An in vitro selection strategy for conferring protease resistance to ligand binding peptides

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One drawback to the use of peptides as therapeutics has been their susceptibility to proteolysis. Here, we have used an in vitro display technology, CIS display, to enhance the proteolytic resistance of ligand-binding peptides by selection of protecting motifs from a large peptide library. The premise to this selection was that certain linear peptides within a library could form structures capable of preventing the access of proteases to defined cleavage sites without affecting ligand binding. A diverse 12-mer peptide library was inserted between a FLAG epitope motif and a thrombin cleavage site and this construct was fused to the bacterial initiator protein RepA for CIS display selection. After five rounds of selection, protection motifs were isolated that were capable of preventing proteolytic cleavage of the adjacent thrombin site. Some of the selected peptides were also resistant to more promiscuous proteases, such as chymotrypsin and trypsin, which were not used in the selection. The observed resistance to thrombin, trypsin and chymotrypsin translated into increased resistance to plasma proteases in vitro and to an increase in circulating half-lives in rats. This method can be applied to enhancing the in vivo stability of therapeutic peptides.

Keywords: CIS display/in vitro selection/peptide/protease resistance/stability

Introduction

The susceptibility of peptides to proteases is a major obstacle to the development of peptides for therapeutic applications (Sato et al., 1996). Peptides are commonly vulnerable to cleavage by the three major classes of proteases: the amino- and carboxy-exopeptidases that cleave at each terminus of the peptide and the endopeptidases that attack internal sites (Rawlings and Barrett, 1993; Rawlings et al., 2008). A notable example of a protease-sensitive peptide with consequently limited therapeutic potential is GLP-1, which has a half-life of less than 2 min, largely due to the degradation by dipeptidyl peptidase IV (Mentlein et al., 1993, Kieffer et al., 1995). Significant efforts to increase the protease resistance of this peptide have been made in order to make it suitable for therapeutic use. Identification of protease cleavage sites in a peptide’s sequence is often the first step in a rational design approach to solve the problem through the incorporation of chemical modifications, non-natural amino acids, d-amino acids, acetylation of the amino terminus or amidation of the carboxyl terminus. However, despite advances in rational design, it often remains unclear how non-contiguous amino acids contribute to stability within a peptide or protein sequence.

In order to address the limitations of rational design, combinatorial methods have been developed for selecting bioactive peptides from libraries. These combinatorial libraries have either been created chemically (Gordon et al., 1994; Lebl and Krchnáš, 1997; Houghten et al., 1999) or by using biological systems such as phage display or bacterial display (Smith, 1985; Cwirla et al., 1990; Devlin et al., 1990; Scott and Smith, 1990; Smith and Scott, 1993; Lowman et al., 1997; Bessette et al., 2004). Both of these methods are limited by the size of the libraries that can be generated, which are typically within the 10⁶–10⁹ range. Larger libraries would be expected to enable the selection of peptides with more optimal properties and in vitro display techniques such as mRNA, ribosome and CIS displays (Mattheakis et al., 1994; Roberts and Szostak, 1997; Odegrip et al., 2004), have therefore been devised to allow the generation and interrogation of libraries that are three to four orders of magnitude more diverse than phage display libraries. Phage and ribosome display methods have been previously described in which combined selection for binding and protease resistance has resulted in the preferential enrichment of stably folded protein structures (Kristensen and Winter, 1998; Sieber et al., 1998; Matsuura and Plückthun, 2003; Bai and Feng, 2004). These selections were predicated on the hypothesis that folded proteins are more resistant to protease attack than less stable or poorly folded variants; the retained ligand binding by a displayed protein in the presence of proteolytic enzymes thus allows recovery of the genetic material encoding only stably folded protein variants. However, these techniques have not been applied to short peptide sequences (<25 residues) mainly because peptides have previously been thought to be incapable of adopting structures resistant to proteolytic attack.

