($\Delta 90-97/+90$ KRIPRGDMPK) in Figure 1A contains the number of peaks expected from the digest, as suggested by Table 2. Fragments 5 and 7 (Table 2) are contained in the 14.5-min peak (see above), fragments 3 and 6 would be expected to elute unretained in the injection peak, and fragments 1 and 4 are expected to be disulfide bonded into a single molecule. This suggests only 3 major peaks (fragments 5+7, 1+4, and 2), as observed.

Figure 1B shows the reaction mixture from iodoacetylation of the endo-LysC digest of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK). Two of the new peaks correspond to the known positions of N^{α} -RIPRGDMPK and N^{α} -LQITR (compare Fig. 2B with Fig. 1B). The other new peaks were not characterized.

Figure 1C shows the reaction mixture obtained by incubating this iodoacetylation reaction mixture at pH 10 for 22 h. Included in the HPLC peaks are minor peaks at the elution position of N^{α} -RIPRGDMPK cyclized via its Met and a major peak corresponding to N^{α} -RIPRGDMPK cyclized via its C-terminal Lys (compare Fig. 1C with Fig. 2D). Analysis of these RP-HPLC chromatograms indicates that iodoacetylation and cyclization in the peptide mixture from the endo-LysC digest occur with yields for each chemical step comparable to the yields for the reactions of the purified peptides (data not shown).

Although the cyclization reaction can be carried out on the mixture of iodoacetylated peptides, we found it important to remove the iodoacetate side product prior to the cyclization. Although HPLC purification of the desired peak would easily eliminate the iodoacetate, such a requirement would also eliminate the potential for carrying out the procedure with maximum convenience and/or throughput. As a compromise we devised a batchwise adsorption-desorption protocol for the peptide iodoacetylation reaction mixture using bulk C¹⁸ resin, and this proved to be an efficient means of removing the iodoacetate (see Materials and methods).

The IC₅₀'s for the $\alpha_{11b}\beta_3$ receptor of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK) and the acyclic and cyclic peptides, both purified and generated in situ, are given in Table 1. In this assay, simple RGD-containing peptides generally exhibit IC₅₀'s of 100 nM or higher, while the snake venom disintegrins exhibit IC₅₀'s of 1 nM or lower (Lee et al., 1993). The IC₅₀ of the scaffold-chimera REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK), which contains the RGD sequence found in kistrin, is 24 nM. The acyclic RGD-containing peptide RIPRGDMPK released by proteolysis of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK) is less active (IC₅₀ = 50 nM) than the protein from which it came. Cyclization of RIPRGDMPK improves its activity by about 5-fold compared with the acyclic peptide, and 2–3-fold compared with the scaffold-peptide hybrid.

The IC₅₀ of the crude endo-LysC digest of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK) is 40 nM, within assay variation of the value found for the pure peptide. Furthermore, the IC₅₀ of the cyclized digest, 8 nM, is within assay variance of the value found

for the purified cyclic peptide. These results confirm the HPLC result of efficient, specific cyclization of the protease-released RIPRGDMPK, even in the context of contaminating peptides. The 5-fold enhancement of peptide activity upon cyclization is as evident in the products of the crude digest as it is for the purified peptides.

We were concerned that residual protease activity in the digest might compromise biological assays, e.g., by digestion of the biotinylated fibrinogen used in the competition binding assay. Using *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide as a substrate, we detected a small amount of endo-LysC activity remaining in the crude REI-V_L digest. This could be eliminated, however, by heating the sample for 20 min at 65 °C. Alternatively, residual protease activity was also eliminated as a result of the adsorption-desorption cleanup with the bulk C¹⁸ resin. In any case, we also showed that residual protease does not interfere with the binding assay, since there is no significant difference in the IC₅₀'s of the REI-V_L digest before or after elimination of the residual protease activity (data not shown).