In order to test the ability of CIS display to address this problem, we created a peptide library comprising an N-terminal epitope for the M2 anti-FLAG antibody, a 12-residue random peptide library and a thrombin cleavage sequence. Following in vitro transcription and translation (ITT) in a bacterial S30 lysate mixture, protein–DNA complexes were incubated with thrombin and then recovered by panning against M2. In this way, only those peptides that had motifs capable of protecting the thrombin recognition sequence from cleavage and therefore retain the FLAG epitope, would be recovered. A number of motifs were identified that were protective against thrombin attack and many of these were also able to confer protection to the activity of other proteases that had not been included in the selection. Some of these were also resistant to proteolytic degradation in human and rat plasma and showed increased stability in vivo.
Materials and methods

Library construction

All enzymes were purchased from New England Biolabs (NEB Ltd., Hitchin, UK). All PCR reactions contained 12.5 pmol of each of the primers, 2.5 units of Taq DNA polymerase, 250-μM dNTP (Roche Diagnostics Ltd., Burgess Hill, UK) and 1× ThermoPol Buffer (NEB Ltd., Hitchin, UK) per 50-μl PCR. PCRs were carried out on a Techne Techgene PCR machine for one cycle of 2 min at 94°C, followed by 20–30 cycles at 94°C, 10 s; at 60°C, 30 s; at 72°C, 1–2 min, followed by a final extension of 5 min at 72°C. Library DNA template was generated via PCR from a plasmid containing the tac promoter using synthetic oligonucleotides, TRL. (GGCGTACCGGCTAGACTAGAACCGCGCCGGATCGAGGACC[VNN]12GTGCCAGTAATCATCAAACTTGTAGTC) (Eurogentec SA, Seraing, Belgium) and M13F (GTAAAACGACGGCCAG) (Sigma Genosys, Haverhill, UK). This oligonucleotide has 12 NNB codons each encoding 48 codons, including all 20 amino acids and 1 stop codon. The repA-CIS-ori construct was generated via PCR from the R1 plasmid, using primers BSPREPAFOR (CTGGAGATGGCATCAAGGGCCCCAACTGATCTTCACCAAACGTATTACC) (Molecular Solutions Europe Ltd., London, UK) and ORIREV (TGCATATCTGTCTGTCCAGGG) (Sigma Genosys Ltd., Haverhill, UK). The two DNA constructs were then digested and ligated as previously described (Odegrip et al., 2004). The amount of full-length ligated product was quantified on an agarose gel, giving a library size of >10^12 molecules. This template was further amplified by PCR using primers M13F and ORIREV. In this way, a FLAG-tagged peptide library, followed by a thrombin cleavage sequence fused to RepA, was generated according to Fig. 1.

Selection of stabilising peptides

ITT was performed in an Escherichia coli S30 lysate system, strain SL119 (Lesley et al., 1991; Lesley, 1995), for up to 1 h at 30°C. A total of 15 μg of library template was added to a total 250 μl of S30 lysate reaction in the first round; in subsequent rounds, 5 μg of library DNA was added in 100 μl of S30 lysate reaction. ITT was then diluted 10-fold with blocking buffer (2% BSA, 0.1-mg/ml herring sperm DNA, in PBS) and 0.1 units of restriction grade human thrombin (Merck Chemicals Ltd., Nottingham, UK) added. Samples were incubated at 25°C for 2 h. One micrograms of biotinylated M2 anti-FLAG antibody (Sigma Aldrich Company Ltd., Gillingham, UK) was added and incubated at 25°C for 30 min. Streptavidin-coated paramagnetic beads (M280, Invitrogen Ltd. Paisley, UK) were added and incubated for 5 min at 25°C. Beads were washed six times with sterile PBS/0.1% Tween 20 (PBST) and twice with sterile PBS. In subsequent rounds, the concentration of thrombin and incubation temperature were increased to 0.5 units and 37°C.

Fig. 1. Schematic diagram of the design of the thrombin resistance library and the resultant protein–DNA complex. (a) The 12-mer library is shown between the FLAG epitope (shaded, derived from a previous selection against the M2 antibody) (Odegrip et al., 2004) and the thrombin cleavage site (GPRS underlined). These are N-terminal to the RepA protein. Potential chymotrypsin (closed triangles) and trypsin (up arrows) cleavage sites are shown within this sequence. (b) The full-length double-stranded DNA library construct showing the tac promoter, FLAG epitope, the library construct, RepA and the 3′ untranslated regions, cis and ori, necessary for CIS activity of RepA. After ITT, FLAG-Library-GPRS-RepA fusion peptides are displayed on their encoding DNA templates. Cleavage with thrombin would cause the RepA-DNA component to be lost when captured by anti-FLAG antibody if no protection occurred.
respectively. Bound protein–DNA complexes were eluted from the beads in 1× ThermoPol PCR buffer (NEB Ltd., Hitchin, UK) at 65°C for 5 min. Template DNA was recovered via PCR using nested primers as described previously (Odegrip et al., 2004).

**ELISA screening of selected peptides on phage**

After each round of selection, recovered DNA was digested with *Not*I and *Nco*I and cloned into a similarly digested M13 gpIII phagemid vector, transformed into *E.coli* TG1 cells and plated on 2% glucose, 2×TY, 100 μg/ml ampicillin plates. It was necessary to clone the output into a phage in order to separate the polyclonal output into individual colonies for screening. These colonies were grown for the production of phage particles as described (Scott and Smith, 1990). Phage supernatants were then treated with either human thrombin or trypsin-agarose (all purchased from Sigma Aldrich Company Ltd.) at 0.65 units/ml for 3 or 6 h at 37°C. Biotinylated anti-FLAG M2 antibody (Sigma Aldrich Company Ltd.) was captured onto high-capacity streptavidin-coated (Streptawell) 96-well plates (Roche Diagnostics Ltd., Burgess Hill, UK) at 0.2 μg/ml ampicillin plates. It was necessary to clone the output into a phage in order to separate the polyclonal output into individual colonies for screening. These colonies were grown for the production of phage particles as described (Scott and Smith, 1990). Phage supernatants were then treated with either human thrombin or trypsin-agarose (all purchased from Sigma Aldrich Company Ltd.) at 0.65 units/ml for 3 or 6 h at 37°C. Biotinylated anti-FLAG M2 antibody (Sigma Aldrich Company Ltd.) was captured onto high-capacity streptavidin-coated (Streptawell) anti-FLAG M2 antibody (Sigma Aldrich Company Ltd.) at 0.65 units/ml for 3 or 6 h at 37°C. Biotinylated anti-FLAG M2 antibody (Sigma Aldrich Company Ltd.) was captured onto high-capacity streptavidin-coated (Streptawell) 96-well plates (Roche Diagnostics Ltd., Burgess Hill, UK) at 1 μg/ml. Plates were washed once with PBS and then blocked with 4% low-fat milk protein in PBS for 1 h at 25°C. The horseradish peroxidase-conjugated (HRP) anti-M13 secondary antibody (GE Healthcare UK Ltd., Chalfont St., Giles, UK) was diluted 1:5000 in 2% milk protein in PBS. ELISA assays were performed as described (McGregor and Robins, 2001). The assay was developed with SureBlue 3,3',5,5' tetramethyl benzidine (TMB) peroxidase substrate (Insight Biotechnology, Middlesex, UK) and read at 450 nm.

**ELISA screening of selected peptides**

Peptides identified from the phage screen were synthesised with a C-terminal biotin modification (Pepsan Presto B.V., Lelystad, The Netherlands). Peptides (approximately 80 nM) were then incubated with chymotrypsin/trypsin-agarose at 0.65 units/ml or human thrombin at 0.5 units/ml for 3 h at 37°C. Chymotrypsin/trypsin-agarose was removed via centrifugation and supernatants were then incubated in streptavidin-coated 96-well plates for 30 min at 25°C. The plates were then washed once with PBST and once with PBS before being blocked with 4% milk protein in PBS for 1 h at room temperature. The plates were then washed twice with PBST and twice with PBS. After washing, the HRP-conjugated anti-FLAG M2 antibody (Sigma Aldrich Company Ltd.) at 0.2 μg/ml in 2% milk protein in PBST was added. The antibody-conjugate was incubated on the plate for 30 min before washing four times with PBST and twice with PBS. The plates were developed with TMB substrate and then read at 450 nm.