Discussion

The iodoacetylation and cyclization of RIPRGDMPK excised from REI-V_L (Δ90-97/+90 KRIPRGDMPK) by endoproteinase LysC occur with an overall isolated yield of about 65%. This yield is competitive with the yields of more conventional routes to cyclic peptides and suggests that this reaction sequence may prove a viable alternative cyclization procedure even for chemically synthesized peptides (Wood & Wetzel, 1992a). The reaction is especially useful, however, for peptides derived from natural sources—either by proteolysis of proteins or by recombinant DNA synthesis—for which there are few viable opportunities to build in protecting groups.

The high selectivity of cyclization for the C-terminal Lys of RIPRGDMPK is probably primarily due to the Pro residue at position 3 selectively retarding cyclization via Met. In principle, peptides containing more than 1 nucleophilic side chain are capable of generating a mixture of products and, as Table 3 shows, the variation in relative reaction extents can be significant depending on sequence. This is a potential limitation on the generality and predictability of the chemistry. However, only Lys, Met (Wood & Wetzel, 1992a), and Cys (Robey & Fields, 1989) are capable of very high reaction rates. Cys can be reversibly protected via mixed disulfide formation (Wetzel et al., 1990), and Met can be reversibly protected by mild oxidation to the sulfide (R. Wetzel, unpubl. results), both via aqueous phase chemistry (Means & Feeney, 1971). Use of a Lys-specific protease assures that each fragment will contain only 1 Lys. In scaffold-phage applications, in which the sequences to be cyclized will be randomly generated, codon-bias mutagenesis protocols might be used to disfavor Lys residues in the randomized peptide interior, while at the same time fixing invariant Lys perforations at the flanks. The competitive alkylation reaction might also prove useful in studies of peptide conformational mobility.

The chemical and biological evidence presented in Figure 1 and Table 1 indicates the high efficiency of the 2-step cyclization procedure—even when conducted in the presence of proteolysis products. The remarkable degree to which the cyclization chemistry is successful when conducted on mixtures of peptides presumably derives from the combination of the high preference for acylation of α-amino groups at pH 6 (Wetzel et al., 1990)

and the highly efficient intramolecular alkylation of nucleophilic side chains compared to their iodoacetylation and intermolecular polymerization (Robey & Fields, 1989). It seems likely that this reaction will prove general, allowing in situ cyclization of all but the longest endo-LysC digest products of many proteins. Peptide sequences as long as 20 amino acids have been cyclized by this aqueous phase procedure (R. Wetzel, unpubl.). In analogy to the cyclization of installed cassettes, it should also be possible to cyclize endo-LysC protease fragments from any protein in situ. If so, this would offer opportunities for rapid preliminary analysis of protein ligands for loop epitopes.

The low IC₅₀'s of both the RGD-containing scaffold REI-V_L (Δ90-97/+90 KRIPRGDMPK) and the cyclized RIPRGDMPK suggest that in this construction the scaffold neither greatly detracts from the activity of the installed peptide (e.g., by steric or electronic effects from neighboring groups) nor does it contribute to the peptide's active conformation any more than a simple covalent crosslink does. Imposition of a loop conformation by either means provides a modest benefit to affinity. That the activities of these 2 molecules are so similar may be due in part to the detrimental impact of loop insertions in REI on global folding stability (Helms & Wetzel, manuscript in prep.). It is possible that a particularly favorable and well-populated RGD conformation is responsible for the additional receptor affinity of kistrin and other disintegrins compared to scaffold-chimera-like REI-V_L (Δ90-97/+90 KRIPRGDMPK). If so, it may be possible to identify other REI-V_L/RGD scaffold mutants that duplicate this conformation and activity.

Materials and methods

General

REI containing the sequence KRIPRGDMPK substituted at positions 90-97 (REI-V_L [Δ90-97/+90 KRIPRGDMPK]) was purified from periplasmic inclusion bodies as described by Chan et al. (manuscript in prep.). IC₅₀ values for inhibition of fibrinogen binding to the α_{11b}β₃ receptor were determined in a microtiter plate assay by a modification (Lee et al., 1993) of the published procedure (Smith et al., 1990). The IC₅₀ values were determined from a 4-parameter curve fit using the software Softmax (Molecular Devices, Inc.), and the concentration of inhibitor at 50% binding was recorded.

Endoproteinase LysC digestion

Endoproteinase LysC was purchased from Boehringer Mannheim Biochemicals. Endo-LysC was added to a protein solution, typically 0.1–0.4 mg, in 1 M urea, 50 mM NH₄CO₃, pH 8.0, at 0.003 units/μg of substrate. Digests were conducted at 37 °C for 18 h. The peptides were purified by RP-HPLC using a Vydac C⁴ (250 × 4.6 mm) column. Peptides were eluted with a linear gradient by mixing 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). A 1% to 74% solvent B gradient was used at a constant flow rate of 1.0 mL/min over 29 min. Peptides were dried down in a Speed-vac and resuspended in an appropriate volume of 10 mM NaPO₄, pH 7.5, and analyzed in the α_{11b}β₃ assay.

Iodoacetylation and cyclization

Iodoacetylation and cyclization were performed as previously described (Wood & Wetzel, 1992a; Lee et al., 1993). The iodo-

acetylation and cyclization with isolation of the intermediate products were as follows: REI-V_L ($\Delta 90-97/+90$ KRIPRGD MPK) was digested with endo-LysC, and the peak containing the $\alpha_{11b}\beta_3$ affinity was purified by RP-HPLC, dried down in a Speed-vac, and resuspended in 100 mM MES, pH 6.0 (100 μ L). Iodoacetylation was carried out at 0 °C with the addition of 1.3 μ L of 0.25 M iodoacetic anhydride, followed, after vortexing and 3 min at 0 °C, with a second 1.3- μ L aliquot of anhydride. The iodoacetylated product was then purified by RP-HPLC and the peptide, in the H₂O/CH₃CN/TFA eluant, was adjusted to pH 10 with 1 M sodium borate, pH 10.8. The reaction was allowed to stand for 22 h at room temperature (RT). The cyclization product was then purified by RP-HPLC, dried in a Speed-vac, and resuspended in an appropriate volume of 10 mM NaPO₄, pH 7.5, for analysis in the $\alpha_{11b}\beta_3$ assay.

Iodoacetylation and cyclization of the endo-LysC digest without isolation of the intermediates were as follows: 0.4 mg of REI-V_L ($\Delta 90-97/+90$ KRIPRGDMPK) was digested with Lys-C in a total volume of 1.0 mL. When complete, as judged by analytical RP-HPLC, the digest was adjusted to pH 6 and iodoacetylated as above, except that there was only 1 addition (10 μ L) of 0.25 M iodoacetic anhydride. The reaction mixture was transferred to a 1.5-mL Eppendorf tube containing 5% (w/v) pre-equilibrated Waters C¹⁸ reverse-phase bulk resin. Pre-equilibration was accomplished by washing the resin with 50% acetonitrile/50% water/0.1% TFA followed by 2 washes of 5% acetonitrile/95% water/0.1% TFA. The reaction mixture containing the C¹⁸ resin was vortexed and allowed to stand at RT for 5 min. The mixture was centrifuged at 14,000 RPM for 1–2 min and the supernatant removed. Then 0.4 mL of 50% acetonitrile/50% water/0.1% TFA was added to the resin, vortexed, and allowed to stand at RT for 5 min. The reaction was centrifuged and the supernatant containing the peptides removed. An aliquot was removed for analysis on RP-HPLC, and the remaining reaction mixture was adjusted to pH 6 with 1 M sodium borate, pH 10.8. The reaction was allowed to stand for 22 h at RT, after which an aliquot was removed for RP-HPLC analysis. The reaction mixture was then analyzed in the $\alpha_{11b}\beta_3$ assay.

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