**Resistance to plasma proteases**

Peptides that showed the best resistance to plasma were synthesised and purified (EZBiolab Inc., Carmel, USA) to 90% purity as judged by mass spectrometry and HPLC analyses. These peptides were then tested in duplicate at 10 μg/ml in 90% Sprague–Dawley citrate treated rat plasma (Harlan UK Ltd., Belton, UK) for stability according to the above protocol. Samples were taken at regular time intervals and snap frozen in dry ice. The plasma samples were then assayed in duplicates by diluting 5 μl into 45-μl PBS in wells of streptavidin plates (Roche Diagnostics Ltd.) and incubated for 15 min. The plates were blocked and washed as above. The same procedure was to test for protease resistance in human plasma (blood type A–, ex blood bank, National Blood Service, Cambridge, UK). Half-lives were determined by fitting concentrations to a single-phase decay algorithm in GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, U.S.A.).

**In vivo stability in rats**

The purified peptides were dissolved at 10 mg/ml in saline or in 50-mM NaCl and administered at 10 mg/kg by intravenous injection into the tail vein of female Sprague–Dawley rats. Six blood 150-μl samples were taken via *vena saphena* from each animal at 2, 15, 30, 45, 60 and 90 min. The blood samples were collected into pre-chilled tubes containing EDTA and 4 μl of protease inhibitor I (Roche Diagnostics Ltd.), dissolved in 1 ml of saline and placed on wet ice. The samples were centrifuged at ~1500 g at 4°C for 10 min, and supernatants were then frozen on dry ice (performed by Active Biotech AB, Lund, Sweden).

The samples were assayed by diluting 5 μl of plasma into 45 μl of PBS into Streptawell plates and assayed as described above. Four animals were used for peptides A12B, THR-6 and THR-7, three were used for THR-3 and each sample was assayed in duplicate. Concentrations were determined by extrapolation from standard curves of peptides assayed on the same ELISA plate. Half-lives were determined as above.

**Results**

**Library construction and selection**

A PCR construct was produced comprising a FLAG epitope followed by a completely randomised 12-mer region that was C-terminally linked to the thrombin cleavage sequence, GPRS (Fig. 1). This construct was then appended to the repA gene (containing the 3’ untranslated regions CIS and ori) to generate a library for CIS display selection. Approximately 10^12 library members were incubated in a bacterial ITT mixture to make peptide-RepA-DNA fusion complexes for incubation with thrombin and subsequent selection against the anti-FLAG antibody M2. When exposed to thrombin, only those library members that contained motifs that could protect the thrombin site from enzymatic attack would be expected to retain the N-terminal FLAG tag leading to their post-selection recovery. Thus, soluble human thrombin was used at increasing concentrations during the cycles of CIS display selection to exert evolutionary pressure for the preferential enrichment of thrombin-resistant FLAG-tagged peptides.

**Thrombin resistance**

Following five rounds of selection, the peptide sequences were cloned into phage in order to screen individual clones. These were tested in an ELISA to determine the retention of their binding to M2 after incubation with thrombin (Fig. 2). A peptide that contained a weak thrombin cleavage site (MPRM) that had previously demonstrated less than 20% activity of untreated signal in thrombin resistance assays was

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In vitro selection of protease-resistant peptide ligands
used as control (peptide A1: HYDYKFDDYWHSA
PRMS, Odegrip et al., 2004).

DNA sequencing revealed that most of the selected clones, which were analysed, had retained the thrombin cleavage site and the selected peptides contained a high incidence of polar and/or proline residues. The remaining peptides contained mutations within the thrombin recognition site. Twenty-six of the best phage peptide clones were sequenced (Table I) and these were shown to retain greater than 40% of their untreated M2-binding activity following incubation with thrombin. Fourteen peptides retained greater than 70% of their untreated M2-binding activity after 3 h treatment with thrombin (Fig. 2).

**Multiple protease resistance**

In order to determine whether the thrombin resistance of the selected peptides was due to specific binding interactions with the protease itself, peptides were exposed to the alternative proteases trypsin and chymotrypsin prior to assaying their binding to M2. Trypsin predominantly cleaves peptide chains at the carboxyl side of lysines and arginines. It would therefore be capable of cleaving both the FLAG epitope after lysine (K3) and the thrombin cleavage site after arginine (R24) present within all library members, (Fig. 1; Keil, 1992). Chymotrypsin predominantly cleaves peptides at the carboxyl side of the aromatic residues tyrosine, tryptophan and phenylalanine and it would therefore be expected to cleave the library at multiple sites, including at least five sites within the FLAG epitope (Fig. 1). Therefore, resistance to these wider spectrum proteases would require a selected motif to provide proteolytic resistance throughout the entire peptide and not just to that part of the peptide that contains the thrombin cleavage site.

Thrombin-resistant peptides expressed as pIII phage fusions were incubated with thrombin, trypsin and chymotrypsin for 6 h at 37°C. Retained binding to M2 was then measured by ELISA and compared with untreated samples (Fig. 2). At least 50% of the peptides assayed showed measurable resistance to trypsin despite the presence of at least two potential cleavage sites within these peptides. Less

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**Table I.** Sequences of the peptides identified as thrombin resistant in the initial screen

<table>
<thead>
<tr>
<th>Proline rich</th>
<th>Polar</th>
</tr>
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<tbody>
<tr>
<td>B5-</td>
<td>D11-</td>
</tr>
<tr>
<td>HDGRPQHHHPRGPRS</td>
<td>PTSHSTRPDGPRS</td>
</tr>
<tr>
<td>A5-</td>
<td>B3-</td>
</tr>
<tr>
<td>NHPNTNPDGPRS</td>
<td>PNPNFQTNHDGPRS</td>
</tr>
<tr>
<td>A10-</td>
<td>H1-</td>
</tr>
<tr>
<td>VNFQTTASQAHGPRS</td>
<td>TNFSKDEQPDDGPRS</td>
</tr>
<tr>
<td>B8-</td>
<td>B9-</td>
</tr>
<tr>
<td>HPNNHHHDQDGPRS</td>
<td>QKNHDTREDGDGPRS</td>
</tr>
</tbody>
</table>

**Mutated thrombin cleavage site**

| C4- | B8- | B9- |
| SAMFGDGGGSNSGS | HPNNHHHDQDGPRS | QKNHDTREDGDGPRS |

**Controls**

| A1- | A12B- |
| HYDYKFDDYWHSA | DFKDDYWHGSGS |

**GPRS = Thrombin cleavage site**

These peptides were expressed as phage pIII fusions. The GPRS thrombin site is highlighted in black and proline residues are underlined. Three clones were identified that had a mutated GPRS sequence. The weak thrombin cleavage signal MPRM is also highlighted in control A1.
resistance was observed to chymotrypsin, but 12 of 26 peptides demonstrated signals that were at least 2-fold higher than that produced by the chymotrypsin-sensitive control peptide, A12B.

Free peptide analysis

Nine peptides, which were first selected as N-terminal fusions to RepA and showed resistance to proteases when assayed as M13 phage pIII fusions in ELISA, were synthesised chemically with an N-terminal FLAG epitope and C-terminal biotin tag (Table II). These peptides were chosen at random to determine whether the fusion partner (phage or RepA) protected the peptides from proteolysis. A negative FLAG control peptide (A12B, DYKFDDYWHGSGGSLCATTLGPRS) was also included that contained a thrombin cleavage site but also a glycine-serine rich linker sequence that is known to be resistant to proteolysis (Huston et al., 1988; Whitlow et al., 1993). Two peptides, where the thrombin site had been mutated (peptides THR-8 and THR-9), were also included. Thrombin, trypsin or chymotrypsin, alone or in combination were used to test the stability of the peptides. Whilst all peptides demonstrated resistance to thrombin, varying degrees of resistance to trypsin and chymotrypsin were observed. Peptides THR-6 and THR-7 showed significant resistance to all of the proteases, alone or in combination (Fig. 4).

Resistance to plasma proteases

Preliminary incubation analysis in human plasma showed that three peptides THR-3, THR-6 and THR-7 were most resistant to plasma proteases (data not shown). These peptides had shown considerable resistance to thrombin and/or trypsin and chymotrypsin and were further tested for their

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>THR-1</td>
<td>DYKFDDYWHYPPTCPYTTGPRRS-biotin</td>
</tr>
<tr>
<td>THR-2</td>
<td>DYKFDDYWHSPPRPRPPNPDGPRRS-biotin</td>
</tr>
<tr>
<td>THR-3</td>
<td>DYKFDDYWHITRPDDNHTPDGPRRS-biotin</td>
</tr>
<tr>
<td>THR-4</td>
<td>DYKFDDYWHGSWSDSNANTGPRRS-biotin</td>
</tr>
<tr>
<td>THR-5</td>
<td>DYKFDDYWHHEPQKPGPGPRRS-biotin</td>
</tr>
<tr>
<td>THR-6</td>
<td>DYKFDDYWHSCPNKQDPTGPRRS-biotin</td>
</tr>
<tr>
<td>THR-7</td>
<td>DYKFDDYWHTDCSHNPTDFGPRRS-biotin</td>
</tr>
<tr>
<td>THR-8</td>
<td>DYKFDDYWHHPIHIIHHSKQSGSGS-biotin</td>
</tr>
<tr>
<td>THR-9</td>
<td>DYKFDDYWHSMPPGSGSGSGSGSG-biotin</td>
</tr>
<tr>
<td>THR-10</td>
<td>DYKFDDYWHYTPNPPQSAAGPRRS</td>
</tr>
<tr>
<td>THR-11</td>
<td>DYKFDDYWHNVMPQHNDGPRRS-biotin</td>
</tr>
<tr>
<td>A12B</td>
<td>DYKFDDYWHGSGGSLCATTLGPRS-biotin</td>
</tr>
</tbody>
</table>

The FLAG tag sequence is underlined, the peptide sequence highlighted in grey and the thrombin cleavage signal is highlighted in black.
stability in plasma. The negative control, A12B was again included, as this had shown sensitivity to all three tested proteases. The plasma proteases represent a different challenge for these peptides, with most of those present being aminopeptidases, plasmins and kinin-kallikreins (Aoyagi et al., 1990). The peptides were synthesised with an unmodified N-terminus and a biotin at the C-terminus and would therefore be expected to exhibit particular susceptibility to the action of aminopeptidases in plasma or in tissues.

In rat plasma, the control peptide was degraded rapidly, having a half-life of 26 min (0.44 h, Fig. 5). All three stabilised peptides outperformed the control having half-lives of 8.97, 3.45 and 7.07 h for peptides THR-3, -6 and -7, respectively. This corresponds to an improvement in stability exhibited by these peptides of between 7- and 20-fold. Similar results were obtained with human plasma where the half-life of A12B was 1.69 h compared with the half-lives of THR-6 and THR-7 being 8.80 and 14.72 h, respectively. THR-3 did not reach a half-life in the time tested (46 h) (data not shown).

In vivo study of protease resistance

The demonstration of in vitro stability does not necessarily translate into in vivo stability as some important proteases are membrane bound and not therefore present in plasma. In order to test in vivo stability, peptides were injected into rats and their presence in the bloodstream of the animals was monitored over a short period of time (90 min). We assumed that losses due to renal clearance would be very similar for all the peptides and that the relative concentrations of peptide observed in the blood would therefore indicate resistance to proteolysis. In the test, all peptides were detected 2 min after injection (Fig. 6). However, at the 15-min sample point, control peptide A12B had been eliminated to below detectable levels. This fast clearance made it impossible to calculate a half-life for A12B. By contrast, peptides THR-6 and THR-7 were observed up to 45-min post-injection and their respective half-lives were calculated at 13.6 and 14.8 min, respectively. For THR-6 and THR-7 the stability in vivo correlated well with stability in plasma, whereas THR-3, which had shown the best plasma stability in vitro, appeared to be rapidly cleared in vivo. The stability of THR-3 in vivo more closely correlated with the in vitro assays in which trypsin or chymotrypsin was used (Fig. 4).

THR-6 contains a single cysteine and THR-7 contains a pair of cysteine residues within the amino acid sequence whereas THR-3 is cysteine free. To determine whether the difference in stability might be caused by dimerisation and the formation of protease-resistant complexes, the peptides were analysed by gel filtration on a Superdex peptide 10/300 column. All the peptides appeared to migrate as monomers, including the control, A12B (data not shown). This suggests that they do not form inter-molecular disulphide bridges or other aggregates and therefore there may be some other structural features within these peptides that limit the access of proteases to their cleavage sites.

Discussion

Despite the potential benefits of peptides as therapeutic agents, proteolytic sensitivity is often cited as a key reason for the rejection of this class of biologics as a source of new drugs. In this study, we used CIS display to isolate peptide motifs capable of protecting adjacent enzyme substrate sequences from proteolytic degradation. This was achieved using a simple randomised 12-mer peptide library comprised entirely of L-amino acids and without any chemical modification of amino acid side chains or peptide bonds. The simple procedure of selecting the most resistant peptides from a large, diverse, library after exposure to thrombin resulted in many 12-mer motifs that exhibited resistance to
thrombin and also to alternative proteases (chymotrypsin and trypsin). This was a striking result given that these alternative enzymes were absent during the selection process yet their cleavage sites were known to be present in both the thrombin substrate sequence and in the FLAG epitope that was used for library recovery. Therefore, some of the selected motifs were capable of protecting cleavage at sites both N- and C-terminal to their own locations and they were capable of protecting more than one protease cleavage site concurrently.

In the majority of the thrombin-resistant peptides, a preference for prolines and/or polar residues (particularly asparagine, serine and threonine) was observed. This suggests the possibility of hydrogen bond formation with the main chain and therefore formation of stabilising secondary structure. However, NMR analysis revealed no evidence of sheet, turn or helical structure in the peptides analysed (not shown). It is possible that peptides have been selected on the basis that they can adopt a restricted repertoire of structures that are unfavourable to protease recognition, rather than forming stable secondary structures.

Matsuura and Plückthun (2003) have described a similar approach in using thermolysin for the evolution of folded proteins by ribosome display. However, this approach has not previously been applied to the selection of stable linear peptide sequences. Here we have shown that CIS display can facilitate the selection of linear peptides that are protected from proteolysis by a mechanism that is not related to secondary or tertiary structure. Enrichment of peptides with high incidence of polar and proline residues correlates well with the known stabilising properties of serine/threonine and proline in natural peptides and proteins. Serine is common in the linker regions of proteins and is favoured with glycine as a protease-resistant motif in recombinant proteins (Huston et al., 1988; Whitlow et al., 1993; Howard et al., 2004). Likewise, threonine is also common in linker regions and is paired with proline in the Pro-Thr box that links the two domains of endoglucanase of Cellulomonas fimii ( Shen et al., 1991). Proline is abundant in many biologically active peptides and is known to protect against proteolysis. In particular, two adjacent proline residues possess a high degree of resistance to any human proteolytic enzyme when they are not positioned terminally ( Vanhoof et al., 1995). Indeed, Ala-Pro-Pro motifs at the amino-terminus of short peptides have been demonstrated to be more stable in various cell extracts ( Vanhoof et al., 1995; Walker et al., 2003). Therefore, it is not surprising that these amino acids are present within a library that links the FLAG epitope and the RepA protein, but it is surprising that these sequences can impart protease resistance to neighbouring regions containing up to nine amino acids.

In order to further test the peptides, they were exposed to the extended cocktail of proteases present in plasma. The control peptide showed limited resistance to plasma proteases whilst the stabilised peptides were more stable. In an extension to this test, the peptides were assayed in vivo as there would be more potential for digestion from proteases present on the surface of membranes and from active serum proteases. Here, again, all three stabilised peptides were more resistant than the control peptide, THR-6 and THR-7 more so than THR-3. It was noted that THR-3 appeared to have the greatest resistance in plasma but its in vivo stability correlates more closely with the results from incubation with trypsin and chymotrypsin. It is possible that the plasma stability assay gives a false indication of the activity of blood as some of the intrinsic proteases are inactivated through the addition of anticoagulants and that more challenges exist in the in vivo environment.

Whilst this model system demonstrates that CIS display is capable of selecting for protease-resistant peptides, and that their observed in vitro stability appears to translate to prolonged half-life in vivo, it would be desirable to use this methodology to improve the stability of therapeutic peptides. We have tested protection motifs presented in this paper in fusion with an active peptide and it appears that the proteolytic peptides need to be selected in context with the adjoining peptide for protective effect. Although the protective sequences themselves appear not to be transferable, the method can be translated to the stabilisation of therapeutic peptides and we have since selected a protection motif with an active peptide (10 amino acids in length), maintaining activity of that peptide in an inositol phosphate accumulation assay, whilst enhancing stability in human plasma (unpublished data). Indeed, the library design can also be adapted to select for changes in the wild-type peptide sequence that can also enhance stability. In order to further improve the selection method, even more promiscuous enzymes could be used to further enhance the in vivo stability. Others have used leucine aminopeptidase or microsomal aminopeptidase as a way of aggressively mimicking the activity of plasma ( Galati et al., 2003) and it is possible that this would yield even more stable peptides if such proteases were used in CIS display selections. In addition, it is possible to combine the combinatorial approach that we have presented here with modification of the peptide backbone at sensitive sites. The use of N-terminal d-amino acids, in order to protect from aminopeptidase digestion, cyclisation or even the incorporation of non-natural amino acids could further improve the robustness of the selected peptides ( Sela and Zisman, 1997; Galati et al., 2003; Murakami et al., 2006; Kang and Suga, 2008).

The stability of peptides to intrinsic proteolysis is an important barrier to overcome in the development of peptides as therapeutics ( McGregor, 2008). However, there are other issues that are beyond the scope of this experiment but are highlighted in our in vivo data. Although we have improved the pharmacokinetics of the peptides, the residence times are still short in comparison with larger proteins such as albumin or immunoglobulins and the rapid loss of peptide in all samples is probably indicative of clearance mechanisms in addition to proteolysis. Albumin and immunoglobulins have extended half-lives due to their recycling through the neonatal IgG-Fc receptor, FcRn and slower kidney clearance because of their larger hydrodynamic diameter ( Ghetie and Ward, 2002). Fusion or binding to IgG1 Fc domains, antibodies or to albumin has been successful in extending in vivo half-lives of peptides and protein products ( Makrides et al., 1996; Goldenberg, 1999; Osborn et al., 2002; Dennis et al., 2002; Dumont et al., 2006; Nguyen et al., 2006; Woodnutt et al., 2008) and similar increases in in vivo half-life can be conferred through the addition of PEG or carbohydrates to peptides and proteins, as demonstrated with Pegasys©, a recombinant IFN-α-2a and Hematide™, a PEGylated erythropoietin receptor agonist ( Harris and Chess, 2003; Bunn, 2007) or through extending the polypeptide
chain with a glycine-rich homo-amino-acid polymer (Schlapschy et al., 2007). A combination of such technologies with the selection approach described in this paper and chemical methods of stabilising peptides improves the prospects for peptides as a credible therapeutic class.

References


Received January 6, 2009; revised August 10, 2009; accepted August 11, 2009

Edited by Dr Philipp Holliger